Title
Abstracts 2019 Joint IOCV XXI and IRCHLB VI

Permalink
https://escholarship.org/uc/item/1zp421bn

Journal
Journal of Citrus Pathology, 6(1)

Authors
IOCV, Organization
IRCHLB, Committee

Publication Date
2019

DOI
10.5070/C461045425

Joint Conference of the
INTERNATIONAL ORGANIZATION OF CITRUS VIROLOGISTS XXI
and
IRCHLB VI
INTERNATIONAL RESEARCH CONFERENCE ON HUANGLONGBING
MARCH 10-15, 2019
RIVERSIDE, CALIFORNIA, USA

2019 Joint IOCV XXI & IRCHLB VI Abstracts

Abstracts presented at the Joint 21st Conference of the International Organization of Citrus Virologists (IOCV) and the 6th International Research Conference on Huanglongbing (IRCHLB), in Riverside, California, USA, March 10-15, 2019 are now available on line at the **Journal of Citrus Pathology (JCP)** (http://journalofcitruspathology.com/).

The abstracts are arranged in the order they were presented at the conference and labeled (O: oral or P: poster). The conference session each abstract was presented at is also noted (see example below). The conference program can be found at: https://escholarship.org/uc/item/61r2d3wc.

The abstracts are published in the **Special Section: Proceedings of the Joint IOCV XXI and the IRCHLB VI,** in the **Volume 6, Issue 1, 2019** of the **Journal of Citrus Pathology (JCP)** for citation purposes (https://escholarship.org/uc/iocv_journalcitruspathology/6/1). The abstracts are published as submitted. They were formatted but not reviewed or edited by the Journal of Citrus Pathology.

Recommended format for citing abstracts, using the example abstract below, is as follows:
Vidalakis, G., Lavagi, I., Osman, F., Bodaghi, S., Greer, G., Christiano, R. (2019). Advancements at the Citrus Clonal Protection Program (CCPP) and National Clean Plant Network (NCPN), U.S.A. [abstract]. *Journal of Citrus Pathology*, 6(1):1-2. Retrieved from https://escholarship.org/uc/iocv_journalcitruspathology/6/1

Abstract Example:

IOCV-01: IOCV History and Citrus Programs Oral Presentations

IOCV-O1-01

Advancements at the Citrus Clonal Protection Program (CCPP) and National Clean Plant Network (NCPN), U.S.A.

Georgios VIDALAKIS[1], Irene LAVAGI[1], Fatima OSMAN[2], Sohrab BODAGHI[1], Greg GREER[1] and Rock CHRISTIANO[1]

[1]University of California, Riverside, USA, [2]University of California, Davis, USA

Abstract: The availability of pathogen tested, true-to-type propagative material is critical for successful citrus production. In the United States, various programs at the national, state or regional level have addressed these needs. In California, the Citrus Clonal Protection Program (CCPP) at UC Riverside is a cooperative program with the United States Department of Agriculture…

IOCV-01: IOCV History and Citrus Programs Oral Presentations

Shoot-tip grafting *in vitro*: forty-five years improving citrus health worldwide

Luis NAVARRO

Instituto Valenciano de Investigaciones Agrarias (IVIA), Moncada, Valencia, Spain.

Abstract: Control of graft and vector-transmitted pathogens requires the establishment of new plantings with healthy plants. To accomplish this objective methods to recover healthy plants from infected ones are required. The technique of shoot-tip grafting *in vitro* (STG) was developed to recover citrus plants free of all pathogens and without juvenile characters to overcome the limitations of nucellar embryony and thermotherapy. This was accomplished at the University of California Riverside at Professor T. Murashige's tissue culture laboratory (Department of Plant Sciences), with the collaboration of C.N. Roistacher (Department of Plant Pathology) for pathogen indexing. Research was carried out from February 1973 to June 1974. The last experiment was started on March 1974, exactly 45 years ago from this Conference. STG basically consists in grafting 0.1-0.2mm long shoot tips composed of the apical meristem plus three leaf primordia, isolated from growing flushes of infected plants, on 2- week-old *in vitro* growing seedling rootstocks and culturing the grafted plants for 1-2 months before transplanting to the greenhouse. The original procedure is still used today with very minor modifications. It allows the recovery of 40-50% successful grafts transplanted with over 90% survival. More than 90% of micrografted plants are usually free of the pathogens infecting the source plant. The main modification of the technique has been the use of budsticks cultured *in vitro* to produce flushes for shoot-tip isolation. This allows using the procedure for importation of genotypes following quarantine regulations.

Non-Technical Summary: STG has been used in most citrus producing countries to recover thousands of healthy plants utilized to propagate hundreds of millions of certified nursery plants. The consequence is that in many countries non-vector transmitted pathogens are just disappearing from commercial plantings. Furthermore, exchange of genotypes among different countries is being done without phytosanitary risks. An additional application is the conservation of healthy germplasm that can be lost if infected. STG is probably the single research development that had a higher impact in the citrus industry worldwide.

IOCV-O1-01

Advancements at the Citrus Clonal Protection Program (CCPP) and National Clean Plant Network (NCPN), U.S.A.

Georgios VIDALAKIS[1], Irene LAVAGI[1], Fatima OSMAN[2], Sohrab BODAGHI[1], Greg GREER[1] and Rock CHRISTIANO[1]

[1]University of California, Riverside, USA,
[2]University of California, Davis, USA

Abstract: The availability of pathogen tested, true-to-type propagative material is critical for successful citrus production. In the United States, various programs at the national, state or regional level have addressed these needs. In California, the Citrus Clonal Protection Program (CCPP) at UC Riverside is a cooperative program with the United States Department of Agriculture, the California Department of Food and Agriculture, and the citrus industry of California as represented by the California Citrus Nursery Board and the Citrus Research Board. The purpose of the CCPP is to provide a safe mechanism for the introduction of citrus varieties into California from any citrus-growing area of the world. This mechanism includes disease diagnosis (e.g. bioindexing and laboratory) and pathogen elimination-therapeutics (e.g. shoot- tip grafting) followed by maintenance and distribution of true-to-type citrus propagative materials. From 2012 - 2017, the CCPP received 238 introductions, performed therapy on 250 accessions, performed 2,660 bioindexing tests for quarantine release of 76 varieties, performed over 150,000 laboratory diagnostic tests, and distributed 280,769 buds to 1,477 nurseries, growers, scientists, and citrus enthusiasts. The National Clean Plant Network for Citrus (NCPN-Citrus) was officially formed in 2010 and currently has 10 centers in 9 US states and territories, including California, Florida, Arizona, Texas, Louisiana, Alabama, Hawaii, Maryland, and Puerto Rico. NCPN-Citrus centers introduce, test, maintain, and distribute pathogen-tested citrus propagative materials for use by scientists, the general public, and the multibillion dollar USA citrus industry. In a typical year, NCPN-Citrus centers conduct over 75,000 diagnostic tests, distribute over 600,000 units of clean materials, perform therapeutics on hundreds of accessions, maintain hundreds of foundation plantings of budwood sources, and foster various citrus extension programs. In conjunction with the other NCPN specialty crops, NCPN-Citrus develops outreach programs to educate and train industry personnel and the public on the importance of using pathogen-tested materials.

Non-Technical Summary: The need for pathogen-tested citrus propagative materials is recognized as fundamental to the establishment and maintenance of a sustainable and profitable citrus industry. The presence of graft-transmissible pathogens such as viruses, viroids or bacteria in citrus propagative materials can be deleterious to tree survival and fruit production for both existing and future citrus plantings. Here we report a successful story of the collaborative power of industry, university, and regulatory agencies to provide pathogen tested, true-to-type propagative material in California and the U.S.A.

IOCV-O1-02

Post Entry Quarantine (PEQ) of citrus in South Africa

Anna JOOSTE, Zama THELEDI, Ngcebo HLALELE

ARC-Tropical and Subtropical Crops, Private Bag X11208, Nelspruit, 1200, South Africa

Abstract: The disease management unit at the Agricultural Research Council's Tropical and Subtropical Crops, Nelspruit campus (ARC-TSC) is a role player in the mandate of the Citrus Improvement Scheme (CIS) to ensure the supply of pathogen-free propagation material to the South African citrus industry. The Post Entry Quarantine (PEQ) function, assigned by the Department of Agriculture, Forestry and Fisheries (DAFF) to the ARC- TSC, is critical to ensure no foreign pathogen introductions via imported budwood. Budwood imports are subjected to shoot tip grafting (STG), to eradicate graft transmissible pathogens, *i.e.* viruses, viroids and bacteria. Once STG is complete, the material is indexed on biological indicators and molecular tests to detect various pathogens including citrus viroids, such as *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CDVd), *Citrus bent leaf viroid* (CBLVd), *Citrus bark cracking viroid* (CBCVd) and *Citrus viroid V* (CVd V). In addition, tests are done for citrus viruses and diseases, including *Citrus tatter leaf virus* (CTLV), *Citrus psorosis virus* (CPsV), Impietratura disease and the associated pathogens of African Citrus Greening and HLB, *viz.* '*Candidatus*' Liberibacter africanus and '*Ca*' L asiaticus, respectively. Depending on the country of origin, more pathogens may be screened for. Seed imports are subjected to grow out tests and subsequent molecular detection for pathogens as per the quarantine pathogen list, including *Citrus leaf blotch virus* (CLBV), depending on the country of origin. The role of the ARC-TSC lab in the PEQ of citrus will be discussed, statistics of citrus imports to South

Africa and STG releases will be shown and emphasis on quarantine pathogens and the importance to comply with PEQ standards will be highlighted.

Non-Technical summary: Post Entry Quarantine (PEQ) is an important function of the Citrus Improvement Scheme (CIS) in South Africa. The Post Entry Quarantine (PEQ) function, assigned by the Department of Agriculture, Forestry and Fisheries (DAFF) to the ARC-TSC, is critical to ensure no foreign pathogen introductions via imported budwood, seed or tissue culture material. Statistics for imports of the past years will be discussed and the importance to comply with PEQ standards will be highlighted.

IOCV-O1-03

Citrus germplasm collection, evaluation and utilization for the rehabilitation and development of the citrus industry in the Philippines

Juliet M. OCHASAN[1], Nancy T. ASPURIA[1], Rogelio G. CUSTODIO[1]

[1] Bureau of Plant Industry – Baguio National Crop Research Development and Production Support Center, Baguio City, Philippines

Abstract: Citrus (*Citrus sp.*) is an important fruit commodity in the Philippines, particularly the local variety called calamansi *(× Citrofortunella microcarpa)*, but also pummelos (*C. grandis*) and mandarins (*C. reticulata*). Along with the decimation of the citrus industry in the early 1980's, most of the important local citrus germplasm was also lost. Hence, the Bureau of Plant Industry (BPI) in Baguio City is making deliberate efforts to collect important local citrus germplasm as well as introduced varieties for the rehabilitation and further development of the citrus industry in the Philippines. To date, there are about 103 varieties belonging to 14 citrus genera, as well as hybrids and related genera in the citrus germplasm collection. These are housed in two separate greenhouses, one greenhouse for the pathogen-free collection while the other containing mostly local collections which may be infected with any of the graft-transmissible diseases. These different varieties, particularly the introduced ones, are being evaluated for their performance under local conditions. After the evaluation trials, those varieties that passed the variety selection standards are registered under the National Seed Industry Council (NSIC). At present, there are 14 registered citrus varieties. These varieties are now being propagated by nurseries for the rehabilitation and expansion of the citrus industry. Meanwhile, the local varieties are undergoing disease indexing and disease elimination.

Non-technical summary: To sustain the rehabilitation efforts of the citrus industry in the Philippines, there is a need for a continuous effort to collect and evaluate citrus varieties. These varieties should be made available to citrus nurseries as registered and pathogen-free budwoods for further multiplication.

IOCV-O1-04

Using Tissue Print Blotting Based qPCR for the Detection of Viroids in Citrus Germplasm

Amanda RAWSTERN[1,2], Esteban RODRIGUEZ[2], Georgios VIDALAKIS[1], John HARTUNG[3], MaryLou POLEK[2]

[1] Department of Plant Pathology, University of California, Riverside, USA, [2] USDA-ARS National Clonal Germplasm Repository for Citrus and Dates, Riverside, USA, [3]USDA- ARS Molecular Plant Pathology Laboratory, Beltsville, USA

Abstract: Viroids are widespread and can be readily proliferated via contamination from mechanical damage or from propagation of infected, symptomless budwood. They can

impact tree health by causing problems such as bark scaling, leaf epinasty, stunting, and yield reductions. They also cannot be eliminated via thermotherapy due to their high heat tolerance and must be sanitized through shoot tip grafting, so testing for them is required for germplasm banks to choose the appropriate therapy. Tissue print blotting extraction and qPCR has been shown to be more cost effective and enable higher throughput testing than traditional nucleic acid purification for Huanglongbing (HLB) and Citrus tristeza virus (CTV), however it has not been used to detect viroids or multiple pathogen infections (Bertolini et al 2008, Bertolini et al 2014, Fu et al 2018). Our study demonstrates that tissue print blot extraction qPCR using single primer mixes can be used to detect either singly infected or multiply infected viroid pathogens in citrus germplasm collections and have Ct values not statistically significantly different than using nucleic acid purification from plant tissue at the same dilution level (paired t test ; p = 0.063). Elution of RNA from nitrocellulose membranes followed by qPCR was compared to tissue ground RNA extraction qPCR by testing 36 positive trees for Group I, Group II, Group III, and Citrus exocortis viroids. Sections of stems were transversely cut and the cut surfaces were pressed onto nitrocellulose membranes to collect the liquid exudate. Five overlapping prints were cut into small pieces and soaked in glycine buffer then placed in a 95°C water bath for 10 minutes to help release the viroids from the nitrocellulose membrane. We are currently exploring the effect of titer levels on the sensitivity of the tissue print blotting extraction method by testing plants with known weaker infection levels as well as dilutions of positive controls.

Non-Technical Summary: Citrus viroids of groups 1-3 and Citrus exocortis viroid can be detected using tissue print blotting extraction followed by qPCR. Tissue print blotting extraction is a crude extraction method that is 90% less expensive than tissue grinding extraction protocols. The Ct values are not significantly different from samples extracted through tissue grinding so it provides comparable results in plants with high titer levels. This method, although non-regulatory, is a rapid and cost-effective means to screen large field plantings and protective structures. We are currently exploring the effect of titer levels on the sensitivity of the tissue print blotting extraction method by testing plants with known weaker infection levels and dilutions of positive controls to determine if the protocol works on a variety of infection levels.

Citations:

Bertolini, E., Moreno, A., Capote, N., Olmos, A., de Luis, A., Vidal, E., Perez-Panades, J., and Cambra, M. (2008). Quantitative detection of *Citrus tristeza virus* in plant tissues and single aphids by real time RT- PCR. *European Journal of Plant Pathology, 120,* 177-188.

Bertolini, E., Felipe, R., Sauer, A., Lopes, S., Arilla, A., Vidal, E., Mourao Filho, F., Nunes, W., Bove, J., and Lopex, M. (2014). Tissue-print and squash real-time PCR for direct direaction of '*Candidatus Liberibacter'* species in citrus plants and psyllid vectors. *Plant Pathology, 63,* 1149-1158.

Fu, S.M., Liu, H.W., Liu, Q.H., Zhou, C.Y., and Hartung, J. (2018). Detection of 'Candidatus Liberibacter asiaticus' in citrus by Concurrent Tissue Print based qPCR and Immunoassay. Manuscript submitted for publication.

IOCV-O1-05

Using Next generation sequencing (NGS) to characterize Australia's living pathogen collection

Grant CHAMBERS[1], Anna ENGLEZOU[1], John WEBSTER[1], Daniel BOGEMA[1], Nerida DONOVAN[1]

[1]NSW Department of Primary Industries, Menangle, Australia

Abstract: Major graft-transmissible citrus diseases, such as huanglongbing (HLB), are not known to occur in Australia. However, there are several endemic graft-transmissible viruses and viroids which are managed using uninfected propagation material. NSW DPI maintains a living pathogen collection to evaluate and develop diagnostic protocols, and as controls for testing Australian citrus germplasm. To evaluate the potential of NGS, high quality small or total RNA extracts from this collection were sequenced using Illumina HiSeq or NextSeq technologies. The NGS data was manually analysed in-house, using CLC workbench 6, Geneious, and an adaption of the *VirusFinder* pipeline (Wang *et al*. 2013); and also analysed with web-based automated pipelines. Data analysis confirmed the presence of 7 viroid and 6 virus species, including several Citrus tristeza virus strain variants. Some viruses and viroids detected were known to be present within the samples, whereas others were only revealed using NGS. Citrus viruses and viroids previously unreported in Australia were detected, and the presence of a novel citrus viroid, tentatively named CVd-VII, was confirmed. The data enabled the generation of draft genomes for a number of viruses and viroids; some potentially unique to Australia.

Non-technical summary: Endemic viruses and viroids of citrus, and new species not known to be present in Australia, were confirmed in an Australian living pathogen collection using advanced sequencing methods. This technology greatly enhances the diagnostic tool kit for robust testing of citrus germplasm before supply to industry.

Citations:

Wang Q, Jia P, Zhao Z (2013) VirusFinder: software for efficient and accurate detection of viruses and their integration sites in host genomes through next generation sequencing data. PLOS ONE 8(5)

IOCV-O1-06

Next generation sequencing of small RNAs from citrus plants with single and mixed infections of viruses and viroids

Grazia LICCIARDELLO[1], Rosario FERRARO[2], Giuseppe SCUDERI[1], Marcella RUSSO[1], Antonino CATARA[2]

[1]Agrobiotech soc coop, Catania, Italy,[2] Science and Technology Park of Sicily, Catania, Italy.

Abstract. Detection of citrus viruses and viroids by next generation sequencing (NGS) and bioinformatic analysis of small RNAs extracted from the bark of five citrus plants, displaying different complexity of infections is reported. Three sources were young budlings growing in greenhouse (SG29, Bau282, HU-PSTS) and two were collected from an old sweet orange Tarocco tree in open field (P3R1, P3R3). The libraries were multiplexed, clustered, and sequenced on an IlluminaHiSeq 2000 with a single-read 50 cycles sequencing protocol. Unpaired reads by NGS, were indexed and aligned with full genome sequences of 11 citrus viruses and 6 viroids. The alignments were evaluated by QualiMap. SG29 and Bau282 libraries generated about 7M of reads mapping with CTV-VT genomes and 5M mapping with CTV-T30, respectively. The coverage of full length genome sequences was > 45X. HU- PSTS library generated about 18M of reads mapping on CTV-VT genomes, 7M reads mapping with CTV-T3 and 12M with CTV- T68.Additionally, a consistent number of reads allowed to reconstruct the genomes of two viruses (CYVCV and CTLV, with 100% and 62% fraction of reference, and two viroids HSVd and CDVd, both with 100% fraction of reference. The results were confirmed by bioindexing and PCR tests. P3R1 and P3R3 libraries were obtained from two different branches of the same tree, one was CTV- ELISA positive and one negative. NGS data revealed the same profile of reads, different in number. P3R1 showed 7M of reads mapping with CTV-VT genomes and P3R3 only 2M. In addition, a number of reads in the range of 15,000 and 37,000 aligned with the genomes of CEVd, HSVd and CDVd, whereas reads below 10,000 showed a poor coverage with CBLVd, CVd-IV and CVd-V. RT-PCR search for the three viroids was negative. Results highlight the need of sampling and confirmation tests before the adoption of the technology.

Non-technical summary. Citrus are historically affected by a large number of viruses and viroids and the number is increasing after the developments in next- generation sequencing technologies and bioinformatics. Since then a growing interest for their application to forensic and quarantine services or certification of propagation materials has been reported. The results confirm the potential of metagenomic analysis to screen simultaneously multiple infections in plants but more data are needed to be adopted.

IOCV-O1-07

Next generation sequencing as a routine diagnostic tool for citrus variety introduction at the Citrus Clonal Protection Program

Tyler DANG[1], Isaac MENCHACA[1], Sohrab BODAGHI[1], Irene LAVAGI[1], Fatima OSMAN[2], Georgios VIDALAKIS[1]

[1]University of California, Riverside USA,
[2]University of California, Davis USA

Abstract: The Citrus Clonal Protection Program (CCPP) is responsible for the introduction, maintenance, and distribution of pathogen-tested citrus propagative materials in California and via the National Clean Plant Network (NCPN) for the U.S.A. Next generation sequencing (NGS) is a powerful technology that has the potential to transform citrus diagnostics. In this study, we tested whether NGS can perform equally well or better than the widely used quantitative polymerase chain reaction (qPCR) for the detection and identification of known graft- transmissible pathogens of citrus. We compared the sensitivity and specificity of NGS (Illumina, HiSeq 4000) to qPCR (SYBR® Green and fluorescently labeled probes) using citrus samples infected with different virus, viroid, and bacterial pathogens from the CCPP disease bank. The initial NGS sequencing results showed that citrus budwood RNA samples treated with Ribo- Zero (Illumina, San Diego, CA) increased the pathogen detection sensitivity by generating more reads (16,874,673.5 vs 1,430,869.5) and contigs (77,069.5 vs 1,382.5) than the non-treated samples. NGS and qPCR detected all targeted pathogens in single and mixed infections. However, in several samples, short NGS contigs indicated the presence of citrus pathogens not detected by qPCR. To eliminate the possibility of cross contamination and sequencing errors and to assess the biological validity of the NGS data, bioindexing experiments are in progress. Actual CCPP variety introductions, from different time periods and citrus growing regions of the world are also in use for diagnostic validation of NGS. These experiments are critical for the proper implementation of NGS as routine diagnostic tool for CCPP, because they will correlate NGS number and length of contigs, with the risk of undetectable pathogen levels by qPCR and will help to evaluate benefits such as decreased greenhouse footprint, labor, time, and cost for variety introductions.

Non-technical summary: It is important for the CCPP to introduce citrus varieties and distribute pathogen-tested propagative materials. We are investigating whether NGS is as practical and reliable diagnostic tool as the currently used qPCR and bioindexing, for detecting known graft-transmissible pathogens of citrus.

Implementation of NGS technologies, will potentially reduce citrus variety introduction time, labor, and cost.

IOCV-01: IOCV History and Citrus Programs Poster Presentations

IOCV-P1-01

Finding an alternative to CF-11 cellulose for dsRNA and viroid extraction in citrus

Marylou POLEK[1], **Esteban RODRIGUEZ**[1], Amanda RAWSTERN[1,2], Sohrab BODAGHI[2], Georgios VIDALAKIS[2]

[1]USDA-ARS National Clonal Germplasm Repository of Citrus and Dates, Riverside, USA, [2]Deparment of Microbiology and Plant Pathology, University of California, Riverside, USA

Abstract: In order to release citrus cultivars from quarantine, clean-stock programs such as the USDA Agricultural Research Service (ARS) National Clonal Germplasm Repository for Citrus and Dates (NCGRCD) and the Citrus Clonal Protection Program (CCPP) must complete approved laboratory diagnostic assays for known graft- transmissible pathogens. Some of these pathogens have RNA genomes; e.g. citrus tristeza virus, citrus tatter leaf virus, and citrus exocortis and hop stunt viroids. A long-used diagnostic method for citrus diseases caused by RNA-type pathogens is biological indexing. This is a valuable method for citrus quarantine programs because it is pathogen sequence and titer independent, but typically takes over 8 months to complete. Morris, et al. (1979) developed a more rapid pathogen sequence independent laboratory assay for the detection of the double stranded RNA (dsRNA), produced during the replication cycle of the RNA-type pathogens. Furthermore, detection of viroid RNA species by sequential polyacrylamide gel- electrophoresis (sPAGE), under denaturing conditions (Bustamante et al. 1986), like dsRNA, is the only molecular detection method that is viroid sequence independent. All these sequence independent pathogen detection methods have become the cornerstones for citrus quarantine programs because they provide the safety net against misdiagnoses due to sequence mutations, pathogen evolution, or poorly characterized viral genomes. The dsRNA and sPAGE protocols have worked well for many years and are approved by Federal and State regulations. However, the major reagent used in these methods, Cellulose CF-11, has been discontinued and an alternative had to be identified. CF-11 was compared to other types of cellulose; these include purified and medium fiber products. The purified form was found to yield equivalent results when used in the approved diagnostic methods of both viruses and viroids.

Non-Technical Summary: CF-11 cellulose has been effective for viroid RNA and virus dsRNA extractions for many years. Due to the discontinuation of CF-11, either a new method or source of cellulose is needed. We report on the evaluation of various products to determine the best alternative to detect RNA genome pathogens in citrus in a molecular yet sequence independent manner.

Citations:

Morris, T.J., Dodds, J.A. (1979) Isolation and Analysis of Double-Stranded RNA from Virus-Infected Plant and Fungal Tissue. Phytopathology 69: 854-858

Rivera-Bustamante, R. F., R. Gin and J. S. Semancik (1986). "Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous-pH system." Analytical Biochemistry 156: 91-95.

IOCV-P1-02

Import of Citrus into the European Union: a French quarantine to control the health risks

CALADO G., LEGRAND P., RENVOISÉ J.-P., and GERBAULT J.-E.
ANSES, Plant Health Laboratory, Quarantine Unit, 6 rue Aimé-Rudel, 63370 Lempdes, France

Abstract: The introduction of plants of Citrus for planting is prohibited for phytosanitary reasons in all member States of the European Union according to Directive 2000/29/EC, which details the protective measures against the introduction of plant pests and their spread. However, derogation for import has therefore been allowed for scientific work and varietal selections by Directive 2008/61/EC. The post-entry quarantine station of the French Plant Health Laboratory, belonging to the French Agency for Food, Environmental and Occupational Health and Safety (ANSES), establishes the health status of Citrus plants introduced and their compliance with the regulatory requirements. Furthermore, this station is the National Reference Laboratory for regulated Citrus viruses. Tests and observations on Citrus plants are carried out in containment facilities to avoid any contamination of the environment in the case of presence of a pest according to European Union phytosanitary rules and phytosanitary standards of the European and Mediterranean Plant Protection Organization. For that purpose, different Citrus quarantine pests (virus, viroids, and endophytic bacteria) are kept in collection to allow development and validation of methods and to provide positive controls for laboratory analyses. Only plant material free from any regulated pests is released on the territory of the European Union. From 2010 to 2018, about 80 Citrus varieties were received at the quarantine station on behalf of French and other European scientists and breeders.

Non-Technical summary: In order to prevent the spread of citrus pests into the European Union, the import of plant material is regulated according to European phytosanitary regulations. Citrus varieties for scientific research or varietal selections may be introduced thanks to the French post-entry quarantine station. Plant material is tested in containment facilities to ensure its phytosanitary status and is released if recognized free of regulated pests.

IOCV-P1-03

Propagation System of Virus-free Citrus Nursery Tree in Guangxi, P. R. China

Chongling DENG, Binghai LOU**,** Shengqiu LIU

Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops, Guilin, Guangxi 541004, P. R. China

Abstract: Guangxi is one of the main citrus production provinces in P. R. China. Until 2017, Guangxi was the largest citrus producing province, having 441,333 hm^2 of citrus growing area with about 6.87 million tons of citrus fruits valued at more than 3.1 billion dollar. The achievements in the development of the citrus industry of Guangxi are closely correlated to the construction of a propagation system of virus-free citrus nursery trees in Guangxi, where citrus Huanglongbing has been occurring for more than 80 years. The core techniques in the propagation system of virus-free citrus nursery tree in Guangxi were mainly developed by Guangxi Academy of Specialty Crops, previously known as Guangxi Citrus Research Institute. Currently, the number of virus-free citrus nursery trees propagated by Guangxi has reached up to 20 million every year.

Non-Technical summary: Guangxi is the biggest citrus production province in P. R. China and has established an excellent propagation system of virus-free citrus nursery tree. Currently, the number of virus- free citrus nursery trees propagated by Guangxi has reached up to 20 million every year.

IOCV-P1-04

Streamlining Citrus Clonal Protection Program (CCPP) citrus diagnostics using multiplex qPCR

Fatima OSMAN[1], Sohrab BODAGHI[2] and Georgios VIDALAKIS[2]
[1]University of California, Davis, CA, USA; [2]University of California, Riverside, CA, USA.

Abstract: The Citrus Clonal Protection program (CCPP) is the citrus germplasm introduction and distribution program for the state of California. CCPP materials are distributed to California citrus nurseries, growers, researchers, governmental institutions, and citrus enthusiasts as well as to many USA citrus producing states and territories via the National Clean Plant Network (NCPN). CCPP is currently using over 23 individual PCR assays for the detection of all known graft-transmissible pathogens of citrus, prior to the release of a new citrus variety from quarantine. These assays are currently being consolidated into high-throughput, multiplex, real-time quantitative PCR based assays to streamline CCPP diagnostic pipeline while increasing specificity, efficiency and reducing cost, time and labor. These multiplex qPCR assays aim to detect all citrus pathogens, endemic or exotic to CA, mandated in the USDA/CDFA approved CCPP protocols and will be compared side-by-side with Next Generation Sequencing (NGS) based assays currently under investigation. So far, singleplex qPCR assays for the following citrus pathogens; Citrus tristeza virus (CTV), Citrus psorosis virus (CPsV), Citrus leaf blotch virus (CLBV), *Candidatus* Liberibacter species (CL spp.), *Spiroplasma citri*, Citrus tatter leaf virus (CTLV), Citrus vein enation virus (CVEV), Citrus yellow vein associated virus (CYVaV), Citrus variegation virus, Citrus leaf rugose virus, Citrus leprosis virus, Citrus sudden death-associated virus, Citrus yellow mosaic viruses, Citrus chlorotic dwarf virus, Citrus yellow vein clearing virus, and the 8 known citrus viroid species have been developed. Several multiplex qPCR assays are at different stages of development/ validation for the simultaneous detection of pathogens such us a) CTV, CPsV and CLBV (Osman et al. 2015, J. Virol. Meth. 220: 64–75), b) citrus viroids (Vidalakis & Wang 2013, US Pat. 20130115591, Osman et al. 2017, J. Virol. Meth. 245: 40–52), c) CL spp. and *S. citri* and d) CVEV, CYVaV and CTLV.

Non-Technical summary: In California, the Citrus Clonal Protection Program (CCPP) has been constantly trying to improve diagnostics for citrus germplasm, moving towards high throughput molecular assays. In doing so, the CCPP will streamline the diagnostic process of new citrus varieties introduction and nursery budwood tree sources testing reducing time and cost.

Citations:

Osman, F., Hodzic, E., Kwon, S, Wang, J., and Vidalakis, G. (2015). Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of *Citrus Tristeza Virus*, *Citrus psorosis virus*, and *Citrus leaf blotch virus*. *Journal of Virological Methods*, *220*, 64-75.

Vidalakis, G. and Wang, J. (2013). Molecular method for universal detection of citrus viroids. US Patent Publication number 20130115591.

IOCV-P1-05

The Texas certified virus-free budwood program – 20 years on.

J. V. DA GRAÇA & M. VAN NESS

Texas A&M University-Kingsville Citrus Center, Weslaco TX, USA

Abstract: Following the detection of *Citrus tristeza virus* (CTV) in a Texas citrus nursery in 1992, the Texas legislature passed a bill in 1996 establishing a mandatory citrus budwood

program. The main commercial citrus grapefruit and sweet orange varieties were indexed for the main pathogens, and were subjected to shoot-tip grafting. In 1998, the first foundation trees were established, and increase trees were propagated from them. At the time, only nurseries in the southern portion of the state where commercial citrus production occurs were required to obtain budwood from the program. Certified budwood of other varieties was obtained from the CCCP to provide certified budwood of non-commercial varieties. The discovery of Huanglongbing (HLB) in Florida prompted Texas to protect its budwood source using insect-resistant screen. New foundation trees were established in a screenhouse, and a US federal grant was obtained to construct a screenhouse complex for additional foundation trees and increase budwood trees. In 2013, all commercial nurseries in the south Texas were required by law to propagate citrus in screenhouses, and in 2018, this was extended across the state to include nurseries which propagate citrus primarily for the homeowner market. The collection now houses approx. 150 varieties, and supplies an average of 250,000 buds annually to the 14 currently certified citrus nurseries in Texas. All trees in the collection are tested annually by PCR for CTV and HLB, and for other viruses and viroids every 3-5 years.

Non-Technical Summary: To reduce the risk of spreading graft-transmissible pathogens, the State of Texas launched a mandatory certified pathogen-free budwood program in 1998. In the past 20 years, the program has evolved into a statewide program where all citrus propagation in the state has to be conducted in insect-resistant screenhouse using budwood which originated from the foundation trees maintained by the Texas A&M University-Kingsville Citrus Center.

IOCV-02: De novo Discovery of Citrus Viruses Oral Presentations

Identification of novel viruses and viroids in citrus hosts by high-throughput sequencing: methodologies, opportunities, challenges and impact

Francesco DI SERIO[1] **and Mengji CAO**[2]

[1]Institute for Sustainable Plant Protection, National Research Council, Bari, Italy
[2]Citrus Research Institute, Southwest University/Chinese Academy of Agricultural Sciences, Beibei, Chongqing, China

Abstract:
Largely improving the capability of sequencing and characterizing nucleic acids, high-throughput sequencing (HTS) technologies have strongly increased the chances of identifying virus- and viroid- derived sequences in plants. On the one hand, HTS offers interesting opportunities in routine diagnosis that, however, must raise to significant challenges, including the need of developing standard protocols, infrastructures and expertise to store and analyze the data. On the other hand, HTS allows the identification of novel viruses and viroids, provided that sequences of related viruses/viroids are available in databases. This means that viruses/viroids with genomes largely divergent with respect to those already knownmay escape detection, especially if appropriate controls and search methodologies are not applied. The low titer of the viruses, their uneven distribution, as well as the presence of inhibitors in the extracts may also impair the sensitivity of HTS-mediated identification of known and novel viruses, especially in woody plants, such as citrus, thus requiring additional efforts to adjust the extraction methods, the library preparations and/or the pipelines adopted for the bioinformatic analyses of the sequencing data. These issues together with the results of case studies, in which different HTS methodologies have been tested for their capability of detecting citrus viruses and viroids, will be examined. The identification of novel viruses by HTS may have consequences on the taxonomy of viruses that, in some instance, has

been already updated to include viruses discovered by HTS. The need of providing biological data on the novel viruses identified by HTS will be also discussed, paying major attention to the relevance of the information requested for assessing the risk these infectious agents may pose.

Non-Technical Summary:
High-throughput sequencing (HTS) technologies offer novel opportunities for identification and characterization of known and novel viruses and viroids infecting plants. However, the application of this methodology needs expertise and infrastructures. Novel viruses identified by HTS need to be further characterize also from a biological point of view.

IOCV-O2-01

Two negative-stranded RNA viruses infecting citrus are representative members of the novel genus *Coguvirus*

F. Di SERIO[1], B. NAVARRO[1], M. MINUTOLO[2], S. ZICCA[1], M. SAPONARI[1], De STRADIS[1] and D. ALIOTO[2]

[1]Istituto per la Protezione Sostenibile delle Piante, Consiglio Nazionale delle Ricerche, Bari, Italy.; [2]Dipartimento di Agraria, Università degli Studi di Napoli Federico II, Portici (Na), Italy.

Abstract: Citrus concave gum-associated virus (CCGaV) and citrus virus A (CiVA) are two negative stranded RNA (nsRNA) viruses recently identified in citrus plants. They have a bipartite genome, composed of an RNA1 encoding the RNA-dependent RNA polymerase in the negative strand and an ambisense bicistronic RNA2 coding for the nucleoprotein and the putative movement protein. Due to the unique structural and phylogenetic features with respect to other nsRNA viruses, these viruses have been proposed to be the representative members of a new genus (*Coguvirus*), whose final approval by the International Committe for Taxonomy of Viruses is pending. Molecular signatures in the viral genomic RNAs and in the encoded proteins, together with phylogenetic tree reconstructions based on Maximum Likelihood and Bayesian Markov Chain Monte Carlo analyses support a modular genome evolution for these viruses that possibly originated from an invertebrate-infecting ancestor. While CCGaV has been associated with a severe disease (citrus concave gum disease) first described in the early 1930s, the possible association of CiVA with symptoms needs further investigation. To this aim a multiplex RT-PCR protocol to detect simulataneously the two viruses has been developed and validated. Results of a preliminary field survey in Southern Italy (Campania and Apulia Region) showing the presence of both viruses in several citrus species, in both single and mixed infections, will be presented.

Non-Technical Summary: Two novel viruses have been identified in citrus trees. One of this virus has been associated with a citrus disease (concave gum disease), the other one has not been associated to any disease so far. The two viruses are closely related to each other from a molecular point view so that they have been proposed to be two novel species in the same new genus. The available molecular information allowed to develop specific multiplex detection methods that will be useful for further investigation on epidemiology and pathogenesis of these infectious agents.

IOCV-O2-02

Complete sequence of three phlebo-like viruses associated to Cristacortis, Concave gum and Impietratura diseases

Karelia VELÁZQUEZ, Alba HERVÀS, José Antonio PINA, Pedro MORENO, José GUERRI, Luis NAVARRO, **Mari Carmen VIVES**

Instituto Valenciano de Investigaciones Agrarias (IVIA), Centro de Protección Vegetal y Biotecnología, 46113 Moncada, Valencia.

Abstract: Cristacortis- (C), Concave gum- (CG) and Impietratura- (I) affected trees show characteristic and differential symptoms in field trees, whereas in Dweet tangor and sweet orange indicator plants they induce similar symptoms, consisting of chlorotic flecking and oak leaf pattern. To identify the causal agent associated to these diseases, the small RNAs fraction, extracted from Etrog citron plants infected with isolates C-601 from Corsica, CG-204 from California or I-501 from Spain, was analyzed by deep sequencing. The assembly of sRNAs allowed obtaining the sequence of three bipartite negative strand RNA viruses that were present in infected plants, but not in plants healthy or infected with other citrus pathogens. The genomic organization of these three viruses resembled that of viruses CGW2, isolated from a field tree with concave gum symptoms, and Citrus Virus A isolate W4, not associated with any specific pathology. Surprisingly, C-601 RNAs revealed 99% and 71% sequence identity with CGW2 and W4, respectively, whereas CG-204 and I-501 showed 74% and 96% identity with CGW2 and W4, respectively. CG-204 and I-501 RNAs shared 97% sequence identity. Specific primers from RNA 2 of each viral sequence were used to detect these viruses in different C, CG and I isolates from the IVIA citrus virus collection and in field trees showing characteristic concave gum or impietratura symptoms. In CG and I isolates from the virus collection and in all symptomatic field trees, both GC-204 and I- 501 sequences were detected, but not the C- 601 sequence. In C isolates only the presence of C-601 sequence was detected. Additionally, C-601, CG-204 and I-501 isolates have been inoculated in Orlando tangelo plants grafted on sour orange to determine if CG-204 or I-501 can induce cristacortis specific symptoms.

Non-Technical summary: There are some graft-transmissible citrus diseases such as cristacortis, concave gum and impietratura that, although known for many years in different citrus growing areas, their causal agent is still unknown. We have used next generation sequencing to identify three new viruses in infected plants. We are trying to double-check symptom specificity of our isolates. Obtaining specific sequences of each pathogen is essential to develop specific and rapid molecular detection methods for the control of these diseases.

IOCV-O2-03

Investigations into an oak leaf inducing agent detected in citrus in Texas

J.W. PARK[1], M. KUNTA[1], E. LOUZADA[1], M. GONZALEZ[1], and J.V. DA GRACA[1]
[1]Texas A&M University-Kingsville Citrus Center, Weslaco, TX 78599, USA

Abstract. Three graft-transmissible citrus diseases of unknown etiology, concave gum, impietratura and cristacortis, all induce oak- leaf patterns in young leaves of certain varieties under cool conditions. The development of these symptoms in indicator seedlings is used for biological indexing. None of these diseases has been reported in Texas, but oak leaf symptoms were observed in six young navel trees out of 42 plants derived via embryo recovery from chimeric fruits from a pathogen-free Cara Cara navel tree. Biological indexing of the parent tree gave negative results, but the oak leaf agent was graft-transmitted from the young plants to Madam Vinous sweet orange. Total RNA was extracted from two plants showing oak-leaf patterns and a healthy Madam Vinous sweet orange plant. Next generation sequencing was conducted on Illumina HiSeq4000 by 2x150bp using ribosomal RNA-depleted total RNA fractions, from which ca. 48.4 Gb per sample were obtained. The raw sequencing reads was trimmed and mapped to the sweet orange genome (v1.1) using CLC Genomics Workbench. After mapping sequencing reads to

the sweet orange genome, the unmapped reads were uploaded to VirFind to investigate the presence of any viral sequence specific to concave gum tree samples. VirFind confirmed the presence of viral sequences showing homology to concave gum-associated Phlebo-like virus in the plants showing oak-leaf patterns. *De Novo* assembly of unmapped reads of each sample followed by Local Blastn with CG-associated contigs obtained from VirFind identified two Phlebo-like viral RNA sequences of 6702 nt (CGTX RNA1) and 2705 nt (CGTX RNA2) in length from both plants, each of which has 72% and 73% sequence identity to Citrus concave gum- associated virus RNA1 and RNA2, respectively. Full-length CGTX RNA1 and RNA2 sequences were cloned and sequenced to confirm the NGS data. The source of this agent is unknown, but since the young plants were initially in a shadehouse, an arthropod vector cannot be ruled out; mechanical and rootstock seed transmission are also possible.

Non-Technical Summary: Oak-leaf patterns were observed on six young grafted navel trees, resembling the symptoms induced by concave gum, cristacortis, and impietratura, diseases not known to occur in Texas. The symptoms were graft transmissible. Next generation sequencing was conducted on a nucleic acid extract and found to have a 72- 73% sequence identity to the recently described concave gum associated phlebovirus. The source of this agent is currently unknown, and further studies on its relationship to known diseases and possible origins continue.

IOCV-O2-04

Detection of a South African variant of a bunya-like virus infecting citrus

Rachelle BESTER[1,2], Maryam KARAAN[1], Glynnis COOK[3] and Hans J MAREE[1,3]

[1]Department of Genetics, Stellenbosch University, Stellenbosch, South Africa [2]Agricultural Research Council, Infruitec-Nietvoorbij: Institute for Deciduous Fruit, Vines and Wine, Stellenbosch, South Africa [3]Citrus Research International, Nelspruit, South Africa

Abstract: Symptoms of psorosis-like diseases in citrus have been observed worldwide for decades, however the causal agent has remained elusive. In this study, high- throughput sequencing (HTS) of citrus psorosis virus (CPsV) negative citrus trees, but displaying typical symptoms of psorosis- like diseases, including an oak leaf pattern on biological indicator hosts, revealed the presence of a bunya-like virus with the highest sequence identity to the recently identified citrus virus A (CiVA) (Navarro et al., 2018). This virus was first identified to infect citrus in Italy and is the second member of a new genus within the order *Bunyavirales*. The South African isolates of CiVA share 95% sequence identity to the Italian CiVA isolate and 63-70% identity to the other member of the new genus, citrus concave gum-associated virus (CCGaV) (Navarro et al., 2017). The identification of CiVA in South Africa and Italy highlights the need to assess diversity and potential geographic grouping. The generation of sequence information is essential for the development of sensitive and specific detection assays to aid citrus sanitation and certification. Additional research into the disease aetiology is also needed to determine if CiVA contributes to psorosis-like diseases or other symptomology in infected citrus trees.

Non-Technical Summary: Psorosis-like diseases will keep spreading if no diagnostic test is available to identify infected plant material. For the development of an effective detection assay to improve management strategies in commercial citrus orchards, the genome sequence of the disease-causing virus needs to be available. In this study, high- throughput sequencing (HTS) led to the identification of a new variant of citrus virus A (CiVA) that may be associated with psorosis-like symptoms.

Citations:

Navarro, B., Minutolo, M., Stradis, A.D., Palmisano, F., Alioto, D., Serio, F.D., 2017. The first phlebo-like virus infecting plants: a case study on the adaptation of negative-stranded RNA viruses to new hosts. *Mol. Plant Pathol. 19, 1075–1089*. https://doi.org/10.1111/mpp.12587

Navarro, B., Zicca, S., Minutolo, M., Saponari, M., Alioto, D., Di Serio, F., 2018. A Negative-Stranded RNA Virus Infecting Citrus Trees: The Second Member of a New Genus Within the Order Bunyavirales. Front. *Microbiol. 9*. https://doi.org/10.3389/fmicb.2018.02340

IOCV-O2-05

A series of novel bipartite negative-strand RNA viruses surfaced in the order *Bunyavirales* highlighting a conspicuous evolutionary diversity.

Song ZHANG [1, 2], Qiyan LIU [1, 2], Yuanjian QIU [1, 2], Changyong ZHOU [1, 2*], **Mengji CAO** [1, 2*]

[1]National Citrus Engineering Research Center, Citrus Research Institute, Southwest university, Chongqing, China, 400712, [2]Academy of Agricultural Sciences, Southwest University, Chongqing, China, 400715

Abstract: Genomes of two watermelon crinkle leaf-associated viruses (WLCaV-1 and -2) were bipartite other than tripartite based on our recent results in 2017; almost at the same time, we discovered two counterparts from citrus tentatively named citrus leaf flecking-associated viruses (CLFaV-1 and -2, which are close to citrus concave gum associated virus and citrus virus A); quite recently we identified the another one infecting oriental paperbush (*Edgeworthia chryantha* Lindl.). All these five viruses comprise RNA1 encoding RNA dependent RNA polymerase (RdRp) and the ambisense RNA2 encoding coat protein (CP) and movement protein (MP) in different ORF orientations. Two opposite transcription termination signals (TTS) in the intergenic region (IR) of RNA2 that trending to form a long stem-loop structure was identified by RACE technique. Other common features shared included highly conserved stretches at the both genomic termini that making the reverse complement. Sequence comparisons showed those RdRp (60–79% amino acid sequence identity) were the most conserved, followed by CP (41–66%) and MP (38–59%). The MP was likely to belong to 30K superfamily for a typical secondary structure. Phylogenetic analysis of RdRp and CP indicated that these viruses were clustered in the order *Bunyavirales* with a distinct branch generated beside the family *Phenuiviridae*, whereas in the tree built for MP they got closest to the genus *Ophiovirus*, suggesting an ancient recombination event in negative-strand RNA viruses kingdom resulted in the beginning of this differentiation. Further work in the biology of CLFaV-1 has been done, including grafting of virus-infected *Citrus clementina* to different citrus cultivars, and we identified several of which as indicator plants for the vivid vein- clearing and oak-leaf pattern in young leaf. Besides, RT-PCR protocols for specific detection of CLFaV-1 with the generic primers were also developed.

Non-technical summary:

A unique sort of novel bipartite negative- strand RNA viruses infecting different plant hosts including citrus were successively identified. Bioinformatics analysis suggested that these viruses belong to the order *Bunyavirales*, representing for a novel genus. We established the detection methods for one of them, the citrus-infecting virus CLFaV-1, in respects of indicator plant and RT-PCR, and the works would form the basis to better prevent the damage of citrus production caused by the virus.

IOCV-02: De novo Discovery of Citrus

Viruses Poster Presentations

IOCV-P2-06

Molecular characterization of a novel citrivirus infecting citrus using next-generation sequencing

Mengji CAO [1, 2*], Song ZHANG [1, 2], Qiyan LIU [1, 2], Zhongan LI [1]

[1] National Citrus Engineering Research Center, Citrus Research Institute, Southwest university, Chongqing, China, 400712;

[2] Academy of Agricultural Sciences, Southwest University, Chongqing, China, 400715;

Abstract: A novel citrus virus isolate with a genome arrangement typical of the genus *Citrivirus*, was identified by next-generation sequencing. This virus of its first discovery in the citrus cultivar Haruka was obtained with full genome nucleotides (8,697 nt) as excluding poly A tail. Three inferred ORFs (1,

2 and 3) in the genome are respectively responsible for expression of putative replicase protein (RP), movement protein (MP) and coat protein (CP), with the 3' untranslated region (UTR), MP and CP region attributing that pointing to be an isolate of *Citrus leaf blotch virus* (CLBV), whereas 5' UTR and RP region should belong to an unknown citrivirus species other than CLBV. Therefore, with respect to a close sequence correlation between the virus and CLBV, we gave it a tentative name of Citrus leaf blotch virus-2 (CLBV-2). However, in the phylogenetic trees constructed from sequence comparisons between whole-genome nucleotide and RP amino acid sequences, CLBV and CLBV-2 were separated into different clades with the distinct evolutionary directions, suggesting a distant relationship. Evidences, recombination analysis supporting plus the discovery of another slightly varied CLBV-2 isolate CN-2 (8,698 nt; 92% whole genome sequence identity) from citrus cultivar Hyuganatsu, demonstrate that the occurrence of the novel virus resulted from a recombination across species level in the Citrivirus is a stable transition, on the base of which we propose the CLBV-2 should be another potential species classified into the genus. The experiment of inoculating CLBV- 2 to *Nicotiana benthamiana* is not successful yet.

Non-technical summary: This study identified and characterized a novel citrivirus (CLBV-2) in citrus in China. Sequence, phylogenetic and recombination analysis allowed the proposal of classifying CLBV-2 as a new species in the genus *Citrivirus*. However, its biological characteristics were still not determined though CLBV-2 was associated leaf chlorotic blotching on citrus.

IOCV-P2-07

Studies on a novel virus-like disease affecting pummelo in Hawaii

Alejandro OLMEDO-VELARDE[1], Michael MELZER[1]

[1] Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, Honolulu, USA.

Abstract: In 2012, pummelo trees with sectoral dieback and foliar symptoms including chlorosis, corkiness and necrosis of veins were observed in the Manoa Valley in Honolulu, USA. In 2018, similar symptoms were observed on pummelo in a citrus demonstration garden center located in Pearl City, USA. In both cases, symptomatic leaves tested negative using molecular and/or serological assays for all viruses, viroids, and fastidious prokaryotes that cause or are associated with similar symptoms in citrus. Transmission electron microscopy revealed the presence of numerous isometric particles which were often associated with inclusion bodies. Although no discernible double- stranded RNAs (dsRNA), a hallmark of

RNA viral infection, were consistently isolated from symptomatic leaves and petioles, a randomly amplified library was constructed from dsRNA extracts and subjected to high-throughput sequencing. Bioinformatic analyses on these data is currently underway. Additionally, non-specific amplification of circular DNA was performed by rolling circle amplification (RCA), producing an amplicon. The RCA product still needs to be characterized. Ongoing experiments and analyses of the HTS are expected to help in the characterization of the virome associated to the pummelo presenting the symptoms mentioned above.

Non-Technical summary: On the island of Oahu, Hawaii, pummelo trees presenting sectoral dieback, and unique foliar symptoms including chlorosis, corkiness, and vein necrosis, symptoms have been observed. Using molecular and/or serological diagnostic assays, the possibility of infection by several characterized citrus pathogens was discarded. Thin sections of symptomatic leaves observed under transmission electron microscope revealed abundant presence of isometric particles associated with inclusion bodies. A rolling circle amplification product was obtained and still needs to be characterized. High-throughput sequencing studies are underway to identify and characterize any virus or virus-like agents associated with the pummelo.

IOCV-03: Citrus Leprosis Oral Presentations

Citrus leprosis-associated viruses: diversity and interactions with host plants and mite vectors

Juliana FREITAS-ASTÚA[1,2]; Pedro L. RAMOS-GONZÁLEZ[2]; Camila CHABI-JESUS[2,3]; Gabriella D. ARENA[4]; Mikhail O. LEASTRO[2]; Aline D. TASSI[1,3]; Elliot W. KITAJIMA[3]

[1]Embrapa Mandioca e Fruticultura/CNPMF, Cruz das Almas, BA, Brazil

[2]Instituto Biológico/IB, São Paulo, SP, Brazil

[3]Escola Superior de Agricultura Luiz de Queiroz/Esalq, Piracicaba, SP, Brazil

[4]Centro APTA Citros Sylvio Moreira, Cordeirópolis, SP, Brazil

Abstract: Although described more than a century ago, citrus leprosis (CL) is considered an emerging disease due to its recent introduction into new areas and to the increasing diversity associated with its growing list of causal agents. The cytoplasmic type of leprosis (CL-C) is caused by two species of the genus *Cilevirus* [bipartite ss(+) RNA]: citrus leprosis virus C (CiLV-C), prevalent in Latin America and composed of two distinct clades, and citrus leprosis virus C2 (CiLV-C2), whose genome shares only 55% nt identity with CiLV-C, and is reported in citrus only in Colombia. The nuclear type of the disease (CL-N) is caused by members of the genus *Dichorhavirus* [bipartite ss(-)RNA], family *Rhabdoviridae*. Originally reported in Florida, USA, in late 1800s, there are currently four accepted or tentative species of dichorhaviruses that can cause CL-N. Orchid fleck virus (OFV-citrus) occurs in commercial orchards in Mexico and Colombia, while citrus leprosis virus N (CiLV-N), citrus chlorotic spot virus (CiCSV), and citrus bright spot virus (CiBSV) are the causal agents of CL-N in Brazil; so far detected only in marginal areas of citrus production in the country. CiLV-N, CiCSV and CiBSV share less than 65% nt sequence identity with OFV-citrus. All CL-C and CL-N associated viruses are transmitted in a persistent manner by different species of *Brevipalpus* mites. The geographic distribution of CL viruses correlates with the presence of the vector species. CiLV-C and *B. yothersi* appear cooperate in the manipulation of host plants in their favor. OFV-citrus affects several *Citrus* spp., while other CL viruses appear restricted to fewer species. *C. sinensis* is their preferred host. The conspicuous

symptoms of necrotic and chlorotic lesions occur in leaves, stems and fruits, and are likely the outcome of a hypersensitive-like response of incompatibility, at least for CiLV-C. This virus interacts with and remodels some organelles in the cells, which can lead to the observed symptoms.

Non-Technical summary: Citrus leprosis (CL) is the most important viral disease currently affecting citrus production in some Latin American countries. Although this disease has been known for more than a century, only in the last years at least six accepted or tentative species of *Brevipalpus*- transmitted viruses, classified into the genera *Cilevirus* or *Dichorhavirus,* have been characterized. Here, we provide and update on what has been identified on CL viruses regarding their diversity and interactions with their host plants and mite vectors.

IOCV-O3-01

The first case of Citrus Leprosis-N in South Africa

Glynnis COOK, Rochelle CLASE, Chanel STEYN and Wayne KIRKMAN

Citrus Research International, Nelspruit, South Africa

Abstract: Citrus Leprosis (CL), one of the oldest citrus diseases, is known to occur in South and Central American countries, but has not previously been reported on citrus in South Africa. CL is caused by several RNA viruses, commonly referred to as either the cytoplasmic type (CL-C), or the nuclear type (CL-N). Symptoms similar to that of CL were observed in Valencia and navel orchards in the Eastern Cape province of South Africa. A CL- N virus belonging to the *Dichorhavirus* genus was detected in samples from all affected sites. The virus is transmitted by *Brevipalpus californicus* (flat mite) and infestation of these mites was observed on fruit, stems and branches at all find sites. Sequence data indicated that the associated virus is a strain of orchid fleck virus (OFV), with closest sequence identity (99%) to the OFV_cym07 isolate on cymbidium orchids (*Cymbidium spp*.) (Kondo *et al.* 2017) and not to OFV strains previously reported on citrus (Roy *et al.* 2014). Full genome sequences of OFV from affected citrus and OFV of *Brassia* sp (orchid), sampled in South Africa, were determined. Analysis of the N gene (nucleocapsid protein), G gene (glycoprotein) and L gene (RNA-dependant RNA polymerase) showed that the OFV from citrus and orchids in South Africa belong to subgroup I of OFV. The infection in citrus is thought to possibly originate from orchids. A response plan to contain and eradicate the disease is being implemented.

Non-Technical Summary: Citrus Leprosis (CL) was found in South Africa in the Eastern Cape province. The virus was identified as the nuclear type (CL-N) of citrus leprosis and found to be most similar to that found in orchids from Japan. These virus genomes differ significantly from OFV previously found on citrus in Mexico. The infection on citrus in South Africa is thought to possibly originate from orchids.

Citations:

Kondo, H., Hirota, K., Maruyama, K., Andika, I. B. and Suzuki, N. 2017 A possible occurrence of genome reassortment among bipartite rhabdoviruses. J. Virol. 508:18-25.

Roy, A., Stone, A., Shao, J., Otero-Colina, G., Wei, G. *et al.* 2014 Identification and molecular characterization of nuclear citrus leprosis virus, a member of the proposed *dichorhavirus* genus infecting multiple citrus species in Mexico. Phytop. 105. 10.1094.

IOCV-O3-02

A comparative cytopathology of leprotic lesions caused by *Brevipalpus*-transmitted viruses on *Citrus* spp. leaf tissues

Elliot W. KITAJIMA[1], Renato B.

SALAROLI[1], Juliana FREITAS-ASTÚA[2,3], José Evando A. BESERRA JR.[4], Gabriel OTERO-COLINA[5], Renata F. CALEGARIO[6], João Paulo R. MARQUES[1], Aline D. TASSI[1]

[1]LFN/ESALQ/USP, Piracicaba, SP, [2]Instituto Biológico, São Paulo, SP, [3]Embrapa Mandioca e Fruticultura, Cruz das Almas, BA, [4]Univ.Fed.Piauí, Teresina, PI, [5]Colegio de Pósgrado, Texcoco, Mexico, [6]Univ.Fed.Paraná, Curitiba, PR, Brazil

Abstract: It is now known that the so-called citrus leprosis syndrome (CL) in *Citrus* spp. may be caused by several distinct viruses transmitted by *Brevipalpus* mites (BTV). Different species of *Brevipalpus* are involved in their transmission. So far the following viruses have been identified: *Cilevirus*- CiLV- C, CiLV-C2; *Dichorhavirus*- OFV-citrus, CiLV-N, CiCSV, CiBSV. Cileviruses are the so-referred to as cytoplasmic type of BTV: short-bacilliform, membrane bounded virions accumulate in the cytoplasm, within endoplasmic reticulum cisternae and a dense, vacuolated inclusion (viroplasm) is consistently present in the cytoplasm. Genus *Dichorhavirus* is known as the nuclear type of BTV. They induce a large electron lucent inclusion in the nucleus. Naked, short rod-like virions are present in both nucleus and cytoplasm, frequently associated with membranes of the nuclear envelope or endoplasmic reticulum. Although being quite distinct taxonomically, anatomical changes observed in tissues of leprotic lesions reveal similarities as the presence of hypertrophic cells in the spongy parenchyma, and occasionally hyperplasy in the palisade parenchyma. Areas of necrotic tissues appear interspersed in the lesion. A common feature is the absence of viral infection in the vascular region, especially of phloematic cells, which may explain why BTV do not cause systemic infection, being rather localized. These conclusions are based on the TEM examination of more than 700 samples, collected from 11 countries, mostly in American continent. In Brazil collections were made in 20 of the 27 states, and in the state of São Paulo, from 51 municipalities. Although most of samples are from *Citrus* sp., more than 30 non-*Citrus* plant species were included in these studies.

Non-Technical summary: Citrus leprosis symptoms may be caused by several distinct viruses, all of them transmitted by tenuipalpid mite *Brevipalpus*, of varied species. They belong to two different types of viruses [cytoplasmic (BTV-C)- *Cilevirus*: CiLV-C and CiLVC-2; nuclear (BTV-N)- *Dichorhavirus:* OFV-citrus, CiLV-N, CiCSV and CiBSV based on their localization in infected cells and alterations they induce. Although being taxonomically distinct, these viruses cause similar changes in infected leaf tissues as cell enlargement and abnormal cell replication, and the absence of infection in the vascular region. Ultrastructural studies may detect BTV-C or BTV-N infection, but definite identification of the virus species requries additional, specific assays, such as RT-PCR.

IOCV-O3-03

Detection of a new citrus strain of *Orchid fleck virus* associated with Citrus leprosis syndrome in Mexico

Avijit ROY[1], Andrew STONE[2], Gabriel OTERO-COLINA[3], John S. HARTUNG[4], William L. SCHNEIDER[2], Gang WEI[1], Charles BELANGER[1,5], Benjamin A. ADDUCCI[1,5], Vessela MAVRODIEVA[1], Ronald H. BRLANSKY[6], and Mark K. NAKHLA[1]

[1]USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705 USA, [2]USDA-ARS, Fort Detrick, MD, U.S.A.; [3]Colegio de Postgraduados, Campus Montecillo, Texcoco, Estado de Mexico,

Mexico; [4]USDA-ARS, Beltsville, MD, U.S.A.; [5]Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695 USA, 6University of Florida, Lake Alfred, FL, U.S.A.

Abstract: The scenario for Citrus leprosis (CiL) disease has changed since application of High throughput sequencing (HTS) for virus discovery. Currently, we define CiL as a disease complex or syndrome associated with any of 10 known viruses that belong to the genera Cilevirus, Higrevirus and Dichorhavirus. Recent reports of nuclear *Citrus leprosis virus* (CiLV) from Brazil, Colombia, Mexico and Panama combined with the rapid spread of cytoplasmic CiLV in Central America and Mexico suggest that CiL represents a real threat to the US citrus industry. USDA-APHIS-PPQ has utilized HTS to identify known or undescribed viruses associated with leprosis syndrome and thus to help prevent introduction of exotic CiLVs in the US. In 2013, we reported the complete genomic sequence of a citrus strain of *Orchid fleck virus* (OFV) from Mexico. In 2014, a total of 25 nuclear CiLV suspected symptomatic plants from the state of Querétaro in Mexico were tested. RT-PCR with OFV-citrus specific primers was used to amplify and sequence the complete nucleocapsid (N) gene (1353 nt). Alignments of the N gene sequences showed that five of the 25 nuclear CiLV isolates shared less than 81% sequence identity with either OFV-1 or - 2 (Accession No. AB244417 and LC222629) or with the citrus strain of OFV (KF209275), and formed a separate branch after cluster analysis. HTS using the Illumina platform was performed to determine the complete sequence of the novel OFV-citrus. The complete genomic sequence of RNA1 of isolate S9 differs considerably (~16-18%) from OFV-citrus and other OFV isolates. However, the sequence of RNA2 is 99% identical to OFV-citrus. Collectively, phylogenetic and recombination analyses suggested that the observed OFV-citrus variation is an outcome of genome reassortment events that may facilitate genetic diversity among OFV, OFV-citrus subgroups and other undetected strains.

Non-Technical summary: Until the last decade we assumed that Citrus leprosis (CiL) disease was associated with two unrelated virus taxa; one replicated in the cytoplasm and another replicated in the nucleus. The CiL disease associated with the nuclear CiLV was endemic in Florida from the late 19th century but has not been recorded after 1968, whereas a completely different type of nuclear CiLV was discovered in Mexico in 2013. To determine the variability among CiLVs infection on different citrus host species, samples received from Mexico were tested. The results revealed the presence of a novel citrus strain of OFV in Mexico that exists either in single or in mixed infections of citrus with previously described OFV-citrus strain.

IOCV-03: Citrus Leprosis Poster Presentations

IOCV-P3-08

Citrus leprosis virus C in the Americas: an insight into the genetic diversity of its population.

Camila CHABI-JESUS[1,2]; Pedro L. RAMOS-GONZÁLEZ[2]; Matheus P. BARRO[3]; Ricardo HARAKAVA[2]; Renato B. BASSANEZI[4], Alecio S. MOREIRA[4,5]; Elliot W. KITAJIMA[2], Juliana FREITAS- ASTÚA[2,5]

[1]Escola Superior de Agricultura Luiz de Queiroz/Esalq, Piracicaba, SP, Brazil.

[2]Instituto Biológico/IB, São Paulo, SP, Brazil.

[3]Universidade Paulista/UNIP, São Paulo, SP, Brazil.

[4]Fundo de Defesa da Citricultura, Araraquara, SP, Brazil.

[5]Embrapa Mandioca e Fruticultura/CNPMF, Cruz das Almas, BA, Brazil.

Abstract: Citrus leprosis virus C (CiLV-C) is the main causal agent of citrus leprosis (CL), a disease threatening citrus yields from Mexico to Argentina. CiLV-C is a bipartite (+) ssRNA virus and the type member of the free- floating genus *Cilevirus.* In nature, CiLV-C is impeded from moving systematically within host plants and as a result produces local chlorotic or necrotic spots. New infection loci, even on a single leaf, exclusively rely on mites of the genus *Brevipalpus*. Two CiLV-C strains, CRD and SJP, occur in the Southwest region of Brazil. Their genomes show up to 85% of nucleotide sequence identity, though the 5'-end of the RNA2 molecules share an extremely conserved stretch, likely as a consequence of recombination. To understand the molecular epidemiology of CiLV-C, we aim to assess the genetic variability of its population in the Americas over a period of 86 years. One full and three partial sequences of the CiLV-C genomes were obtained from herbarium specimens collected in the state of São Paulo (1932-1975). Additionally, 101 complete sequences of CiLV-C putative coat protein gene (*p29)*, recovered from samples collected in commercial citrus orchards in São Paulo during 2017, and CiLV-C sequences retrieved from the GenBank, were incorporated into the analysis. Collectively, the dataset amasses 132 sequences, representing isolates from Mexico to Argentina collected between 1932 and 2017. The ML tree of the *p29* gene confirmed the segregation of the CiLV-C population into two clades: CRD and SJP. The haplotype diversity for both subgroups was found to be 0.99, whereas the nucleotide diversity (π) for each clade was 0.009 and 0.008, respectively. The non-synonymous/ synonymous substitution ratios were 0.074 and 0.076 suggesting purifying selection at the *p29* gene polymorphic sites. The π-value for the entire CiLV-C population was approximately 10x greater than within each clade. Moreover, the complete genome from the isolate conserved from 1975 showed 99% of nucleotide sequence identity with the CRD clade type-member, demonstrating that the low degree of variability within the clade might not be restricted to the *p29* gene. Low variability of CiLV-C population and the co-existence of two well-defined clades led us to launch two hypotheses: viruses of the two clades evolved either (*i*) independently, outside the citrus agroecosystem, or (*ii*) along with the non-permissive CL pathosystem (local infection, HR-like response, vector transmission), which entails to the stochastic fixation of divergent haplotypes through highly frequent bottlenecks. New analyses might shed light on our current understanding of CiLV-C population dynamics and how we can cope with the disease the virus produces.

Non-Technical Summary: Citrus leprosis is the leading viral disease affecting the Brazilian citriculture. Its main causal agent, the Citrus leprosis virus C (CiLV-C), is distributed throughout Latin America. It is well-know that a better understanding of viral populations might improve the efficiency of control measures. In order to assess the diversity of CiLV-C in the Americas, viral sequences from herbarium specimens collected during the period of 1932-1975 were obtained and analyzed together with other samples recently collected from the state of São Paulo. Altogether, the analysis of the 132 samples representing CiLV-C isolates gathered from 1932 to 2017 indicated that the CiLV-C population is subdivided into two well-defined clades, which show an extremely low variability. Data suggest at least two putative hypotheses concerning the CiLV-C evolution: viruses of the two clades evolved either (*i*) independently, outside the citrus agroecosystem, or (*ii*) along with the non- permissive CL pathosystem (local infection, HR-like response, vector transmission) which entails to the stochastic fixation of divergent haplotypes through highly frequent bottlenecks. New analyses might shed light on our current understanding of CiLV-C population dynamics and how we can cope with the disease the virus produces.

IOCV-P3-09

Development and validation of molecular assays for detection of Citrus leprosis viruses

Gang WEI[1], Benjamin A. ADDUCCI1, Avijit ROY[1], Charles BELANGER[1,2], Vessela MAVRODIEVA[1], Mark K. NAKHLA[1]
[1]USDA-APHIS-PPQ-CPHST, Beltsville, MD 20705 USA
[2]Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695 USA

Abstract: Citrus leprosis is known to be a disease complex associated with any of 10 reported viruses that belong to two distinct types of Citrus leprosis viruses (CiLV): a cytoplasmic (C) type and a nuclear type (N). In order to prevent the reemergence of CiLV into the US, we have being engaged on development and validation of molecular assays for detection of CiLV since 2010. Our work has evolved with the discovery of new CiLVs and its strains. First, we developed specific real-time RT-PCR (qRT-PCR) to detect CiLV-C, CiLV-C2 and Orchid fleck virus-citrus strain 1 (OFV-Citrus 1) with a plant internal control. Later we combined the individual assays into a multiplex qRT-PCR assay for detection of these three viruses. To differentiate the citrus and hibiscus strain of CiLV-C2 we developed a triplex qRT-PCR for detection of CiLV-C2, a hibiscus infecting cilevirus (CiLV-C2H) with a plant internal control since we detected CiLV-C2H in a co- infection with CiLV-C2 in Colombia. qRT- PCR assays are very sensitive and fast however they require complex equipment and well train personnel. For field use, we developed a CiLV-C2 specific Loop- Mediated Isothermal Amplification (LAMP) assay coupled with a fast and easy sample preparation protocol that can be run at plant inspection stations, ports of entry or packing houses. The assays have been validated for sensitivity, specificity, repeatability and intermediate precision to ensure the fitness for intended use. ISO-type work instructions have been developed for internal use or released to a network of screening laboratories (NPDN, State Department of Agricultures and federal). For confirmatory assays and production of CiLV-free citrus and hibiscus plants, we have developed two conventional multiplex RT- PCRs for the detection of CiLV C and N types. Generic and specific primer pairs can be used for detection and identification of known or undescribed cytoplasmic and nuclear CiLVs.

Non-Technical summary: Since 2010, we at Beltsville Lab have being engaged in development and validation of molecular assays for detection of CiLVs to prevent reemergence of Citrus leprosis disease into the US. So far, ten work instructions have been developed, validated and approved with more assays are under development and/or validation.

IOCV-P3-10

Prospecting of genes related to the RNAi pathway in *Brevipalpus yothersi*, a vector of citrus leprosis virus C

Luana Aparecida ROGERIO[1], Maria Andreia NUNES[1], Diogo Manzano GALDEANO[1], Marcos Antonio MACHADO[1], **Valdenice Moreira NOVELLI**[1]

[1]Sylvio Moreira Citrus Center, Agronomic Institute (IAC) Cordeirópolis, São Paulo, Brazil
Abstract: Citrus leprosis is considered as the main viral disease of Brazilian orange orchards, commonly caused by citrus leprosis virus C (CiLV-C) and transmitted by *Brevipalpus yothersi* (Acari: Tenuipalpidae). RNA Interference technology (RNAi) is a powerful strategy for studies of functional genomics and has been used as a tool for the control of pests and plant diseases. The aim of this study was prospect endogenous RNAi pathways of false

spider mite and perform the phylogenetic comparison of these functional mechanisms with arthropods. Through analyzing *B. yothersi* genome data, we explored the RNA biogenesis and were identified sequences encoding RdRp, Loquacius, Drosha, Pasha, Argonaute 2, Argonaute 3 and Dicer, which are crucial proteins for RNAi pathways. After validation of these sequences, comparative phylogenetic analysis revealed that false spider mites have a similar RNAi machinery relative to insects and other mites. This study provides evidence of RNAi machinery to false spider mites and could be further explored to develop RNAi- based management strategies to control of *B. yothersi*. Financial support Fapesp 2016/21749-8; INCT-Citros (Fapesp 2014/50880-0 and CNPq 465440/2014-2).

Non-Technical summary: The citrus leprosis control has been done basically with acaricides, increasing the costs of production and the risks of environmental damages. More efficient and sustainable strategies are highly desirable in the management of diseases and the use of techniques in molecular biology is an alternative that has been investigated for citriculture. Here we investigated RNAi pathways of false spider mite, the main vector of the citrus leprosis. It is expected on future that interfering RNA (RNAi) technology can be used for the control of vector.

IOCV-04: Citrus Viroids Oral Presentations

Citrus viroid diseases Nuria DURAN-VILA

Instituto Valenciano de Investigaciones Agraria (IVIA). Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA). Presently retired.

Abstract: The exocortis disease was first described in 1948 as a bark scaling disorder affecting trees growing on the *Poncirus trifoliata* rootstock. When it was demonstrated that exocortis was a graft transmissible disease, it was believed to be caused by a virus or a virus-like agent until when in early 1970s, VIROIDS were discovered/defined as a new class of plant pathogenic agents (as infectious low molecular weight RNAs). At that time nucleic acid technologies were extremely limited and transmission assays, availability of alternative herbaceus hosts and biological properties were the major information used for the characterization of *Citrus exocortis viroid* (CEVd) as the causal agent of the exocortis disease. The use of 'Etrog citron' for biological indexing and the adoption of new nucleic approaches allowed the identification of additional viroid-like RNAs being widespread in virtually all commercial citrus cultivars. With the information available today, in terms of sequencing data and biological properties including the performance of field grown trees, the first five viroids identified in the 1980s fit the viroid species concept and they are presently named *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) with specific variants causing the cachexia-xyloporosis disease, *Citrus bent leaf viroids* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd). With the molecular technologies available today, three additional viroids have been identified in citrus, *Citrus viroid V* (CVd-V), *Citrus viroid VI* (CVd-VI, former CVd- OS) and *Citrus viroid VII* (CVd-VII) but their effect on field grown trees is still unknown. On the other hand, the causal agent of a number of citrus pathologies such as «quebra galho» of Tahiti lime, gummy bark, gum pocket-gummy pitting, Kassala disease, of which the viroid etiology has been taken into consideration, remains unproven.

Non-Technical Summary: Today we are here to share the available information regarding the molecular and biological properties of citrus viroids. Since its creation in 1957, IOCV conferences offer the opportunity to exchange all kinds of information as well as to initiate collaborations to further understand the behavior and control of citrus diseases.

IOCV-O4-01

Probing the viroid RNA sequence for non-vital regions: Areas that can be altered

without loss of viroid viability and pathogenicity

Carlos RODRIGUEZ[1], Gregor BLAHA[1], Sohrab BODAGHI[1], Georgios VIDALAKIS[1]

[1]University of California Riverside, Riverside, CA, U.S.A.

Abstract: In order to gleam information on viroid RNA biology in host cells, we wanted to study the viroid throughout its lifecycle *in planta* and not only *in vitro*. We are experimenting with a biochemical system capable of identifying viroid sequence regions that are amenable to change without compromising its ability to infect or replicate. This is challenging because much like a ribozyme, the RNA sequence is what allows the viroid to take the necessary shape for interaction with plant cell systems. The entire RNA sequence of hop stunt viroid (HSVd) was probed by transposon mutagenesis, which allowed testing every position in the viroid sequence at once instead of manually picking areas to test. We used a plasmid DNA which contained the viroid sequence and transcribed it into infectious RNA. The use of transposase recognized flanking regions of a specific DNA sequence (i.e. entraceposon DNA) and subsequently catalyzed the insertion of the entraceposon DNA into the target DNA (here being the HSVd-coding plasmid). The entraceposon DNA fragment contained an antibiotic resistance marker and restriction enzyme cut sites that allowed selecting only for the plasmids that experienced a transposition event and then cut out the majority of the inserted fragment, leaving only a 15bp addition. Because there is only one random 15bp insertion event per plasmid we were able to create a HSVd population which consisted of a diverse pool of variants originating from the same viroid. Transcription of these altered DNA sequences which code for HSVd+15bp will be tested for their ability to infect plants, autonomously replicate, and become pathogenic. This experiment will give us some information on viroid sequence and structure but primarily it will identify an RNA region that is receptive to the addition of a short fluorophore binding aptamer that would allow for viroid tracking *in planta*.

Non-Technical Summary: Viroids are single stranded, circular RNA pathogens that range in size from 300-450 nucleotides. These pathogens can cause stunted growth and reduced yields in plants however very few viroid-RNA biology studies have been performed in plants. With the presented biochemical method we will be able to label and study viroid-RNAs in their natural state inside plants.

IOCV-O4-02

Global transcriptomic analysis reveals insights into the response to *Citrus exocortis viroid* in citron (*Citrus medica* L.)

Yafei WANG [1,2], Sagheer ATTA[3], Shuai LI[1,2], Changyong ZHOU [1,2]*, **Mengji CAO** [1,2]

[1] National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing, China; [2] Academy of Agricultural Sciences, Southwest University, Chongqing, China; [3] Department of Plant Protection, Faculty of Agricultural Sciences, Ghazi University, Dera Ghazi Khan, Pakistan

Abstract: *Citrus exocortis viroid* (CEVd) is the causal agent of citrus exocortis disease. As it seriously affected citron indicator plant, we employed CEVd-infecting citron as a system to study the feedback regulation mechanism using transcriptome sequencing. The transcriptomes of fresh leaves of healthy and viroid-infected (three months after inoculation) plants were comparatively analyzed, 1,530 differentially expressed genes were detected. As a result of the replication of CEVd in citron, there is significant up- regulation of the genes encoding DCL2

(Dicer-like 2), RDR1 (RNA-dependent RNA polymerase 1), AGO2 (Argonaute 2), AGO7 in RNA silencing pathway specializing in degradation of exogenous nucleic acids. In addition, the genes of disease resistance proteins, pathogenicity-related proteins, and HSP70 (Heat shock cognate 70 kDa protein) were also up-regulated. Moreover, some other up-regulated genes expressing CNGCs (Cyclic nucleotide-gated ion channel), MAPKs (Mitogen-activated protein kinase), CMLs (Calcium-binding protein), FLS2 (LRR receptor-like serine/threonine-protein kinase FLS2), and Rboh (Respiratory burst oxidase homolog protein D) were involving in the basic defense response. In contrast, the IAA (auxin) signal transduction pathway was inhibited, which might lead to plant dwarfing as apparent consequences. Besides, many genes responsible for phytohormone such as JA (jasmonic acid), GA (gibberellin) and BR (brassinolide) signal transduction pathways were changed. Collectively, these results suggested that CEVd infection in citron gave rise to complex changes in gene expression, allow us to better understand the offense- defense interplays of viroid and woody plant.

Non-Technical Summary: We used the CEVd-infected Citron (Citrus medica L) system to detect host genome-wide changes using RNA-seq. We analyzed the response of the woody host to the viroid and revealed a large number of genes involved in the defense response, indicating activation of plant immunity following CEVd infection. Our findings will help to study the detail response mechanisms of woody plants against viroid infections and help to better develop strategies to combat viroid diseases in fruit trees.

IOCV-O4-03

Citrus dwarfing viroid reduces citrus apical shoot growth and alters tree hormonal profile: Pathogen or answer to emerging citriculture challenges?

Irene LAVAGI[1], Roya CAMPOS[1], Rock CHRISTIANO[1], Carol LOVATT[1], Georgios VIDALAKIS[1]

[1]University of California Riverside, Riverside, CA, U.S.A.

Abstract: Citrus dwarfing viroid (CDVd) infection of navel orange trees on trifoliate orange rootstock has been previously reported to reduce canopy volume by approximately 50 %. To understand how CDVd reduces canopy volume, a survey of navel orange tree growth was initiated in an experimental block planted in 1998 at the Lindcove Research and Extension Center (LREC) in the San Joaquin Valley, California. We confirmed that 20 years after planting there was still a significant difference in tree height and canopy volume between CDVd-infected trees and noninfected controls. Since vegetative growth accounts for most of a tree's canopy volume, we monitored vegetative apical shoot growth over two seasons in the CDVd-infected and noninfected control trees. We also surveyed the apical shoot growth of 34-year-old navel orange trees on 'Flying Dragon' trifoliate rootstock, commonly known to produce small-sized trees. We found that the net growth of the apical shoots of CDVd-infected trees was significantly lower (reduced by almost 30 %, $p < 0.01$) than that of the control. We also determined that reduced apical shoot growth caused by CDVd was not significantly different from the effect of 'Flying Dragon' rootstock on growth ($p = 0.8$). This study indicates that the effect of CDVd on apical shoot growth is likely to be the main factor determining the observed reduction in overall citrus tree canopy volume. In the absence of a true citrus dwarfing rootstock, elucidating the CDVd-mediated dwarfing mechanism has the potential to develop citrus dwarfing applications that do not require a transmissible agent.

Non-technical summary: Citrus dwarfing viroid (CDVd) reduces the canopy volume of

navel oranges growing on trifoliate rootstock. A similar reduction occurs when navel oranges are grown on ‘Flying Dragon’ trifoliate rootstock. Apical shoot growth on CDVd-infected trees was reduced by almost 30 % as compared to noninfected trees. However, the reduction in CDVd-infected trees was not significantly different than that of trees grown on ‘Flying Dragon’. The effect of these factors on apical shoot growth is probably the main factor resulting in reduced canopy volume. Elucidated of the mechanism of CDVd-mediated growth reductions may allow the development of citrus dwarfing mechanisms not requiring a transmissible agent.

IOCV-O4-04

First report of Citrus Bent Leaf Viroid and Citrus Dwarfing Viroid in Argentina

Maria F. PALACIOS and Julia FIGUEROA

Centro de Saneamiento de Citrus, Estación Experimental Agroindustrial O. Colombres, Tucumán, Argentina.

Abstract: Citrus Bent Leaf Viroid (CBLVd) and Citrus Dwarfing Viroid (CDVd), from genus Apscaviroid, are two of the widely distributed viroids among the eight citrus viroids detected to date. They have been found to induce reduction in canopy volume and fruit production in citrus trees on trifoliate orange and trifoliate orange hybrids rootstocks. Moreover, citrus trees infected only with CBLVd or in combinations with Citrus Exocortis Viroid (CEVd), Hop Stunt Viroid (HSVd) and CDVd have been associated with a poor development of the root system. Samples of citrus trees showing viroid symptoms collected from citrus orchards in Tucumán, Salta, and Jujuy provinces (NOA region) were initially indexed on Arizona 861- S-1 Etrog citron (*Citrus medica*) and then analyzed by sPAGE. These samples were found to be infected with different viroid species, among them, CEVd and HSVd have been already identified. In order to determine the presence of CBLVd and CDVd, we performed a RT-PCR assay using the specific primers CM2-R (TCGACGACGACCAGTCAGCT) and AP2- F (TCCCCTTCACCCGAGCGCTGC), for CBLVd and AM-R (TCACCAACTTAGCTGCCTTCGTC) and AP-F (CTCCGCTAGTCGGAAAGACTCCGC) for CDVd. Thirty five samples including 11 lemons, 12 oranges, 6 grapefruits, 2 citrumelos and 1 Cleopatra mandarin were analyzed so far. On the basis of amplification of the appropriately sized DNA, CDVd was detected in thirty one samples and CBLVd in the six grapefruits and one lemon. Analysis of the sequence of amplicons revealed 96% and 97% identity with CBLVd GenBank reference sequences, and 96% to 98% with CDVd GenBank reference sequences. This is the first report of CBLVd and CDVd in citrus trees in Argentina. These results indicate the need to work in exhaustive diagnosis in the certification programs for pathogens that represent a threat to citrus groves and therefore to the regional economy.

Non-technical summary: Citrus Bent Leaf Viroid and Citrus Dwarfing Viroid were detected in citrus trees from northwestern region of Argentina, being this the first report of these pathogens in the country. CDVd was predominant, present in all citrus species and in more than 88% of the samples analyzed. CBLVd was detected only in 20% of the samples, and it was present in all grapefruits tested.

IOCV-04: Citrus Viroids Poster Presentations

IOCV-P4-11

Detection and Mechanical Transmission of Citrus Bent Leaf Viroid From Citrus Varieties in Malaysia

Yasir IFTIKHAR[1], YingWei KHOO[2], Tanes MURUGAN[2], Rabi’atul ADAWIYAH[2], Lih

Ling KONG[3] and Ganesan VADAMALAI[2,3]

[1]Department of Plant Pathology, College of Agriculture, University of Sargodha, Sargodha, Pakistan, [2]Department of Plant Protection, Faculty of Agriculture, Universiti Putra Malaysia, Serdang, Selangor, Malaysia, [3]Institute of Plantation Studies, Universiti Putra Malaysia, Serdang, Selangor, Malaysia

Abstract: Citrus viroids are emerging pathogens in citrus plantation and it's a potential threat to the citrus industry worldwide. It is found in low concentration and their expression may be symptomatic or non-symptomatic in different citrus species. Some of the symptoms associated with citrus viroids include wood pitting, bark scaling, leaf epinasty, leaf bending and stunting. Citrus viroids have been classified into four genera of the *Pospiviroidae* family. Citrus exocortis viroid (CEVd), citrus bent leaf viroid (CBLVd), citrus dwarfing viroid (CVd-III), citrus viroid V and VI (CVd-V and CVd-VI), hop stunt viroid (HSVd) and citrus viroid IV (CVd-IV). A study was carried out under the TWAS-UPM fellowship program to detect citrus viroids in citrus growing areas in Malaysia. Leaf samples were collected citrus plants exhibiting viroid-like symptoms and also non- symptomatic plants from five different places viz., Kajang, Muar, Taiping, Sabak Bernam and Kuantan during a limited survey. CBLVd was characterized from citrus varieties in Malaysia showing leaf bending, stunting and also from non-symptomatic trees. CBLVd was detected in *Citrus aurantifolia*, *C. hystrix* and *C. sinensis* through RT-PCR using a CBLVd specific primer. Amplicons of approximately 328 nt was cloned and sequence analysis of the clones showed 100% sequence identity to CBLVd variant, Jp isolate (GenBank Acc no: AB006734), reported from Japan. Six months old seedlings of *C. micocarpa* inoculated with sap extracted from CBLVd positive citrus leaves showed symptoms of smalling of leaves, leaf bending and mild mosaic after 4 to 6 months upon inoculation. The presence of CBLVd in the inoculated seedlings were re-confirmed through RT-PCR using CBLVd specific primers.

Non-Technical summary: Citrus viroids are emerging threat to citrus production. They are widely distributed and potential threat to the citrus industry. Citrus viroids have their role in declining of citrus orchards, either alone or in combination with citrus virus. Origin of the citrus viroids are not well elucidated but they have been detected and characterized from different citrus growing areas of the world.

Citation:

Y.W. Khoo, Y. Iftikhar, T. Murugan, N.A. Roslin, R. Adawiyah, L. L. Kong and G. Vadamalai. 2017. First report of Citrus bent leaf viroid in Malaysia. Journal of Plant Pathology, 99 (1), 287-304

IOCV-P4-12

Transcriptome sequencing reveals novel *Citrus bark cracking viroid* (CBCVd) variants from citrus and their molecular characterization

Yafei WANG [1, 2], Sagheer ATTA [3], Zhiyou XUAN [1,2], Xuefeng WANG [1, 2], Fangyun YANG [1], Changyong ZHOU [1, 2], and **Mengji CAO** 1, 2

[1] National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing, China; [2] Academy of Agricultural Sciences, Southwest University, Chongqing, China; [3] Department of Plant Protection, Faculty of Agricultural Sciences, Ghazi University, Dera Ghazi Khan, Pakistan

Abstract: *Citrus bark cracking viroid* (CBCVd), previously called *Citrus viroid IV*, belongs to the genus *Cocadviroid* within the family

Pospiviroidae. It has been identified as an important causative agent in citrus and hops. In the present work, we obtained the full-length genomes of different variants of all known citrus viroids from Pakistan through transcriptome sequencing. However, the CBCVd variants were found in Pakistan for the first time. These newly discovered variants were provisionally named “CBCVd-LSS” for their low sequence similarity (80.9%–88.9%) with the CBCVd RefSeq sequence (NC_003539). Interestingly, the two most predominant variants had closest sequence identity (90.6% and 87.9%, respectively) with two isolates from hops. Besides, considering geographical relationships between Pakistan and China, we also collected different variants including those reported, in China, for further comparisons. The length of CBCVd isolates from China ranged from 282 to 286 nucleotides, while those from Pakistan ranged from 273 to 277 nucleotides. Based on genetic diversity and phylogenetic relationships, two main clades in CBCVd were identified and further divided into six sub-clades representing for Pakistan, China, and other countries. Furthermore, sequence analysis revealed the nucleotide changes i.e. mutations between these sub-clades, some of those significantly affected the primary and secondary structure of the viroid. In summary, our results indicate that the CBCVd sequences from Pakistan and China were significantly different with respect to genome and secondary structure and Pakistan might be one of the independent geographical origins of CBCVd worldwide.

Non-Technical Summary: Our study identified and provided an initial characterization of novel Pakistani CBCVd variants called “CBCVd-LSS” on citrus cultivars by transcriptome sequencing. They are named for their low sequence similarity to the CBCVd RefSeq sequence (NC_003539). We also reported the molecular characterization of CBCVd from citrus in China and found that the CBCVd sequences from China and Pakistan differed significantly in genome and secondary structure. Based on genetic diversity and phylogenetic analysis, we deduced that Pakistan might be one of the independent geographical origins of CBCVd worldwide.

IOCV-05: Citrus Tristeza Oral Presentations

Development and Evolution of the CTV Vectors

Choaa EL MOHTAR and Bill DAWSON

University of Florida, Citrus Research and Education Center, Lake Alfred, FL 33850.

Abstract: Since first developing a transient expression vector based on *Citrus tristeza virus* (CTV), it has developed into unexpected applications. Initially, an infectious cDNA clone of CTV was produced in order to do reverse genetics to examine functions of the viral genes. Once you have an infectious cDNA, you might as well take some things out or put things in to see what happens. One thing to put in was the green fluorescent protein (GFP) from jellyfish, which allowed us to follow the movement of the virus, which changed our whole perspective. If the virus allows you to express GFP, you now have a vector. Surprisingly, the CTV vector was unusually stable, with most infected citrus trees expressing GFP for more than ten years. With the invasion of HLB into Florida, it became evident that the vector could be used as a bandaid to more quickly manage the disease in the field if an efficacious protein or peptide was found to limit the bacterial pathogen. A patent was licensed to Southern Gardens Citrus Inc. who has been taking the vector through the permitting processes for commercial use. Subsequently, it was discovered that the vector could be used to induce RNAi in psyllids to prevent development of progeny and presently the vector is being used to identify genes for potential mutation by CRISPR technologies.

Non-Technical Summary: Developing CTV vectors has not been trivial and we discovered retroactively that the first infectious clone based on CTV T36 benefited from a series of fortuitous events. The El Mohtar group has been creating new CTV vectors with additional advantages based on the VT, T30, and T68 strains, but without these fortuitous events. Although sequencing is easier and DNAs can be purchased making cloning straightforward on paper, CTV always provides non-intuitive results that will be discussed.

IOCV-O5-01

New Transient Expression Vectors Based on *Citrus tristeza virus* (CTV) Strains VT and T30

Choaa EL MOHTAR, Turksen SHILTS, Cecile ROBERTSON, Carmen BIERMAN and William O. DAWSON

Department of Plant Pathology, University of Florida, Citrus Research and Education Center, Lake Alfred, Fl 33850

Abstract: CTV is endemic in Florida citrus. Most infected trees have CTV isolates composed of more than one genotype, mainly from the T36 and T30 strains. T36 isolates are invisible in commercial citrus unless it is on sour orange rootstock on which trees decline and die. Strain T30 isolates cause no symptoms, even in trees on the sour orange rootstock. Isolates of the VT strain occur sporadically in commercial citrus. Previously, we created an infectious cDNA clone of the type isolate of T36 that was developed into a transient expression vector as a tool for citrus biology and is widely used to express genes and RNAi inducer sequences for activity against the bacterial pathogen of Huanglongbing (HLB) and its Asian citrus psyllid (ACP) vector. Because of the unusual stability of the CTV vector in trees and the relatively short time required for its deployment, it is being considered as an interim measure to manage HLB/ACP in the field. However, this vector has some short- comings. It cannot be used in trees on the sour orange rootstock and therapeutic treatment of field trees already infected with isolates of the T36 strain would prevent superinfection. We report the creation of new CTV-based transient expression vectors from the T30 and VT strains. The T30-based vector can be used on trees on the sour orange rootstock and maintains higher titers in summer heat. The vector based on the rarely found VT isolate is designed for therapeutic treatment of field trees already infected with the isolates from the common isolates of T30 and T36. This now provides a range of tools to be used to combat HLB/ACP under different conditions as well as understand the interaction among/between the different strains in different citrus hosts.

Non-Technical Summary: CTV isolates from citrus hosts exist mostly as a mixture of different genotypes referred to as strains. Each strain/isolate has its own unique biological characteristics in the different host species. In this work, we report our ability to manipulate two new strains of CTV to transiently express a sequence of interest in the citrus phloem tissue. In addition to T36, the new T30 and VT CTV transient expression vectors would complement each other under different conditions to express therapeutic sequences targeting the bacterial causal agent of HLB and the ACP insect vector that spreads it among trees.

IOCV-O5-02

New tools to study viral gene expression and subcellular localization in citrus plant cells

Amit LEVY[1,2], Chunxia WANG[1], Choaa EL-MOHTAR[1,2], Vladimir ORBOVIC[1]

[1] Citrus Research and Education Center, University of Florida, Lake Alfred, USA, [2] Department of Plant Pathology, University of Florida, Gainesville, USA

Abstract: To understand how citrus viruses, such as the *citrus tristeza virus* (CTV), replicate and move inside the plant, it is critical to study the cellular interactions and localization of their encoded proteins. However, so far these studies were only conducted in the non-natural host *Nicotiana benthamiana*, since no efficient transient expression system was available for citrus. Particle bombardment is a physical method to deliver nucleic acid and other biomolecules directly into the cells. The Helios® Gene Gun is a hand-held device that uses a low-pressure helium pulse to accelerate high density, sub- cellular sized particles into a wide variety of targets for *in vivo* and *in vitro* applications. Here we show that this method can be used for robust transient expression in citrus in various tissues. To further expand the toolset for functional assays in citrus, we also generated a new set of transgenic citrus organelle marker lines, with markers to the nucleus, actin and ER. Using these resources, we were able to study the localization of the coat protein of CTV in its natural citrus host.

Non-Technical Summary: In order to understand how viruses behave inside their citrus hosts, it is important to understand their cellular activities. We have optimized a method for robust transient gene expression in citrus, and developed a set of citrus organelle markers. Using these resources, viral protein activity and subcellular localization can be studied in citrus rapidly and in high throughput.

IOCV-O5-03

Variants of the CTV T68 strain differ in stem pitting expression in grapefruit

Glynnis COOK[1*], Beatrix COETZEE[2], Johan T BURGER[2] and **Hans J MAREE**[1,2]

[1]Citrus Research International, Nelspruit, South Africa
[2]Department of Genetics, Stellenbosch University, Stellenbosch, South Africa

Abstract:
Mild strain cross-protection was applied in South Africa as a means to retain the commercial viability of grapefruit that was severely affected by citrus tristeza virus (CTV) stem pitting. An isolate, GFMS12, was effective against natural challenges, but pre- immunised grapefruit trees, planted in humid, hot regions succumbed to severe stem pitting. Some ‘Star Ruby’ grapefruit bud-wood source trees also developed severe stem pitting which was suggestive of a segregation event within the CTV population. Separation of T68 strain variants, derived from the original GFMS12 source, displayed diverse stem pitting severity. Both severe and mild stem pitting T68 isolates were biologically characterised and full-genome sequences determined by Sanger sequencing and independently verified by NGS. NGS reads were mapped to the Sanger sequences and *de novo* assemblies done. The genome of the two variants differed at only 39 nucleotide positions. Variants of the T68 strain were diagnostically differentiated by RT-PCR and Sanger sequencing and their effects on stem pitting in grapefruit were investigated in a glasshouse and in an existing field trial. The existence of quasispecies, best explains the isolation of these genetic variants of T68 from GFMS12. This is the first report of the characterisation of CTV variants of a strain, originating from the same parental population and which display altered stem pitting severity.

Non-Technical Summary:
An understanding of the CTV components involved in stem pitting in grapefruit is required to correctly apply effective cross- protection and to address failures encountered with the management strategy. Different CTV populations of a strain were found to develop and influence stem pitting in grapefruit.

IOCV-O5-04

Preinfection of citrus by RB strains of *Citrus*

tristeza virus (CTV) negatively affected expression of a modified T36 CTV vector

Vijayanandraj SELVARAJ[1], Yogita MAHESHWARI[1], **Subhas HAJERI[2]**, Raymond YOKOMI[1]

[1]USDA, ARS, SJVASC, Parlier, CA; [2]CPDP, CCTEA, Tulare, CA

Abstract: Genetically modified T36 *Citrus tristeza virus* (T36-mCTV) is considered by APHIS to be a biological control organism intended to help manage citrus greening disease without genetically engineering citrus trees in Florida. For research purposes, the T36-mCTV vector, clone 527, which carries a complete green fluorescent protein (GFP) gene and a truncated citrus phytoene desaturase (tPDS) gene, was imported and established in a quarantine greenhouse in California. Since California would be interested to test field application of mCTV, research was conducted to determine if pre- existing California wild-type CTV isolates would affect super-infection of the T36- mCTV vector. *Citrus macrophylla* preinfected with CA wild-type (wt) CTV isolates representing the VT, T30, S1, RB and T36 genotypes were challenge-inoculated with T36-mCTV to examine coinfection and expression of mCTV vector. As expected, CA-wt-T36 prevented superinfection by T36-mCTV by super-infection exclusion phenomenon, and T30, S1, and VT did not prevent establishment or expression of T36-mCTV. Surprisingly, Resistance-Breaking (RB) strains significantly reduced titer and GFP expression of T36-mCTV. Moreover, RB strains prevented photo bleaching symptoms induced by T36-mCTV as compared to control treatments. The data showed that RB strains induced some degree of cross-protection to T36-mCTV, suggesting that pre-existing infection of RB strains can reduce efficacy of the T36-based mCTV gene expression and its potential control of HLB. Therefore, another genotype of a mild mCTV strain, not limited by the RB strain, should be considered for field application.

Non-Technical summary: Modified (m) infectious clones of *Citrus tristeza virus* is showing promise to mitigate HLB in Florida. We obtained a T36-mCTV from Florida carrying several reporter genes and experimentally inoculated it into *Citrus macrophylla* (Cmac) to examine expression of these reporter genes. Replicated treatments included Cmac alone or coinfected with various wild type strains California CTV including T36, VT, S1 and RB genotypes. As expected, T36-mCTV expressed reporter genes alone or in combination with VT, and S1 CTV strains but not with T36 as was predicted by genotype superinfection exclusion by the same genotype. However, limited expression of the T36-mCTV was observed in Cmac coinfected by RB genotypes suggesting that this genotype could reduce efficacy of T36-mCTV to control CLas and ACP.

IOCV-O5-05

Current situation of citrus tristeza disease in the Cuban citriculture

Yilian LLANES-ALVAREZ[1]*, Inés PEÑA-BÁRZAGA[1], Lester HERNÁNDEZ-RODRÍGUEZ[2], Fernando RIVAS-GRELA[2], Lochy Batista-Le RIVEREND[1], Daylé LÓPEZ-HERNÁNDEZ[3], Ronal PACHECO-SÁNCHEZ[4], Victoria ZAMORA-RODRÍGUEZ [1].

[1] Instituto de Investigaciones en Fruticultura Tropical (IIFT), La Habana, Cuba. [2] Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Salto Grande, Salto, Uruguay. [3]Empresa Agroindustrial Ceballos, Ciego de Ávila, Cuba. [4]Instituto de Biotecnología Universidad Nacional Autónoma

de México, Cuernavaca, Morelos, México.

Abstract: Citrus tristeza is the viral disease that causes the highest economic losses to the citrus industry worldwide. In Cuba, since the detection of citrus tristeza virus (CTV), its causal agent, and *Toxoptera citricida* (Kirkaldy), its main aphid vector, a series of measures was implemented to manage the disease. The detection of huanglongbing (HLB) has had a great impact on Cuban citrus, introducing technological changes that influence the epidemiology of CTV. The objective of this work was to determine the current situation of tristeza in Cuba through the partial characterization of the predominant isolates. A CTV collection was developed with isolates collected through a survey in the main Cuban citrus regions for their biological, serological, and molecular characterization. Symptoms induced by the studied isolates in indicator plants were mostly mild. In the serological characterization tests with a panel of monoclonal antibodies, some isolates reacted with the MCA13 MAb, capable of recognizing severe CTV isolates. Phylogenetic analyzes of the predominant sequences of the p20, p23 and p25 genes of the isolates, placed them in individual branches with the homologous sequences of the T30- like CTV genotype. The results suggest the presence of severe isolates among the populations, although the majority of isolates are mild and of the T30 genotype. The present work demonstrates the need to continue surveillance and update disease management strategies to avoid the emergence of new epidemics caused by severe isolates of CTV.

Non-technical summary: Citrus tristeza, caused by the Citrus tristeza virus (CTV), is the most damaging virus disease of citrus cultivation in Cuba. The detection of huanglongbing (HLB) has resulted in changes in cultural techniques that may influence CTV epidemiology. A survey was conducted of the main Cuban citrus producing areas and CTV isolates were collected and characterized biologically, serologically, and molecularly. Most isolates were of a R30-like genotype and produced mild symptoms in biological indicators. Some isolates reacted with the MCA-13 Mab, indicating a more severe isolate. The results indicate the need for continued vigilance in surveying and managing CTV.

IOCV-O5-06

Characterization of California *Citrus tristeza virus* (CTV) strains that react with CTV MCA13 monoclonal antibody

Raymond YOKOMI[1] and Subhas HAJERI[2]
[1]USDA-ARS, SJVASC, Parlier, CA, USA;
[2]CPDP, CCTEA, Tulare, CA, USA

Abstract: In California, reactivity of *Citrus tristeza virus* (CTV) isolates to CTV monoclonal antibody MCA13 is rare and if an isolate tests positive, suggests it may be exotic, potentially severe and, hence, subject to regulatory action. Full-length sequencing of several California MCA13-reactive isolates revealed that they were grouped in three different CTV genotypes: VT, RB, and S1 (1,2). Greenhouse virus indexing showed that the VT strains were virulent but the RB and S1 strains were mild. The Dekopon isolate fell into the CTV Western VT group. RB strains fell in two CTV RB subgroups: Group 1 (similar to New Zealand isolates) and Group 2 (similar to Asian-Pacific isolates). Two isolates formed a new genotype named S1. Isolates from these genotypes were present alone or in combination with other genotypes. Sequence analysis provided strong evidence that the RB and S1 strains originated as recombinants. RT-qPCR primers and probes were designed and validated to identify and differentiate these strains. This research provided important data on the genetic diversity CTV in California and suggested that CTV testing by MCA13 should be supplemented with strain-specific RT-qPCR markers to more accurately determine potential virulence.

Non-Technical summary: Several MCA13-reactive isolates of *Citrus tristeza virus* (CTV) collected from citrus orchards in central

California were characterized by its full- length genome sequence and virulence by greenhouse virus indexing. The Dekopon source plant carried a CTV VT genotype and indexing showed it caused severe seedling yellows and stem pitting. Two other genotypes were found: RB (resistance-breaking) and a new genotype called S1. Both RB and S1 were mild. Molecular markers were developed for RT-qPCR to distinguish these genotypes and generate information which could be incorporated into management strategies to control CTV diseases.

Citations:

Yokomi, R.K, Selvaraj, V., Maheshwari, Y., Saponari, M., Giampetruzzi, A., Chiumenti M., and Hajeri, S. Identification and characterization of *Citrus tristeza virus* isolates Breaking Resistance in trifoliate orange in California. Phytopathology 107:901-908

Yokomi, R, Selvaraj, V., Maheshwari, Y., Chiumenti, M., Saponari, M., Giampetruzzi, A., Weng, Z., Xiong, Z. and Hajeri, S. 2018. Molecular and biological characterization of a novel mild strain of *Citrus tristeza virus* in California. Arch. Virology 163: 1795-1804

IOCV-O5-07

Genotyping CTV isolates by sequential RT-PCR and microarray hybridization in a miniaturized lab-on-chip (LoC) device

Grazia LICCIARDELLO[1], Giuseppe SCUDERI[1], Marcella RUSSO[1] and **Antonino CATARA**[2]

[1]Agrobiotech soc. Coop., Catania, Italy, [2] Science and Technology Park of Sicily, Catania, Italy

Abstract. Surveillance of the genetic diversity of Citrus tristeza virus (CTV) isolates is one of the key points for the management of tristeza disease (1). Biological indexing and deep sequencing of small RNAs, widely considered as the gold standard for assessing the biological and genetic genome phylogeny, are unsuitable and/or too expensive for intensive monitoring. In this study, a commercial miniaturized silicon lab-on-chip (LoC) device (Verechip™, Veredus Laboratories, Singapore) has been customized for fast, simultaneous and automated identification of CTV genotypes. The device, already developed for human and food pathogens (2), integrates a PCR microreactor fluidically connected with a customable microarray area where the hybridization takes place. The CTV LoC system enables sequential RT-PCR and microarray hybridization of amplicons by using a set of 12 primers and 44 probes (x 2 replicates) targeting variable genomic regions of six genes (5'UTR, ORF1a, RdRp, p33, p20 and p23). Briefly, the procedure includes total RNA isolation from bark tissues and young leaves, two quadruplex RT-PCR reactions, microarray hybridization, signal optical detection and spot image analysis, automatically driven by a software. Preliminary tests were carried out in comparison with conventional biological and molecular assays after the *in silico* validation of primers and probes (3). The protocol was standardized by testing eight full genome sequenced CTV isolates representative of genotypes VT, T30, T36, T3 and T68. Subsequent tests of 30 isolates from Italy, China and South Africa confirmed the potential of the system to distinguish local and exotic strains, as well as single or mixed infections, based on the number of probe hybridization, which varies according to the isolate genotype and the differences among the strains. The platform allows to process five samples in less than three hours.

Non-technical summary. CTV is still a limiting factor for the citriculture in many regional areas. The monitoring of its genetic population remains a priority measure to prevent the spread of new or exotic destructive isolates. Unlike available methods, LoC provides a comprehensive and very fast "one step genotyping". It appears suitable for the development of a simple and rapid diagnostic point-of-care, for routine surveillance as well to reveal new outbreaks. Moreover, it helps to expand the knowledge on the diversity of CTV isolates tested.

Citations:

EFSA PLH (EFSA Panel on Plant Health), Jeger M, Bragard C, Caffier D, Dehnen-Schmutz K, Gilioli G, Gregoire J-C, (…..) Candresse T (2017) Scientific Opinion on the pest categorisation of Citrus tristeza virus (non-European isolates). EFSA Journal 2017;15(10):5031, 29 pp.

Teo J, Di Pietro P, San Biagio F et al (2011) VereFluTM: an integrated multiplex RT-PCR and microarrayassay for rapid detection and identification of human influenza A and B viruses using lab-on- chip technology. Arch Virol 156:1371- 1378.

Scuderi G, Lombardo A, Raspagliesi D et al (2016) Development and evaluation of a novel probe microarray for genotyping Citrus tristeza virus using an integrated lab-on-chip device. J Plant Pathol 98:25- 34.

IOCV-05: Citrus Tristeza Poster Presentations

IOCV-P5-13

A survey of *Citrus tristeza virus* RB strainin Gannan district of Jiangxi province, China

Min DING[1], **Aijun HUANG**[1, 2], Yilin XIA[1], **Long YI**[1, 2]

[1]Gannan Normal University, Ganzhou, P.R.China, [2]National Navel Orange Research Center, Ganzhou, P.R.China

Abstract: *Citrus tristeza virus* (CTV) is one of the most economically destructive pathogens that affects citrus trees around the world. Seven distinct genetic lineages or genotypes strains of CTV have been identified. One of them called resistance- breaking (RB) genotype is capable of overcoming the trifoliate-rootstock resistance[1]. The RB strain has been reported in the Provinces of Zhejiang and Hunan in China[2,3]. Ganzhou, also called Gannan district, is the largest navel orange production area in China. The majority of navel orange trees are grown on trifoliate rootstock in Ganzhou. Due to the potential risk to the Gannan navel orange production, the incidence of the RB strain was investigated. A total of 381 samples were collected from navel orange orchards in 10 counties in Gannan. Of these samples, 122 (32.0%) were detected to be positive for CTV. The incidence of CTV in all surveyed counties is between 6.7% and 54.5%. In order to determine whether the RB strain existed in these samples, an RT-PCR strain-specific detection assay was carried out. Fragments of the expected size were amplified in 18 samples (18/381, 4.7%) from 5 of the counties, indicating the presence of the RB strain in Gannan. Further confirmation was executed by deep sequencing one of the suspected RB infected sample. Results showed co-infection of 5 isolates of CTV in the sample, and one isolate showed high nucleotide identity (94%) to RB isolate CA- RB-AT25 (GenBank accession number KU356770). Our conclusion is that the CTV RB strain has spread into the Gannan citrus- growing area. This should be taken into consideration when developing disease management strategies.

Non-Technical Summary: The Gannan district of Jiangxi province is the largest navel orange producing area in China. Trifoliate rootstock which was considered to have a high degree of resistance to CTV is widely used in this area. The CTV RB strain can overcome this resistance, so it may cause serious damage to navel orange production. Our results indicated that the RB strain has occurred in some districts of Gannan. Though the infection incidence is low, it should be paid more attention in CTV management strategies.

Citations:

Dawson TE, Mooney PA. 2000. Evidence for trifoliate resistance breaking isolates of Citrus tristeza virus in New Zealand, Proceedings of the 14th Conference of the International Organization of Citrus Virologists. IOCV, Riverside, CA.

Cheng XF, Wu JX, Ma XY, Fu HF, Wu XY. 2011. First report of *Citrus tristeza virus* in citrus Changshanensis cv. Huyou in Zhejiang, China. Journal of Plant Pathology, 93(S4): 68.

Xiao C, Yao RX, Dai SM, Licciardello G, Catara A, Gentile A, Deng ZN. 2017. Population structure and diversity of citrus tristeza virus (CTV) isolate in Hunan province, China. Archives of Virology, 162(2): 409-423.

IOCV-P5-14

Aggressiveness and genetic diversity of the *Citrus tristeza virus* population in single Uruguayan isolates segregated after single aphid transmission

Lester HERNÁNDEZ-RODRÍGUEZ[1], Ana BERTALMÍO[1], Leticia RUBIO[1], María J. BENÍTEZ-GALEANO[2],Ana ARRUABARRENA[1], Fernando RIVAS[1], Rodolfo ROLÓN[1], Rodney COLINA[2] & Diego MAESO[3]

[1]Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Salto Grande. Camino al Terrible, CP 50000, Salto, Uruguay. [2]Laboratorio de Virología Molecular, Centro Universitario Regional Noroeste (CENUR Noroeste), Universidad de la República, Rivera 1350, CP 50000 Salto, Uruguay. [3]Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Las Brujas. Rincón del Colorado. Canelones, Uruguay.

Abstract: Prevalence of *Citrus tristeza virus* (CTV) isolates causing severe stem pitting symptoms and the high diversity in its genetic composition represent a threat for the development and sustainability of citrus in Uruguay. The goal of this work was to characterize viral components in the CTV population of six field isolates segregated by single aphid (*Toxoptera citricida*) transmission (SAT). Thirty-nine SAT-derived sub-isolates were obtained from six CTV isolates collected at commercial orchards in Uruguay. Only eight of the thirty-nine were MCA13 non-reactive. Six of the eight MCA13 non-reactive sub-isolates and three MCA13 reactive ones, were considered mild CTV isolates defined by the intensity of their reaction on 'Mexican' lime, 'Madam Vinous' sweet orange, 'Duncan' grapefruit and sour orange. Fourteen SAT sub-isolates, including two MCA13 non-reactive, were considered moderate isolates, and the rest were all MCA13 reactive with severe reactions in the four inoculated indicator hosts. Sequence comparisons of the p20, p23 and p25 genes showed a high inter (among all isolates and their derived sub-isolates)- and intra (among each isolate and its derived sub-isolates)- specific genetic diversity among isolates and sub-isolates, with the presence of the CTV strains VT, T68, T3, RB, NC and T36 in mixtures of their complex populations. The results obtained in the molecular analysis, bioassays and serology of the obtained SAT sub-isolates revealed the heterogeneity of the populations inside these six CTV field isolates. These results provide additional biological and molecular information of the complex CTV populations circulating in the country.

Non-technical Summary: Single aphid transmission allowed separation of mixtures followed by the study of CTV populations of Uruguayan field isolates. We identified several viral complexes composed of mild and severe components with high genetic diversity. Molecular and biological properties of the CTV isolates circulating in Uruguay revealed an important risk that could enhance the genetic diversity background of CTV populations.

IOCV-P5-15

Applying *Citrus tristeza* virus clones to understand stem pitting development in citrus

D.J. ALDRICH[1, 2], R. BESTER[1, 2], G. COOK[3], J.T. BURGER[1] and H.J. MAREE[1,3]

[1]Department of Genetics, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

[2]Agricultural Research Council, Infruitec-Nietvoorbij: Institute for Deciduous Fruit, Vines and Wine, Private Bag X5026,Stellenbosch 7599, South Africa

[3]Citrus Research International, PO Box 28, Nelspruit 1200, Mpumalanga, South Africa

Abstract: Citrus tristeza virus (CTV) has greatly influenced the course of the citrus industry. CTV-induced stem pitting causes significant economic losses, negatively impacting grapefruit and sweet orange vigour and yield. The exact mechanisms of stem pitting development in CTV-infected citrus remain unknown. This study will utilise various CTV infectious clone mutants in a reverse genetics approach to study this disease complex in more detail. The specificity of disease induction in citrus, coupled with the spectrum of different stem pitting phenotypes, suggests different host-pathogen interactions leading to stem pitting in different citrus hosts. It is possible that the stem pitting determinants could be in the CTV genome, the plant genome, or likely a complex interaction of both. Stem pits will be studied at the microscopic anatomical level to better understand stem pit morphology and to evaluate the effect of stem pits on the cambium layer and the differentiation of phloem-associated cells. Gene expression data will also be generated to identify the plant’s response to the various CTV infectious clones constructed in this study. Several green fluorescent protein-tagged CTV clone mutants have been constructed thus far and were shown to be infectious in *Nicotiana benthamiana*. These infectious clones will subsequently be applied to study host- pathogen interactions in different citrus species. High-resolution nano-CT scans of stem pits were generated to better understand the aberrant cellular differentiation in these areas. Elucidating the citrus-CTV interactions leading to stem pitting in grapefruit would aid in refining cross protection strategies in South Africa and ultimately limit economic losses.

Non-Technical summary: This study aims to utilize *Citrus tristeza virus* (CTV) infectious clones to elucidate mechanisms involved in CTV-induced stem pitting in citrus, which have yet to be resolved. We aim to study this interaction from the perspective of the virus, by manipulating genes implicated in stem pitting development, as well as the host plant, in order to obtain a more holistic view of the citrus-CTV interactions underlying stem pitting development. This knowledge can be used to refine the application of CTV cross- protection.

Citations:

Moreno, P., Ambrós, S., Albiach-martí, M.R., Guerri, J., Peña, L., 2008. Citrus tristeza virus: a pathogen that changed the course of the citrus industry. Mol. Plant Pathol. 9, 251–268. doi:10.1111/j.1364-3703.2007.00455.x

Tatineni, S., Dawson, W.O., 2012. Enhancement or Attenuation of Disease by Deletion of Genes from Citrus Tristeza Virus. J. Virol. 86, 7850–7857. doi:10.1128/JVI.00916-12

Dawson, W.O., Garnsey, S.M., Tatineni, S., Folimonova, S.Y., Harper, S.J., Gowda, S., 2013. Citrus tristeza virus-host interactions. Front. Microbiol. 4. doi:10.3389/fmicb.2013.00088

IOCV-P5-16

Biological characterization of the first RB (resistance breaking) isolate of *citrus tristeza virus* identified in Uruguay

Leticia RUBIO[1*], Lester HERNÁNDEZ-RODRÍGUEZ[1], Ana BERTALMÍO[1], María J. BENÍTEZ-GALEANO[2], Ana ARRUABARRENA[1], Fernando RIVAS[1], Rodney COLINA[2] and Diego MAESO[1]

[1]Instituto Nacional de Investigación Agropecuaria (INIA), Estación Experimental INIA Salto Grande. Camino al Terrible, CP 50000, Salto, Uruguay.

[2]Laboratorio de Virología Molecular, Centro Universitario Regional Noroeste (CENUR Noroeste), Universidad de la República, Rivera 1350, CP 50000 Salto, Uruguay.

Abstract: *Citrus tristeza virus* (CTV) is the causal agent of the most important viral disease of citrus. In Uruguay, severe CTV isolates with a high genetic variability are prevalent, and symptoms that affect the yield and fruit quality of citrus plants are observed even though resistant rootstocks are used. Recently the presence of the RB (resistance- breaking) genotype was identified, first in partial sequences of p20 p23 and p25 genes, and later a RB sub-isolate was obtained through single aphid transmission from a field isolate. Subsequently, the RB phenotype nature was confirmed by detecting CTV replication in graft-inoculated *Poncirus trifoliate* plants. The presence of the RB strain is relevant since *P. trifoliate* and its hybrids are the most popular rootstocks in Uruguay. The aim of this work was to assess the biological characteristics of the RB local isolate on CTV indicator plants to determine its severity. Bioassays were conducted in a controlled-condition glasshouse. The RB isolate and five other reference isolates, belonging to our local CTV collection, were graft-inoculated onto Mexican lime (ML), sour orange (SO), sweet orange (SW) and Duncan grapefruit (DG). Three seedlings of each indicator species were used and negative and positive controls were included. Symptoms (vein clearing, leaf cupping, vein corking, seedling yellows, stunting and stem pitting) and their intensity were recorded for a year. Results showed that the RB isolate induced mild symptoms such as vein clearing and leaf cupping in ML, compared to severe isolates which induced stem pitting, stunting and seedling yellow in ML, SO and DG.

Non-technical summary: The biological characterization of the RB local isolate in indexing tests, allowed us to classify it as a mild isolate compared to severe isolates in our CTV collection. However, this result does not imply that physiological traits of *P. trifoliata* carrying the RB strain cannot be affected.

IOCV-P5-17

Molecular characterisation of aphid vectors associated with Citrus tristeza virus–infected sweet oranges in Nigeria

Adedapo ADEDIJI[1], **Idara ESSIEN[1]**, Adedoyin ADEYEYE[1], Gabriel ATIRI[1]

[1]Department of Crop Protection and Environmental Biology, University of Ibadan, Nigeria

Abstract: The presence of Citrus tristeza virus (CTV) in Nigeria has been established. However, the relationship with its aphid vectors on citrus hosts grown in various locations is largely unknown. We therefore attempted to characterize the aphid species found on CTV-infected sweet orange plants and also to detect CTV within single aphids. Leaf samples were obtained from CTV- infected *Citrus sinensis* trees in four locations within Nigeria namely Ibadan, Ogbomosho, Benin City and Makurdi. Aphids were also collected from these trees and stored in 95% ethanol. Nucleic acid extracts were extracted from both leaves and aphids while the presence of CTV was tested using specific primers via RT-PCR. The PCR products of the cytochrome oxidase subunit I (COI) gene was amplified from the aphids, cloned and

Sanger- sequenced. Results showed 97– 99% identities with *Toxoptera citricida* COI genes when aphid sequences were matched using BLASTn on the NCBI GenBank database. Nucleotide comparisons also revealed 92.6–96.8% similarities among the COI sequences obtained from all four locations while RT- PCR confirmed CTV within the sweet orange leaves and single aphids tested. Although CTV is transmitted by several aphid species, this study revealed the prevalence of *T. citricida* as the most predominant aphid species on sweet orange in Nigeria. Naturally,
T. citricida remains the most efficient aphid vector of CTV and this information will be useful in understanding the epidemiology of CTV with possible implications for vector control towards overall CTV management in Nigeria.

Non-Technical Summary: Citrus tristeza virus (CTV) is known to be transmitted by several aphid species. Thus, we investigated the molecular characteristics of aphid species found on CTV-infected sweet oranges in Nigeria. Our results confirmed the occurrence of the brown citrus aphid (*Toxoptera citricida*) and also detected CTV in the leaves and within single aphids. This study characterized, for the first time, aphid species on a citrus host in Nigeria. This is vital towards understanding CTV epidemiology in Nigeria for effective disease management.

IOCV-P5-18

Overexpression of miR171b and pre-ctr-miR171b-based artificial microRNA targeting the CTV p23 gene confers stable CTV resistance in *Citrus maxima*

Yun ZHONG, Chunzhen CHENG, Bo WU, Bo JIANG, Guangyan ZHONG

1.Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, Guangdong, China. 2.Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization//Guangdong Province Key Laboratory of Tropical and Subtropical Fruit Tree Research

Abstract: Gene silencing regulated by small interfering RNAs and artificial microRNAs (amiRNAs) is commonly used to enhance plant resistance to pathogenic viruses. In this study, we assessed the utility of amiRNA- mediated gene silencing for improving the resistance of *Citrus maxima* to citrus tristeza virus (CTV). The designed amiRNAs targeted conserved sequences in three CTV RNA silencing suppressor genes. Seven amiRNA-overexpression constructs were subsequently obtained by replacing the miR171b/miR171b* in pre-ctr-miR171b with amiRNA/amiRNA* sequences. Thirteen transgenic *C. maxima* lines were produced following an *Agrobacterium tumefaciens*-mediated transformation. One amiR23-1 transgenic plant was free of CTV at 1 year after inoculations, implying the amiRNA-mediated gene silencing induced CTV resistance in this plant. Additionally, CTV was undetectable in the miR171b-overexpressing transgenic plants at 1 year after inoculations, implying miR171b protected plants from CTV, while also influencing plant growth and development. The data presented herein may provide the basis for the application of miR171b- and amiRNA-mediated gene silencing during the breeding of new citrus varieties exhibiting viral resistance.

Non-Technical Summary: The application of poly-amiRNA-mediated *p23* silencing or the use of several amiRNAs targeting different regions of the CTV genome may further improve the efficiency and stability of the observed CTV resistance.

Citations:

López, C., Cervera, M., Fagoaga, C., Moreno, P., Navarro, L., Flores, R., Peña, L., 2010. Accumulation of transgene-derived siRNAs is not sufficient for RNAi- mediated protection against Citrus tristeza virus in transgenic Mexican lime. Mol. Plant. Pathol.

11 (1), 33–41.

Mahale, B.M., Fakrudin, B., Ghosh, S., Krishnaraj P.U., 2014. LNA mediated in situ hybridization of miR171 and miR397a in leaf and ambient root tissues revealed expressional homogeneity in response to shoot heat shock in Arabidopsis thaliana. J. Plant. Biochem. Biot. 23 (1), 93-103.

Saha, A., Bhagyawant, S.S., Parida, M., Dash, P.K., 2016. Vector-delivered artificial miRNA effectively inhibited replication of Chikungunya virus. Antiviral. Res. 134, 42-49.

IOCV-P5-19

Variation of *Citrus tristeza virus* genotype after sweet orange chip-budding on Mexican lime, and possible role of genotype T30 in virus transmission by *Aphis gossypii*

Rocío CAMPS[1], Nicola FIORE[2], Natalia RIQUELME[1], Wilson BARROS[1], **Ximena BESOAIN[1]**

[1]Facultad de Ciencias Agronómicas y de los Alimentos, Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso, Chile.

[2]Facultad de Ciencias Agronómicas, Universidad de Chile, Santiago, Chile.

Abstract: *Citrus tristeza virus* (CTV) was detected in Chile affecting only grapefruit and Mexican lime trees in the north of our country in two main Oases and separate from the central region by the Atacama desert. Up to now no decline syndrome has been observed in Chile affecting citrus orchards. CTV has 7 well known genotypes that vary according to the phylogenetic analysis. The population of genotypes in a host, the species or cultivars that affect it and the species of the rootstock used all influence the symptomatology, expression and severity of the disease. The objectives of this work were to verify how the population of the Chilean CTV isolates vary when are transfered from sweet orange trees to Mexican lime plants by grafting patches; and determine if the population of the CTV genotypes can influence the transmission efficiency of *A. gossypii*. The results showed a variation in the population of genotypes of the same isolate detectable by RT-PCR when being transferred from sweet orange to Mexican lime by means of graft patch. Isolates considered as not aggressive when transferred to Mexican lime appeared the genotype VT, previously considered as an aggressive isolte. In the transmission mediated by *A. gossypii*, the population of genotypes of the donor associated with the T30 genotype influenced the transmission by *A. gossypii*, although if it is mixed with the VT genotype. This work Will contribute to the understanding of how genotypes interact between hosts and their effect on the transmission of the virus through their aphid vectors.

Non-technical summary:

Here we report on the genotypic variation of Chilean *Citrus tristeza virus* isolates after transmission by *Aphis gossypii* from sweet orange to Mexican lime plants. The most notable finding was that isolates previously classified as non-aggressive, genotype T30, after transmission to Mexican lime, we found that were mixed with the aggressive genotype VT.

IOCV-P5-20

Wide segregation of Stem Pitting and other CTV symptoms in a hybrid population derived from West Indian lime

Malcolm SMITH[1], Toni NEWMAN[1], Debra GULTZOW[1], David INNES[1], Nerida DONOVAN[2], Grant CHAMBERS[2], Patricia BARKLEY[3], Andrew MILES[4], Beatriz STEIN[5]

[1]Queensland Department of Agriculture and Fisheries, Bundaberg Research Station, Australia; [2]New South Wales Department of

Primary Industries, EMAI, Australia; [3]Retired Citrus Pathologist, Mulgoa, Australia, [4]2PH, Emerald, Australia; [5]EEAOC, Tucuman, Argentina

Abstract: West Indian lime (*Citrus ×aurantifolia*) possesses useful traits for breeding, including a short juvenile period and resistance to some important fungal pathogens. However it has seldom been used in breeding programs because of its extreme sensitivity to CTV. In this work we examine whether CTV symptoms, including stem pitting, vein clearing and seedling yellows segregate in a population of hybrids and could therefore be selected against during the nursery phase, prior to field establishment. Hybrids with Meyer lemon (*C. ×meyeri*) expressed CTV symptoms within 6 months of budding onto infected Rough lemon (*C. ×jambhiri*) rootstocks and showed wide segregation in phenotype. This pattern of segregation was not related to the growth or morphology of hybrids, even though traits such as vigour, thorniness, anthocyanin and petiole articulation also segregated widely. All 89 hybrids replicated CTV, but 24% remained free of stem pitting, 40% showed no vein clearing, and 71% did not develop a seedling yellows reaction. Collectively, just 17% of hybrids were completely free of all 3 symptoms. Replicated re-budding of 13 hybrids that displayed a wide diversity of symptom phenotypes, demonstrated that CTV symptom expression was under strong genetic control and could be accurately phenotyped on single trees within 6 months of budding. This suggests a methodology for the efficient and rapid screening of large segregating populations. Early culling of most hybrids (~80%) based on stem pitting, vein clearing and seedling yellows, should enable breeding efforts to quickly focus on desirable traits transmitted from West Indian lime, while continuing to eliminate any CTV-sensitive hybrids with less obvious symptoms.

Non-Technical summary: West Indian lime (also known as 'Mexican' and 'Key' lime) has many useful characteristics, but has been avoided in breeding programs because it is damaged by Citrus Tristeza Virus. We have demonstrated that not all hybrids inherit this problem from their West Indian lime parent and that it may be possible to quickly identify the small percentage of hybrids that remain healthy. A fast and simple technique is suggested that could enable the efficient use of this valuable germplasm in breeding new and better citrus varieties.

IOCV-06: Citrus Yellow Vein Clearing Oral Presentations

Progress in study of *Citrus yellow vein clearing virus*

Yan ZHOU and Changyong ZHOU

Citrus Research Institute of Chinese Academy of Agricultural Sciences / Southwest University, Beibei, Chongqing 400715, P.R.China

Abstract: *Citrus yellow vein clearing virus* (CYVCV), a member of the genus *Mandarivirus*, was first reported from Pakistan in 1988 in lemon and sour orange, followed from Turkey (2002), India (2003), China (2009) and Iran (2017). Now CYVCV is widely distributed in China, symptomless in most field citrus cultivars, causing a huge loss to the lemon industry. Although it could also induce vein clearing in the young spring flushes of a few cultivars of mandarin, tangerine and pumelo, the symptoms were reduced and even disappeared after the leaves were matured. Sequence alignments reveal that there is a very low level of sequence heterogeneity among CYVCV isolates of different geographic origins and hosts. CYVCV is transmitted through the vegetative propagation of infected buds, scions or rootstocks and by mechanical inoculation of sap extracts onto citrus and herbaceous indicators (*Chenopodium quinoa*, *C. amaranticolor*, *Phaseolus vulgarisvar* and *Vigna unguiculata*). A previous study showed that CYVCV could be transmitted by *Aphis craccivora* and *A.*

spiraecola from lemon to bean, and from bean to bean. Recent studies indicated that under controlled conditions, it was also transmitted among lemon trees by *A. spiraecola*, *Dialeurodes citri* and contaminated tools. To date, various detection techniques have been addressed, including biological indexing, immune- chromatographic strip, loop-mediated isothermal amplification, PCR-based techniques and droplet digital PCR assay. CYVCV ultrastructure and associated cytopathology in lemon were also documented. Furthermore, the development of infectious clone lays the foundation for further studies on viral gene functions and virus-host interactions. Basically, the practical measures of controlling citrus Huanglongbing have been adapted to control CYVCV damage to lemon industry in China, and tools for grafting and picking were also required to be decontaminated.

Non-Technical Summary: CYVCV is an emerging citrus virus and now considered to be the most serious pathogen affecting lemon production in a few countries such as Turkey and China. As an opening guidance for CYVCV session, the recent research progresses on its occurrence history, distribution, host range, transmission, diagnosis, genetic variation and control measures were reviewed.

Citations:

Chen, H. M., Li, Z. A., Wang, X. F., Zhou, Y., Tang, K. Z., Zhou, C.Y., Zhao, X.Y., & Yue, J. Q. 2014. First report of *Citrus yellow vein clearing virus* on lemon in Yunnan, China. Plant Disease, 98: 1747.

Loconsole, G., Önelge, N., Potere, O., Giampetruzzi, A., Bozan, O., Satar, S., De Stradis, A., Savino, V., Yokomi, R.K., & Saponari, M. 2012. Identification and characterization of *Citrus yellow vein clearing virus*, a putative new member of the genus *Mandarivirus*. Phytopathology, 102: 1168-1175.

Cui, T.T., Bin, Y., Yan, J.H., Mei, P.Y., Li, Z.A., Zhou, C.Y., & Song, Z. 2018. Development of infectious cDNA clones of *Citrus yellow vein clearing virus* using a novel and rapid strategy. Phytopathology, 108: 1212-1218.

IOCV-O6-01

Evidence for non-transmission of Citrus yellow vein clearing virus by seed

Q. WANG, Y. L. WANG, Z. YANG, M. J. CAO, Y. J. LIU, W. Z. HU, and **Y. ZHOU**

Citrus Research Institute, Southwest University, Chongqing, 400712, P. R. China

Abstract: In 2009, a new citrus viral disease caused by *Citrus yellow vein clearing virus* (CYVCV) was discovered in China and now CYVCV is considered to be the most serious disease of lemon production in China (Zhou et al. 2017). Despite CYVCV being frequently detected in the seed tissues, there is a lack of evidence that CYVCV can be transmitted through seed. Because in a previous study the numbers of seeds tested were low, we further investigated the possibility that CYVCV is transmitted through seed. In November 2016, healthy appearing seeds were collected from mature fruit of 17 CYVCV-infected Eureka lemon (*Citrus limon*) trees at the Citrus Research Institute, Southwest University, China. Approximately 1500 seeds were disinfected with 1% 8- hydroxyquinoline, thoroughly washed with distilled water, and dried at room temperature. Then the seeds were planted in plastic pots with a sterilized soil mix (1/3 sand, 1/3 chaff, 1/3 peat) and seedlings were maintained in an insect-proof greenhouse at 20 – 25 °C. Six and 12 months after germination, total RNAs were obtained from 1267 Eureka lemon seedlings and assayed by RT-PCR with a specific primer pair (sense: 5’-TACCGCAGCTATCCATTTCC-3’ and antisense: 5’-GCAGAAATCCCGAACCACTA-3’) designed from the coat protein gene of CYVCV (Chen et al., 2014). However, CYVCV was not detected in any of the seedlings and none of the seedlings showed

symptoms. In fact, these seedlings looked like and grew at the same rate as control seedlings from healthy mother plants. The present study confirmed that CYVCV is not transmitted through seed.

Non-Technical Summary: Despite CYVCV being frequently detected in the seed tissues, there is a lack of evidence that CYVCV could be transmitted through seed. Because in a previous study, the numbers of seeds tested were low, we further investigated the possibility that CYVCV transmission through seed. However, CYVCV was not detected in seedlings from CYVCV-infected trees and none of the seedlings showed symptoms. In fact, these seedlings looked like and grew at the same rate as control seedlings from healthy mother plants. The present study confirmed that CYVCV is not transmitted through seed.

IOCV-O6-02

Identification of *Dialeurodes citri* as a vector of *Citrus yellow vein clearing virus* in China

Y. H ZHANG[1], C. H LIU[1], **Q. WANG[1]**, Y. L WANG[1], S. HE[2], C. Y ZHOU[1] and Y. ZHOU[1]

[1]Citrus Research Institute, Southwest University, Chongqing, 400712, P. R. China; Academy of Agricultural Sciences, Southwest University, Chongqing 400715, China.

[2]Anyue Lemon Science and Technology Institute, Anyue, Sichuan 642300, China

Abstract: In 2009, a new citrus viral disease caused by *Citrus yellow vein clearing virus* (CYVCV) was discovered in China and now CYVCV is widely distributed in the field. CYVCV is transmissible by grafting and is spread by aphids from lemon to bean, and from bean to bean. However, until now no vector has been shown to transmit CYVCV from citrus to citrus. In this study, after a 24 h acquisition access period (AAP), CYVCV was tested in *Dialeurodes citri* (Ashmead), *Panonychus citri* McGregor, and *Aphis citricidus* (Kirkaldy) by quantitative RT-PCR. After an AAP of 48 h, groups of adults of *D. citri*, *P. citri*, and *A. citricidus* were given a 48 hour inoculation access period on 'Daidai' sour orange seedlings. Three, six, and twelve months post-inoculation by *D. citri*, CYVCV was detected in the receptor plants, and the mean incidence of infected trees was 31.9 %,
39.1 % and 39.1 %, respectively. CYVCV was not transmitted to citrus by *P. citri* or *A. citricidus*. This is the first report of the ability of *D. citri* to transmit CYVCV from infected to healthy citrus under laboratory conditions.

Non-Technical summary: *Citrus yellow vein clearing virus* (CYVCV) was transmitted by *Dialeurodes citri* and the incidence of transmission ranged from 31.9% to 39.1% depending on the sampling time. Therefore, *D. citri* (Ashmead) is probably important for citrus to spread CYVCV in China. In contrast, CYVCV was not transmitted by *Panonychus citri* or *Panonychus citri*.

IOCV-06-03

Infectivity Analysis of CYVCV Infectious cDNA Clones on Different Citrus Cultivars

Yu BIN, Zhen SONG, Jianhong YAN, Yu DUAN, Cuihua LIU, Zhimin MA, and Changyong ZHOU

Citrus Research Institute, Southwest University, Chongqing 400712, P. R. China.

Abstract: In order to analyze the infectivity and simulate the infection process of *Citrus yellow vein clearing virus* (CYVCV) on different citrus cultivars, yeast homologous recombination system was used to construct the full-length genome cDNA infectious clone of CYVCV（pCY-CYVCV-AY221）Nine different citrus cultivars were inoculated with pCY-CYVCV-AY221. The symptoms of the inoculated seedlings were recorded every 25 days. DTBIA

method was used to detect the infectious rate of pCY-CYVCV- AY221 on different citrus cultivars. The total RNAs of the CYVCV-positive plants tested by DTBIA were extracted on the 25th, 50th, 75th and 100th day after infiltration. The RT- qPCR was performed to monitor the relative accumulation of CYVCV. Symptom records suggested that Eureka Lemon [*Citrus limon* (L) Burm. f.] and Feminellolemon [*C. limon* (L) Burm. f.] showed the most serious symptoms with severe yellow vein clearing and leaf distortion in different time periods. Daida (*C. aurantium* L.), Cuixiangtianyou [*C. grandis* (L.) Osbeck], Jincheng (*C. sinensis* Osbeck) showed moderate leaf veining and leaf distortion, and mild vein clearing symptoms were observed on the young leaves of other cultivars which weaken or disappear as the leaves aged. The infectious rate of pCY- CYVCV-AY221 on Eureka Lemon, Daidai, Cuixiangtianyou, Feminellolemon, Symons Sweet orange (*C. sinensis* Osbeck), Rusk Citrange [*Poncirus trifoliata* (L.) Raf. ×*C. sinensis* Osbeck], Jincheng, Oota Ponkan (*C. reticulata* Blanco) and Xinsheng Pengan (*C. reticulata* Blanco) were 100% (8/8), 87.5%（7/8）， 87.5%（7/8）， 75%（6/8）， 75%（6/8）， 62.5% (5/8), 50% (4/8), 37.5%(3/8), and 25% (2/8), respectively. Furthermore, RT-qPCR analysis indicated that the relative accumulation of CYVCV in Eureka lemon was the highest, followed by Jincheng, Feminellolemon, Cuixiangtianyou, Daidai, Xinsheng Pengan, Symons Sweet orange, Oota Ponkan and Rusk Citrange on the 100th day after infiltration. In summary, this study reveals the infectious clone of pCY- CYVCV-AY221 has a high infectivity on different citrus cultivars, and its infectious rate can reach 100% on Eureka lemon. Furthermore, RT-qPCR analysis indicated that the relative accumulation of CYVCV had a positive correlation with the severity of symptoms in the tested citrus cultivars.

Non-Technical Summary: We successfully constructed the full-length genome infectious cDNA clones of *Citrus yellow vein clearing virus* (CYVCV). The research result shows that the infectious clone of CYVCV has a high infectivity on different citrus cultivars, and the relative accumulation of CYVCV had a positive correlation with the severity of symptoms in the tested citrus cultivars. This study would be foundation for the subsequent modification and application of VIGS (virus induced gene silencing) vectors and the research of interaction between CYVCV and citrus.

IOCV-O6-04

Rapid construction of infectious clones of citrus virus based on transformation-associated recombination (TAR) in yeast

Zhen SONG, Tiantian CUI, Yu BIN, Jianhong YAN, Yu DUAN, Zhimin MA and Changyong ZHOU

Citrus Research Institute, Southwest University, Chongqing 400712, P. R. China

Abstract: Infectious clone of citrus virus provide a powerful platform to delve deeper into the nature and function of viruses as well as to engineer viral vectors for protein expression or gene silencing. However, it is difficult to obtain such infectious cDNA clones, which not only reflected in assembling process of long cDNA segments, but also in delivering process into citrus plants. In this study, a ternary Yeast-*E.* coli-*A.* tumefaciens shuttle vector was constructed. One to two cDNA segments covering entire genome of *citrus yellow vein clearing virus* (CYVCV) or *Citrus leaf blotch virus* (CLBV) were amplified by long-distant RT-PCR. 15~30 full-length cDNA clones of CYVCV and CLBV were then developed using transformation-associated recombination (TAR) technology. When Eureka Lemon (*Citrus limon* (L) Burm. f.) and Jincheng (*C. sinensis* Osbeck) seedlings were inoculated with selected full-length cDNA clones of CYVCV or CLBV by *Agrobacterium*- mediated vacuum-infiltration (AVI) strategy, high infection rates was seen to be 80-100% and 13-63%, respectively.

Moreover, the progeny of cloned CYVCV or CLBV elicited typical symptoms on the inoculated Eureka Lemon or Dweet tangor that were no difference to those induced by the corresponding wild type ones. These results suggested that the TAR cloning is a powerful and efficient tool to develop full-length cDNA clones of citrus virus, and AVI of citrus seedlings was an efficient strategy for viral cDNA clone delivering.

Non-Technical Summary: Stable infectious full-length genome cDNA clone of CYVCV and CLBV were constructed using TAR cloning method, and were successfully inoculated directly in citrus seedlings by AVI. This study would be foundation for further study of viral gene function of CYVCV and CLBV and for the development of a gene expression or VIGS vector. In addition, it was also testified that combination of TAR and AVI may be a useful strategy to generate infectious clone of plant viruses rapidly, particularly the viruses with large genomes.

IOCV-06: Citrus Yellow Vein Clearing Poster Presentations

IOCV-P6-21

Development of a sensitive and reliable reverse transcription-droplet digital PCR assay for the detection of *Citrus yellow vein clearing virus*

Yingjie LIU[1,2], Yingli WANG[1,2], **Qin WANG[1,2]**, Yanhui ZHANG[1,2], Wanxia SHEN[1], Sgaoguo HE[3], Mengji CAO[1,2], Changyong ZHOU[1,2] , Yan ZHOU[1,2]

[1] National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing 400712, China; [2] Academy of Agricultural Sciences, Southwest University, Chongqing 400712, China; [3] Anyue Lemon Science and Technology Institute, Anyue, Sichuan 642300, China

Abstract: In 2009, a new citrus viral disease caused by *Citrus yellow vein clearing virus* (CYVCV) was first discovered in China. Now CYVCV is considered to be the most serious disease affecting lemon production. In this study, a more sensitive and reliable reverse transcription-droplet digital PCR (RT- ddPCR) assay was developed to detect and quantify CYVCV without references. Specificity of the assay was determined by the failure of amplification of other relevant viruses and viroid. Quantitative linearity, sensitivity and accuracy of RT-ddPCR for the detection of CYVCV were compared to those of real-time RT-PCR. The results showed that both methods had a high degree of linearity ($R^2 = 0.9776$) and quantitative correlation, but RT-ddPCR showed 100 times higher sensitivity than real-time RT-PCR and could be used to detect CYVCV in single arthropod. In summary, the results demonstrated that the RT-ddPCR assay has the potential for the quantitative detection of CYVCV with high precision and accuracy.

Non-Technical summary: The current practices for the control of yellow vein clearing disease are the use of CYVCV-free propagation materials, removal of symptomatic plants and control vectors. This RT-ddPCR may provide a valuable tool for the detection of the latent infections in citrus and will facilitate the implementation of citrus bud-wood certification program to screen plants in nurseries. The assay will also be useful to study etiology of YVCD, interaction of virus-vector and virus-host and epidemics of YVCD.

IOCV-07: Other Citrus Viruses and Diagnostics Oral Presentations

IOCV-O7-01

California Citrus Nursery Stock Pest Cleanliness Program: High throughput diagnostics, laboratory information and

quality management systems

Sohrab BODAGHI[1], Irene LAVAGI[1], Fatima OSMAN[2], Haoping WANG[1], Georgios VIDALAKIS[1]

[1]University of California, Riverside, USA;
[2]University of California, Davis, USA

Abstract: A high throughput nucleic acid extraction protocol for citrus tissue along with diagnostic assays for the universal detection of citrus viroids and the multiplex detection of citrus viruses have been developed by the Citrus Clonal Protection Program (CCPP). These assays have been approved by the California Department of Food and Agriculture (CDFA) for use in the mandatory “Citrus Nursery Stock Pest Cleanliness Program". Since the implementation of the program in 2010, the CCPP has tested over 22,800 samples (~2,800 per year). Prior to 2010, the voluntary citrus nursery program could test ~500 samples per year. As a result, the viroid infection rate in citrus budwood source trees in more than 35 California citrus nurseries was always relatively high (2004 - 2010: 4.3 - 9.5 %). High throughput diagnostics in combination with best management nursery practices have reduced and maintained citrus viroid finds in citrus nurseries to less than 1% (2015 - 2018: 0.5 - 0.8 %). To facilitate effective and sustainable management testing high numbers of citrus samples, the CCPP diagnostic laboratory has initiated the implementation of the “System for Timely, Accurate & Reliable Diagnostics” (STAR-D) of the National Plant Disease Network (NPDN). STAR-D is based on the ISO/IEC 17025 standards customized to plant diagnostic facilities requirements for pathogen detection. Several members of the CCPP diagnostic laboratory have participated in STAR-D training programs and a Laboratory Information Management System (LIMS) has been installed on the University of California, Riverside servers. LIMS customization has been initiated hat will accommodate CCPP citrus diagnostic protocols as well as the management of samples, personnel workload, protocols, instrument calibration and maintenance, audits and corrective actions, reports, and customer communications. These actions have defined the framework for CCPP’s diagnostic services quality management system (QMS) and have paved the path for the lengthy process of full laboratory accreditation.

Non-technical summary: High throughput citrus diagnostics and quality management systems are critical for accurate and reliable disease testing programs that support a healthy, robust, sustainable and profitable citrus industry. We developed laboratory protocols that allow for multiple pathogen testing of large numbers of samples and incorporated new technologies to improve the quality of diagnostic services.

IOCV-O7-02

Examination of a widespread psorosis-like disease in Hawaiian citrus trees

Alejandro OLMEDO-VELARDE[1], Michael MELZER[1]

[1] Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, Honolulu, USA.

Abstract: In 2015, citrus growers on the island of Hawaii reported symptoms of severe bark peeling and gummosis (excessive exudation of sap). Three years later, similar symptoms were seen in the island of Oahu. The affected trees were tangerine, tangelo, grapefruit, and sweet orange. There are several pathogens that are known to cause these types of symptoms in citrus, the primary one being *Citrus psorosis virus* (CPsV), the causal agent of citrus psorosis. With the aim of determining the identity of the causal agent of this psorosis-like disease in both islands, bark and leaf samples from trees

displaying these psorosis-like symptoms were collected from Hawaii and Oahu in 2017 and 2018, respectively. DNA and RNA were extracted from the samples to test for the presence of seventeen viruses (including CPsV), seven citrus viroids, *Lasiodiplodia* sp. (a fungus associated with a gummosis disease in Texas and Mexico), three bacteria, and phytoplasmas using established PCR-based assays. In addition, a lateral flow assay was used to detect the presence of *Phytophtora* spp. in the samples. The samples were found to be negative for all the pathogens, except for *Citrus exocortis viroid*, *Hop stunt viroid* and *Citrus dwarfing viroid* that were sometimes present, but not clearly associated with the symptomatic trees. To determine if a novel viral or subviral agent was present, double- stranded RNA (dsRNA) from leaves, petioles and green bark was performed, revealing the presence of several dsRNAs ranging from 1 to 20 kilobases in size. These results suggest a viral agent is present in the citrus trees and may represent a novel citrus pathogen or pathogen strain. High-throughput sequencing studies are underway to characterize these dsRNAs and determine if they are associated with this psorosis like disease of citrus in Hawaii.

Non-Technical summary: Citrus trees displaying psorosis-like symptoms have been observed on the islands of Hawaii and Oahu. No known citrus pathogens were detected by PCR-based assays, including *Citrus psorosis virus*, the causal agent that is closely associated with citrus psorosis. Ongoing high- throughput sequencing studies will perhaps help to elucidate a virus or virus-like agent associated with these symptoms.

IOCV-O7-03

Citrus vein enation virus encodes P0 and ORF3 to suppress post-transcriptional gene silencing

Shih-hua TAN[1] and Georgios VIDALAKIS[1]

[1]University of California, Riverside, USA

Abstract: Viral proteins have been known to have multiple functions in virus-host interactions. In this study, we identified the P0 and open reading frame 3 (ORF3), a putative coat protein, of citrus vein enation virus (CVEV) as silencing suppressors of host antiviral RNA silencing using transient expression assays on GFP-transgenic *Nicotiana benthamiana* 16c plants. CVEV uses P0 as a local and systemic silencing suppressor while the ORF3 as a systemic suppressor. When the *Potato virus X* infectious vector harboring either CVEV P0 or ORF3 was inoculated on *N. benthamiana*, both constructs promoted viral infection and symptom development, likely through their RNA silencing suppression activities. Mass spectrometry-based immunoprecipitation proteomics identified CVEV P0, which possessed an F-box motif, interacting with the SKP and Cullin to form a multi-protein E3 ubiquitin ligase complex called SCF complex. However, we did not identify protein targets directly related to host silencing pathway for ORF3 which means that the protein may use indirect mechanisms or interact with RNA species. Co-immunoprecipitation confirmed the interaction between P0 and AtSKP1. Both co-immunoprecipitation and pull-down assays confirmed the direct interaction between P0 and AGO1, one of the main components in host RNA silencing pathway. Degradation assays using MG132 to inhibit proteasome activity proved that P0 catalyzed the ubiquitination of AGO1 and destined for it proteasomal degradation. We also observed that the P0 triggered robust hypersensitive response-like cell death in *N. benthamiana* probably through gene-for-gene interaction.

Non-Technical Summary:
CVEV is a newly discovered citrus virus which infects most citrus varieties and is aphid transmissible. CVEV causes woody gall and vein enation on some citrus species and damages young citrus seedlings. In this study, we initiated the CVEV characterization at the molecular level and identified some interactions with the plant

host. So far, we confirmed that CVEV uses P0 and ORF3 as viral silencing suppressors against host antiviral defenses and further discovered the host components interacting with these proteins. This information will contribute to the development of CVEV disease management strategies to benefit the citrus industry.

Citations:

Vives, M. C., Velázquez, K., Pina, J. A., Moreno, P., Guerri, J., and Navarro, L. 2013. Identification of a new enamovirus associated with citrus vein enation disease by deep sequencing of small RNAs. Phytopathology 103:1077-1086.

Fusaro, A. F., Correa, R. L., Nakasugi, K., Jackson, C., Kawchuk, L., Vaslin, M. F., and Waterhouse, P. M. 2012. The Enamovirus P0 protein is a silencing suppressor which inhibits local and systemic RNA silencing through AGO1 degradation. Virology 426:178-187.

Song, Z., Wang, Y., Cui, T., Bin, Y., Yan, J., and Zhou, C. 2018. Identification of an RNA silencing suppressor encoded by citrus vein enation virus. Journal of Plant Pathology

IOCV-O7-04

Rescue of Citrus sudden death-associated virus in *Nicotiana benthamiana* plants from cloned cDNA: insights into mechanisms for expression of the three capsid proteins

Emilyn E. MATSUMURA[1], Helvécio D. COLETTA-FILHO[2], Marcos A. MACHADO[2], Shahideh NOURI,[3] Bryce W. FALK[1]

[1] University of California, Davis, CA, USA, [2] Centro de Citricultura Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis, SP, Brazil, [3] Kansas State University, Manhattan, Kansas, USA

Abstract: Citrus sudden death-associated virus (CSDaV) is a member of the genus *Marafivirus* in the family *Tymoviridae* and has been associated with citrus sudden death (CSD) disease in Brazil. Difficulties in purifying CSDaV virions from infected citrus plants have prevented progress in studying the role of this virus in CSD and in understanding its molecular biology. In this work, we have constructed a full-length cDNA clone of CSDaV driven by the 35S promoter (35SRbz- CSDaV). *Agrobacterium tumefaciens*- mediated inoculation of the 35SRbz-CSDaV in *Nicotiana benthamiana* plants enabled an efficient and fast recovery of high amounts of virions from the agroinfiltrated leaves, which has allowed a better molecular characterization of CSDaV. *In vivo* analyses of mutant versions of the 35SRbz-CSDaV clone revealed the expression strategies used by CSDaV for production of the capsid proteins. We show that CSDaV produces three forms of the capsid protein (CP): the major CPp21 is a product of direct translation by leaky scanning from the second start codon in the subgenomic RNA (sgRNA), while the minor CPs, p25 and p23, are produced by direct translation from the first start codon in the sgRNA and by *trans*-proteolytic cleavage processing derived from the p25 precursor, respectively. Together, these findings contribute to advance our understanding of CSDaV genome expression strategies, which has not been clearly elucidated by previous works, but it is essential for further research. Additionally, construction and characterization of CSDaV infectious clone represents an important step toward studying the role of this virus in CSD and for its use as a tool for citrus biotechnology.

Non-Technical Summary: CSDaV is the virus most associated with CSD, an important citrus disease that has affected sweet oranges in Brazil. However, researchers have faced many challenges in showing the roles of CSDaV in CSD-affected plants, mainly because of the low viral titer and the presence of other viruses that usually hamper the access of the virus of interest in infected plants. To overcome this, we built the first CSDaV infectious clone that is able to quickly locally- infect tobacco leaves, generating high amounts of viral particles that can now be used to infect citrus plants. Characterization of the infectious clone revealed new biological information on the CSDaV, which represents important advances in understanding the role of CSDaV in CSD and also make available a new virus vector to be used as a tool for citrus biotechnology.

Citations:

Müller et al., 2002; Román et al., 2004; Maccheroni et al., 2005; Bassanezi et al., 2007; Matsumura et al., 2016; Matsumura et al., 2017.

IOCV-O7-05

Response of sweet orange to co-infection with '*Candidatus* Liberibacter asiaticus' and *Citrus tristeza virus* at the transcriptional and biological levels

Shimin FU[1], Changyong ZHOU[1], Xuefeng WANG[1], Jonathan SHAO[2], and John S. HARTUNG[2]

[1] Citrus Research Institute, Southwest University, Chongqing, P. R. China

[2] U.S. Department of Agriculture-Agricultural Research Services, Maryland, USA

Abstract: Citrus worldwide is threatened by huanglongbing (HLB) and tristeza diseases associated with '*Candidatus* Liberibacter asiaticus' (CLas) and *Citrus tristeza virus* (CTV), respectively. Both CLas and CTV are restricted to phloem cells and are transmitted by insect vectors. CLas induces symptoms of phloem dysfunction while the mild strain CTV-B2 does not cause any loss to commercial trees. The transcriptomic profile of 'Valencia' sweet orange co-infected by CLas and CTV-B2 was greatly perturbed, including carbohydrate and amino acid metabolism, cell wall modification, transportation, circadian system, hormonal responses, and ribosomal biogenesis. Notably, genes *ZIP1*, *ZIP4*, *ZIP5* encoding zinc transporters and *PP2-B15* in association with phloem protein showed very different expression patterns in response to double infection by CLas and CTV-B2 compared to infection by CLas or CTV-B2 alone. The balancing effects of oppositely regulated genes in response to infection by CLas and CTV-B2 may have reduced phloem damage and symptom development associated with CLas. We found that sweet orange seedlings co-infected by CLas with CTV-B2 survived longer and developed less symptoms than seedlings infected by CLas alone. Further, CLas was more sporadically distributed in a plant co-infected by CLas and CTV-B2 than in a plant infected by CLas alone, based on immunoassay. Therefore, we conclude that defense responses activated by the mild strain CTV-B2 may provide benefit against CLas. The aligned transcriptional data, and biological and cytobiological observations provide a novel notion that mild CTV strains may provide some protection against CLas by limiting its multiplication, spread and HLB symptom development.

Non-Technical summary: Co-infection of citrus with the causal agent of HLB and tristeza changes the physiology of the citrus plant. Citrus seedlings infected by both CLas and CTV-B2 showed less symptoms and survived longer than seedlings infected by CLas alone. CTV-B2 hindered the movement and multiplication of CLas in the plant. This finding may eventually provide a useful tool to protect citrus trees from HLB.

Citations:

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

Shimin Fu, Jonathan Shao, Cristina Paul, Changyong Zhou and John S. Hartung. Transcriptome Analysis of Sweet Orange Trees Co-infected with '*Candidatus* Liberibacter asiaticus', Mild or Severe Strain of *Citrus tristeza virus*. *BMC Genomics*,

2017. 18(1): p.837.

Shimin Fu, Cristina Couin, Changyong Zhou, John S. Hartung. Limited Infection by ‘*Candidatus* Liberibacter asiaticus’ in ‘Valencia’ Sweet Orange trees in the presence of Citrus tristeza virus. *Journal of Integrative Agriculture*, 2018 (accepted).

IOCV-07: Other Citrus Viruses and Diagnostics Poster Presentations

IOCV-P7-22

Coat protein and movement protein of Citrus tatter leaf virus possess RNA silencing suppression activity to suppress host antiviral RNA silencing

Shih-hua TAN[1] and Georgios VIDALAKIS[1]
[1]University of California, Riverside, USA

Abstract: Upon entry into host cells, viral proteins manipulate specific physiological processes or signaling pathways to subvert host immunity. In this study, we identified two citrus tatter leaf virus (CTLV) proteins used to suppress host antiviral RNA silencing. Both coat protein (CP) and movement protein (MP) suppressed RNA silencing in GFP-transgenic *Nicotiana benthamiana* 16c plants. MP was characterized as a local suppressor of RNA silencing while the CP acted as a systemic suppressor. When the *Potato virus X* (PVX) infectious vector harbored either the CP or MP, they both promoted viral infection and symptoms development, likely through their RNA silencing suppression activities. The PVX deletion assay, revealed that the functional regions of each silencing suppressor were located at amino acids 36 to 70 for CP and 112 to 143 for MP and these deletions failed to promote virus infection. The results of mass spectrometry-based immunoprecipitation proteomics showed that both CTLV CP and MP did not interact with components directly involved in host antiviral RNA silencing pathways and they are probably using other indirect mechanisms. We also found that instead of targeting at protein level, CTLV MP interacts with dsRNA species which leads to suppression of host antiviral RNA silencing. These findings identified RNA silencing suppression as a common strategy used by viral pathogens to cause disease and overcome host antiviral defense and may have indicated how CTLV can infect a wide range of hosts including a variety of woody and herbaceous plants.

Non-Technical Summary:
Citrus tatter leaf virus (CTLV) induces chlorosis, leaf deformation and most importantly, a citrus bud-union incompatibility when infecting trees grown on trifoliate or trifoliate hybrids rootstocks. These are commercially important rootstocks in California and around the world which means that a disease management strategy for CTLV is needed to prevent economical loses. In this study, we identified two CTLV proteins (coat and movement) used as viral silencing suppressors against host antiviral defenses which might be one of the main reasons for the virus having a wide host range.

Citations:

Tatineni, S., Afunian, M. R., Hilf, M. E., Gowda, S., Dawson, W. O., and Garnsey, S. M. 2009. Molecular characterization of Citrus tatter leaf virus historically associated with Meyer lemon trees: complete genome sequence and development of biologically active in vitro transcripts. Phytopathology 99:423-431.

Lu, R., Folimonov, A., Shintaku, M., Li, W. X., Falk, B. W., Dawson, W. O., and Ding, S. W. 2004. Three distinct suppressors of RNA silencing encoded by a 20-kb viral RNA genome. Proc Natl Acad Sci U S A 101:15742-15747.

Qiao, Y., Liu, L., Xiong, Q., Flores, C., Wong, J., Shi, J., Wang, X., Liu, X., Xiang, Q., Jiang, S., Zhang, F., Wang, Y., Judelson, H. S., Chen, X., and Ma, W. 2013. Oomycete pathogens encode RNA silencing suppressors. Nat Genet 45:330-333.

IOCV-P7-23

Complete genome sequences and recombination analysis of two citrus mosaic virus isolates.

Song ZHANG [1,2], Jiaxing WU [1,2], Yuanjian QIU [1,2], Changyong ZHOU [1,2*], **Mengji CAO** [1,2*]

[1]National Citrus Engineering Research Center, Citrus Research Institute, Southwest university, Chongqing, China, 400712, [2]Academy of Agricultural Sciences, Southwest University, Chongqing, China, 400715

Abstract: Next-generation sequencing allowed the obtainment of full genome sequences of two Citrus mosaic virus isolates, named as CiMV-SE1 and -GTC1 according to their source host citrus cv. Setoka and Goutoucheng. This is first time that CiMV was found in China, and completed in RNA1. CiMV-SE1 and -GTC1 were positive-strand RNA viruses with bipartite genomes RNA1 (6,758 and 6,770 nt) and RNA2 (5,345 and 5,263 nt) each encodes a polyprotein. RNA1 polyprotein contains helicase, proteinase (Pro) and RNA dependent RNA polymerase (RdRp); RNA2 polyprotein contains movement protein (MP) and coat protein (CP). Sequence analysis revealed extensive identical in genomic 5′ end, 5′ end of coding regions and 3′ end between RNA1 and RNA2 of CiMV similar to Satsuma dwarf virus (SDV), and high sequence conservations no matter in whole, or core coding regions (Pro-Pol i.e. the joint of Pro and RdRp, and CP) when compared with SDV, reinforcing the placement of CiMV as strain in *satsuma dwarf virus* species in the genus *Sadwavirus*. Furthermore, in the phylogenetic trees built upon Pro-Pol and CP regions, SE-1 and GTC- 1 were situated together, while separated into two distinct clades, in CiMV groups paralleling SDV and other extant strains, reflecting an evolutionary diversity within the genus. RDP4 analysis and sequence comparisons predicted that 5′ end coding regions of RNA1 and 2 where the most of recombination events occurred were unstable, and resembled highly in amino acid sequence comprising a nuclear localization signal, suggesting some interplays may exist between two RNAs. Collectively, this study enriched genome as well as evolution information for the genus *Sadwavirus*.

Non-technical summary: CiMV was discovered in China, and sequenced in genomes (isolates SE-1 and GTC-1), for the first time. Comprehensive analysis based on whole genome sequence provided more details in the relationships of members in the *Sadwavirus*. This study also discussed recombination events of CiMV within the genus, providing an insight into the evolution history.

IOCV-P7-24

Development of new tools for on-site detection of *Spiroplasma citri*, causal agent of citrus stubborn disease

Mounira Inas DRAIS[1,2], Yogita MAHESHWARI[3], Vijayanandraj SELVARAJ[3], Leonardo VARVARO[2], Raymond YOKOMI[3], and Khaled DJELOUAH[1]

[1]CIHEAM Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano (BA), Italy; [2]Department of Agriculture, Forests, Nature and Energy, Tuscia University, Viterbo, VT 01100, Italy; [3]United States Department of Agriculture-Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648-9757, USA,

Abstract:

Citrus stubborn disease (CSD) is caused by the bacterium, *Spiroplasma citri*. CDS-affected trees are stunted, low yielding with reduced fruit quality. This research developed innovative serological and molecular tools for on-site

detection of *S. citri* to facilitate large- scale pathogen detection surveys. Firstly, a Direct Tissue Blot ImmunoAssay (DTBIA) that uses a polyclonal antiserum to *S. citri* was developed and validated in a field survey of 112 citrus trees sampled from the Mitidja region, the main citrus-growing region of Algeria. This survey detected two *S. citri*-infected trees by the DTBIA assay in two varietal collections and results were confirmed by molecular assays of a spiralin gene sequence from DNA extracted from infected field trees. The resulting sequence shared 99% identity with the Iranian Fasa I strain (GenBank Accession No.FJ755921.1). In addition, a Loop-Mediated Isothermal Amplification technique (LAMP) was developed. The LAMP assay targeted the spiralin gene and was optimized for crude plant extracts. The LAMP assay showed high specificity and detected *S. citri* DNA to a level of 100 fg/µl with no inhibition by crude plant extracts. Although the LAMP assay was 9 times less sensitive than qPCR with purified DNA templates, an Android-based portable isothermal RNA/DNA amplification system yielded comparable yes/no calls for *S. citri* infection with qPCR and purified DNA templates from the same trees in field validations. These methods allow growers, pest control or diagnostic services to rapidly test for *S. citri* in the field without a laboratory or DNA purification.

Non-technical summary: Citrus stubborn disease (CSD), caused by *Spiroplasma citri*, affects citrus production in the Mediterranean region as well as California and Arizona. CSD symptoms can easily be confused with huanglongbing (HLB). Therefore, methods for large-scale detection of *S. citri* is important for citrus disease management programs. To this end, a direct tissue blot immunoassay (DTBIA) and a Loop-Mediated Isothermal Amplification technique (LAMP) were developed. The DTBIA was validated by field citrus samples from Algeria and the LAMP assay was validated by citrus samples from California.

IOCV-P7-25

Diagnosis of citrus psorosis by real time RT-PCR in Argentina

Melina SIMEONE[1], Claudio GÓMEZ[2], Ana BERTALMÍO[3], Fabián RAMOS[2], Patricia FERNÁNDEZ[2], Esperanza RUIZ[4] and Maria Laura GARCIA[1]

[1]Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET— UNLP, La Plata, Buenos Aires, Argentina; [2]Estación Experimental Agropecuaria, INTA, Concordia, Entre Ríos, Argentina; [3]Instituto Nacional de Investigación Agropecuaria, INIA, Salto Grande, Uruguay; [4]Laboratorio de Investigación y Desarrollo de Bioactivos (LIDeB), UNLP, La Plata, Buenos Aires, Argentina

Abstract: Citrus psorosis virus (CPsV) is the causal agent of the psorosis disease, which has greatly affected citrus crops in Argentina. Control of the disease in our country is achieved through a quarantine and certification program to avoid virus entry and propagation. Currently, the Argentine certification program is based on traditional grafting indexing as the unique diagnostic technique for detection of CPsV. This method is sensitive and reliable, but laborious and expensive, and take at least one year, limiting the number of tests that can be done simultaneously in one certification station, and thus restricting the certification of seed parent plants and the introduction of new varieties. In order to provide an alternative diagnostic technique able to process a large number of samples in shorter times, we are evaluating diagnostic parameters (sensitivity, specificity and likelihood ratios) of real-time reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) to detect a fragment of the RNA3 of CPsV, encoding the coat protein (De Francesco et al., 2015). We have analyzed 226 citrus samples of different species and from different origins (i.e., greenhouses, farms, nurseries) and regions (Northwest of Argentina, Northeast of Argentina and Uruguay) that underwent biological indexing with the RT-qPCR method and compared the results. According to the biological index, 5 of 23 samples from Northwest of Argentina gave false positive results by RT-qPCR. Taken all these samples, the total agreement between these methods was substantial with a Cohen's kappa index of 0.90 ± 0.07. The results were coincident in 98.23%, the sensitivity of RT-qPCR was 1, specificity 0.98 and the BAK was 0.96 ± 0.07.

Non-technical summary: We are evaluating the RT-qPCR protocol using the biological indexing as referent, finding a high coincidence between these methods, indicating that this technology will be likely included in the Argentine normative after analysis of a higher number of samples, and diagnostic parameters were determined (Vidal et al., 2012).

Citations:

De Francesco, Agustina, Norma Costa, Maria Inés Plata and M. Laura Garcia. 2015. Improved detection of Citrus psorosis virus and coat protein-derived transgenes in citrus plants: comparison between RT- qPCR and TAS-ELISA. Journal of Pythopathology 163: 915-925.

Vidal, E., Yokomi, R. K., Moreno, A., Bertolini, E., and Cambra, M. 2012. Calculation of diagnostic parameters of advanced serological and molecular tissue-print methods for detection of Citrus tristeza virus: A model for other plant pathogens. Phytopathology 102:114- 121.

IOCV-P7-26

Incidence of the “Psorosis complex” diseases in seed source mother trees in Northwestern Argentina

Julia FIGUEROA[1], Maria F. PALACIOS[1], Lucas FOGUET[1], Lucas VILLAFAÑE[1] and Beatriz STEIN[1]

[1]Centro de Saneamiento de Citrus, Estacion Experimental Agroindustrial O.Colombres, Tucumán, Argentina.

Abstract: The Citrus Certification Program in Argentina has been mandatory since 2010 and establishes that seed source trees for rootstock propagation have to be free of the “Psorosis complex diseases” (Psorosis A and B, concave gum, blind pocket, impietratura, and cristacortis) due to possible seed transmission. Biological indexing is required and every six years the diagnosis has to be repeated in order to keep the seed source trees within the mentioned program. At the Centro de Saneamiento de Citrus, biological indexing for the Psorosis complex has been done since 2005 with Pineapple sweet orange (*Citrus sinensis* Osbeck) seedlings as indicator plant in a cool greenhouse (18 to 24 ºC). To date, 1324 tests (1053 tests and 271 retesting) have been performed. Results showed 9 positive plants (0,85 %) with typical oak leaf pattern symptoms and with no reaction to the cross protection with Psorosis B. The number of positive plants was: two Cleopatra mandarin (*C. reshni*), one Carrizo citrange (*C. sinensis* × *Poncirus trifoliata*), one X 639 citrandarin (*C. reticulata x P. trifoliata*), three Swingle citrumelo, and two 75 AB citrumelo (*C. paradisi x P. trifoliata)*. Psorosis A and B were not found. Results indicate that in the Northwestern citrus area of Argentina there is a low incidence of diseases of the Psorosis complex in seed source trees and no reinfection have been detected so far in retested plants. This is the first information on the presence of these diseases in seed source trees in Northwestern Argentina. This work will continue with the identification of the diseases present with molecular techniques.

Non-technical Summary: This paper reports a low incidence of Psorosis complex diseases in seed source trees in northwestern Argentina. Biological indexing 1058 plants of different varieties was performed since 2005. Only 0.85 %

were found positive for oak leaf pattern diseases and none for Citrus Psorosis Virus, and no reinfection have been detected so far in the retesting of 271 plants.

IOCV-P7-27

Molecular identification and characterization of *Citrus psorosis virus* in China

Min LI [1,2], Song ZHANG [1,2], Jiaxing WU [1,2], Zhongan LI [1,2], **Mengji CAO** [1,2] *

[1]National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing, China, [2]Academy of Agricultural Sciences, Southwest University, Chongqing, China

Abstract: *Citrus psorosis ophiovirus* (CPsV), the type species of the genus *Ophiovirus*, is graft-transmissible, and destructive to citrus production. In this study, we obtained the complete genome sequences of four CPsV isolates CHN-1, CHN-2, CHN-3 and CHN-UN21 by using transcriptome sequencing (RNA-seq), RT-PCR, cloning and Sanger sequencing approaches. Genome sequence analysis showed that the full length sequences of CHN-1, CHN-2, CHN-3 and CHN-UN21 were 11, 282, 11, 279, 11, 278 and 11, 305 nucleotide (nt) respectively, and were comprised of four open reading frames (ORFs) each in their tripartite genomes. Nucleotide sequence similarity between genomic RNAs of the four CPsV isolates ranged from 81.44% to 95.98%, and their amino acid (aa) sequence similarities were from 88.61% to 99.32%. When coat protein (CP) genes of these four isolates were compared with the other six extant, the nt sequence similarities were from 81.29% to 95.23% while aa sequence similarities were from 86.10% to 99.32%. Phylogenetic analysis indicated that the isolates CHN-1, CHN-2 and CHN-3 clustered into the same branch of evolutionary tree, suggesting they should have the same origin. Whereas, CHN- UN21 related isolates were grouped in a separate clade consistent with a specific leaf ringspot symptom in comparison with the formers, suggesting it should be a new strain of CPsV. During a field investigation, CPsV was detected by RT-PCR in 5 out of 87 accessions (4 in Orah and 1 in Murcott, respectively) from China. To our knowledge, this is the first report of CPsV in China.

Non-Technical summary: CPsV was reported in China for the first time, with four isolates namely CHN-1, CHN-2, CHN-3 and CHN-UN21 characterized. Sequence, phylogenetic and symptom analysis revealed that CHN-UN21 was a novel strain. Although spread of two types of CPsV in China might be limited, it is urgent to take measures to prevent their further dispersal.

IOCV-P7-28

Use of young plants (mini-plants) for biological indexing for citrus graft transmissible pathogens

Richard. F. LEE[1], Manjunath KEREMANE[1], and Chandrika RAMADUGU[2]

[1] USDA ARS, National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA, [2] University of California, Riverside, CA,

Abstract: While there are laboratory based methods for detecting known citrus pathogens, biological indexing is essential for confirmation of the absence of known graft transmissible pathogens (GTP) and to detect possible unknown citrus pathogens. We have developed a biological indexing method using very young (mini) indicator plants, 75-90 days old. Side by side comparisons of 30 accessions using traditional biological indexing methods and mini-plant biological indexing indicate the same level of sensitivity between the two methods.

The use of mini-plants for biological indexing requires much less time to prepare the indicator plants for use and much less greenhouse space. In California we were able to index for graft transmissible pathogens (GTP) requiring cooler temperatures during the summer by placing the mini-plants used for biological indexing near the cooling pads, thus enabling indexing of GTP requiring cooler temperatures to express symptoms on a year round basis. Much greenhouse space is saved as the mini-plants are ready for use for biological indexing within 75-90 days as compared to 8-12 months with traditional indicator plants, they require much less space (24 plants/sq. ft of bench space as compared to three plants/sq. ft using the traditional approach to biological indexing). The use of mini-plant biological indexing will enable citrus clean stock and/or certification programs to be more productive while using less resources.

Non-Technical summary: The protocol for using young plants (75-90 days old) as indicator plants is described. Results from using the young plants as indicators is compared to results using the traditional biological indexing.

IOCV-08: Topics in Citrus Pathology Oral Presentations

Topics in Citrus Pathology

Jim GRAHAM
University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: The ravages of citrus tristeza in South America and California brought together researchers and growers at the Riverside Citrus Experiment Station to learn about this disease and measures to be taken against it. Thirty invitational papers were presented during formal sessions, and field trips to local citrus production areas were conducted. Under the leadership of J.M. Wallace, virologist at the Citrus Experiment Station, the International Organization of Citrus Virologists, IOCV, was born in 1957. The IOCV has held conferences every three years in every major citrus area of the world and published the proceedings. The IOCV was dedicated to the understanding and control of virus and other graft-transmissible diseases. In 2013 at the conference in South Africa, the IOCV decided to establish the Journal of Citrus Pathology. This peer reviewed, on-line publication, gave the IOCV an opportunity to support research on issues pertaining to all citrus diseases, production, and health. Since 2013, the journal has published papers on general citrus pathology but not addressed these topics in the IOCV conference program. Sixty-two years after the first conference, the 21st IOCV is convening citrus pathologists at the inaugural session presenting research on fungal and non-systemic bacterial diseases.

IOCV-O8-01

Gene expression of Guanggan (*Citrus reticulata*) infected by *Phytophthora nicotianae*

Huaxue YAN[1,2], Yun ZHONG[1,2], Bo JIANG[1,2], Birong ZHOU[1,2], Bo WU[1,2], Guangyan ZHONG[1,2]

[1]Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, China, [2]Key Laboratory of South Subtropical Fruit Biology and Genetic Resource Utilization, Ministry of Agriculture, Guangzhou, China

Abstract: Root rot and gummosis caused by *Phytophthora* are the most economically important soil-borne diseases in citrusworldwide. Guanggan (*Citrus reticulata*) has been reported to be strongly resistant to *Phytophthora nicotianae*. To study the mechanisms of resistance to *P. nicotianae* and identify the candidate genes involved in disease resistance in Guanggan, leaves were collected 48 hours after inoculation(?) with *P. nicotianae* and gene expression was analyzed by using next-

generation sequencing. With a filter of false-discovery rate less than 0.05 and fold change greater than 2.0, 446 genes were found to be up-regulated, and 523 genes were down-regulated. The results indicate that *P. nicotianae* infection significantly enhanced the expression of genes involved in cell wall metabolism, secondary metabolite synthesis, starch and sucrose metabolism, plant defense and signaling transduction pathways while it decreased the expression of genes involved in light reactions and protein degradation. The expression of genes involved in ethylene biosynthesis was induced by *P. nicotianae* infection. We speculate that the Jasmonic Acid/Ethylene Signaling Pathway participates in the defense responses. Furthermore, due to the down-expression of genes involved in light reaction and up-expression of genes involved in the starch biosynthesis, the content of small molecule sugars in Guanggan might be decreased during the pathogen infection. Limiting the availability of extracellular sugar could deprive the pathogen of an energy source and restrict delivery of virulence factors. In addition, *P. nicotianae* infection also enhanced the expression of genes involved in disease resistance, such as respiratory burst, *R* genes, pathogenesis- related genes (*PR*). Differentially expressed genes were validated by RT-qPCR in the plants inoculated with the pathogen versus a non-inoculated control.

Non-Technical summary: Our data describes the gene expression changes of Guanggan leaves in response to *P. nicotianae* infection. The results indicate the mechanisms of resistance of Guanggan to *P. nicotianae* involved in various processes of plant defense responses to pathogen attack.

IOCV-O8-02

Microsatellite characterization and marker development for the fungus *Penicillium digitatum*, causal agent of green mold of citrus

Erika VARADY[1, 2], Sohrab BODAGHI [1], Georgios VIDALAKISV[1], and Greg W. DOUHAN [3]

[1] Department of Microbiology and Plant Pathology, University of California, Riverside, USA [2] Department of Molecular Biology and Biochemistry, University of California, Irvine, USA, [3] University of California, Cooperative Extension, Tulare, USA.

Abstract: *Penicillium digitatum* is one of the most important post-harvest pathogens of citrus on a global scale causing significant annual losses due to fruit rot. However, little is known about the diversity of *P. digitatum* populations. The genome of *P. digitatum* has been sequenced, providing an opportunity to determine the microsatellite distribution within *P. digitatum* to develop markers that could be valuable tools for studying the population biology of this pathogen. In the analyses, a total of 3,134 microsatellite loci were detected; 66.73, 23.23, 8.23, 1.24, 0.16, and 0.77% were detected as mono, di, tri, tetra, penta, and hexanucleotide repeats, respectively. As consistent with other ascomycete fungi, the genome size of *P. digitatum* does not seem to correlate with the density of microsatellite loci. However, significantly longer motifs of mono, di, and tetranucleotide repeats were identified in *P. digitatum* compared to 10 other published ascomycete species with repeats of over 800, 300, and 900 motifs found, respectively. One isolate from Southern California and 5 additional isolates from other countries ('global isolates') were used to initially screen microsatellite markers developed in this study. Twelve additional isolates, referred to as the 'local isolates', were also collected from citrus at the University of California Riverside agricultural experiment station and were subsequently used to screen the primers that sequenced well and were polymorphic based on the global isolates. Thirty-six primers were screened and nine trinucleotide loci and one hexanucleotide locus were chosen as robust markers. These loci

yielded 2 to 7 alleles and will be useful to study population genetic structure of *P. digitatum* populations.

Non-Technical summary: Microsatellite markers in the post-harvest pathogen *P. digitatum* were analyzed in the genome of this important pathogen of citrus. The goal was to find a set of molecular markers that were variable enough in the pathogen to be utilized in future studies. Ten markers were found to be variable enough in a set of local isolates collected at a small scale in California as well as within a set of globally collected isolates. These markers will enable future studies to investigate genetic diversity, epidemiology, the potential for recombination/sexual reproduction, and other ecological and evolutionary processes that shape *P. digitatum* populations.

IOCV-O8-03

Exogenous Nicotinamide Adenine Dinucleotide Induces Resistance to Citrus Canker in Citrus

Fernando M. ALFEREZ[2,3], Kayla M. GERBERICH[2], Jian-Liang LI[4], Yanping ZHANG[1], James H. GRAHAM[2] and Zhonglin MOU[1]

[1]University of Florida, Gainesville, USA, [2]University of Florida, Lake Alfred, USA, [3]University of Florida, Immokalee, USA, [4]National Institute Of Health, Research Triangle Park, USA

Abstract: Nicotinamide adenine dinucleotide (NAD) is a universal electron carrier that participates in important intracellular metabolic reactions and signaling events. Cellular NAD can be actively or passively released into the extracellular space, where it is processed or perceived by ectoenzymes or cell-surface receptors. In *Arabidopsis*, extracellular NAD (eNAD) induces immune responses. However, it was unclear whether eNAD signaling is specific for *Arabidopsis* or the Brassicaceae family. In this study, we treated citrus plants with eNAD and tested NAD-induced transcriptional changes and disease resistance. Our results show that NAD induces profound transcriptome changes and strong resistance to citrus canker, a serious citrus disease caused by the bacterial pathogen *Xanthomonas citri* subsp. *citri* (*Xcc*), in a concentration-dependent manner. A total of 660 and 574 genes were up- and down- regulated, respectively, at 4 hr after the NAD treatment, and a large number of defense- related genes were upregulated by eNAD. Furthermore, NAD-induced resistance persists in new flushes emerging after removal of the tissues previously treated with NAD, providing long-lasting protection against citrus canker. Finally, NAD treatment primes citrus tissues, resulting in a faster and stronger induction of multiple salicylic acid pathway genes, including *CsCM1* (*CHORISMATE MUTASE1*), *CsCM2*, *CsICS* (*ISOCHORISMATE SYNTHASE*), *CsPAL* (*PHENYLALANINE AMMONIA LYASE*), *CsNPR1* (*NON-EXPRESSOR OF PATHOGENESIS-RELATED (PR) GENES1*), and *CsPR5*, upon subsequent *Xcc* infection. Taken together, these results suggest that eNAD may be a conserved damage associated molecular pattern in citrus.

Non-technical summary: Treatment with exogenous nicotinamide adenine dinucleotide (NAD) induces profound transcriptome changes and provides long-lasting protection against citrus canker in citrus. The resistance is likely provided by faster and stronger induction of multiple salicylic acid pathway genes in the NAD-treated citrus tissues upon subsequent infection by the citrus canker- causing bacterial pathogen *Xanthomonas citri* subsp. *citri*.

Citations:

Alferez, F.M., Gerberich, K.M., Li, J.-L., Zhang, Y., Graham, J.H., and Mou, Z. (2018). Exogenous nicotinamide adenine dinucleotide induces resistance to citrus canker in citrus. Front. Plant Sci. 9:1472.

Wang, C., Zhou, M., Zhang, X., Yao, J., Zhang, Y., and Mou, Z. (2017). A lectin receptor kinase as a potential sensor for extracellular nicotinamide adenine dinucleotide in *Arabidopsis thaliana*. eLife 6: e25474.

IOCV-O8-04

Metabolic engineering of monoterpene synthases to control important pests and diseases in citrus varieties

Ana RODRÍGUEZ [1,2], Takehiko SHIMADA [3], Berta ALQUÉZAR [1,2] and **Leandro PEÑA [1,2]**

[1] Instituto de Biología Molecular y Celular de Plantas (IBMCP), Consejo Superior de Investigaciones Científicas (CSIC)-Universidad Politécnica de Valencia (UPV), 46022,
Valencia, Spain.

[2] Laboratório de Biotecnologia Vegetal, Pesquisa & Desenvolvimento, Fundo de Defesa da Citricultura (Fundecitrus), Vila Melhado, 14807-040, Araraquara, São Paulo, Brazil.

[3] National Institute of Fruit Tree Science (NIFTS), National Agriculture and Bio-oriented Research Organization (NARO), Shizuoka 424-0292, Japan.

Abstract: Volatile organic compounds (VOCs) are widely emitted from all plant organs. From a biological view, the accumulation (or *de novo* formation) of volatile terpenes at a certain stage of leaf development or fruit maturation correspond with different functions related to plant defense and/or to attraction of specialized beneficial organisms as parasitoids and/or seed disseminators. It is thought that the primary function of secondary metabolites, including VOCs, present in green tissues, including immature fruits, is to defend them against all types of potential consumers. In citrus, VOCs accumulate abundantly in specific cavities, the oil glands, present in all organs except roots. Monoterpenes are the most representative type of volatiles in citrus tissues, their content varying between 50% to almost 100% of the total VOCs depending on organs and genotypes. VOC profiles may be similar, at least qualitatively, or may be variable among close types, with drastic changes in some specific major compounds. D-Limonene represents approximately 95% of the total oil gland content in mature orange fruit while linalool is one of the most abundant compounds of orange leaves and flowers. Similarities or differences in VOC profiles may have consequences in plant interactions with their biotic environment. Frugivores include not only legitimate dispersers such as vertebrates and birds but also less appreciated and more abundant consumers of fleshy fruits, such as insects and microorganisms. We have used this information to generate genetically modified (GM) citrus varieties with either up- or down-regulated expression of specific monoterpene synthases (linalool and D- limonene synthases, respectively) to alter the accumulation of specific VOCs and then induce less attraction to citrus pests (the Mediterranean fruit fly, *Ceratitis capitata*) or resistance to specialized citrus pathogens (such as *Xanthomonas citri* subsp. c*itri* and *Phyllosticta citricarpa*). A review of the latest works showing the different possibilities offered by terpene engineering in generating resistance or repellence (actually less attraction) in citrus plants to microorganisms and pests, respectively, will be presented.

Non-technical summary: It is known that volatile organic compounds (VOCs) produced and emitted from different plant tissues mediate in plant-animal communication for defense against pathogens, deterrence against predators and attraction of frugivores and pollinators. Engineering metabolic changes affecting specific VOC profiles may influence citrus interactions with specific pathogens and pests. We are showing here that the drastic alteration of either D-limonene or linalool contents in fruit peel and green tissues, respectively, from genetically modified citrus trees is a promising method for generating resistance to economically important diseases.

IOCV-08: Topics in Citrus Pathology Poster Presentations

IOCV-P8-29

Leaf and fruit reactions of sweet orange and hybrids varieties under endemic conditions of citrus canker

Sérgio Alves de CARVALHO[1]; Franklin BEHLAU[2]; José BELASQUE JÚNIOR[3]; Rodrigo Rocha LATADO[1]; Mariangela CRISTOFANI-YALI[1], Rui Pereira LEITE-JÚNIOR[4]; Francisco de Assis Alves MOURÃO-FILHO[2]

[1]Centro de Citricultura Sylvio Moreira - Instituto Agronômico - CCSM-IAC, Cordeirópolis, SP, Brazil [2]Fundo de Defesa da Citricultura – Fundecitrus, Araraquara, SP; [3]Escola Superior de Agricultura Luiz de Queiróz – ESALq USP. Piracicaba, SP; [4]Instituto Agronômico do Paraná – IAPAR. Londrina, PR

Abstract: Severe infections of citrus canker caused by *Xanthomonas citri* ssp. citri (ex Hasse) Shaad et al (2006), can cause serious damage to citrus, with premature fall of leaves and fruits. In order to evaluate, under endemic conditions for citrus canker, accessions of the Citrus Germplasm Bank of the CCSM-IAC, and new materials of the breeding programs of IAC, IAPAR and ESALQ, were established in Guairaçá, PR in December 2012. The experiment involved 76 varieties of sweet oranges and hybrids grafted on Rangpur lime, and 3 of them also on Swingle citrumelo. Evaluations conducted from 2014 to 2018, based on leaf and fruit symptoms, indicated variations in the susceptibility of the different materials. A rootstock effect was observed: sweet orange varieties Natal, Iapar 73 and Hamlin on Swingle citrumelo, had 24, 20 and 32% less leaf lesions than trees on Rangpur lime, respectively. Lower incidence of leaf and fruit lesions was observed in the varieties Murcha IAC, IPR Jaffa, IPR 172, Folha Murcha, Natal Murcha, Pera Clone 42, IPR Salustiana, Pera Perão and Pera Morretes.

Non-Technical summary: Citrus canker was kept under control in the State of São Paulo for several decades, through outbreak eradication programs, control of propagation material and quarantine. Currently, after changes in Federal legislation, the State is considered an area under risk mitigation system, and actions are no longer required to eradicate or suppress the disease, but only to prevent its spread to other areas and to the its handling, and use of more tolerant varieties. Here we investigated the field reaction of 76 varieties of oranges and hybrids to citrus canker based on leaf and fruit symptoms, indicating potential commercial use of new varieties.

IOCV-P8-30

Prevalence of citrus viruses and viroids: a snapshot of the Uruguayan citrus industry

María José BENÍTEZ-GALEANO[1], Estefany BERTONI[1], Federico DALMAO[2], Ana BERTALMÍO[3], Leticia RUBIO[3], Fernando RIVAS[3], Diego MAESO[3] & Rodney COLINA[1]

[1]Laboratorio de Virología Molecular, Centro Universitario Regional Noroeste, Sede Salto, Universidad de la República, Uruguay.

[2]Departamento de Matemática y Estadística del Litoral, Centro Universitario Regional Noroeste, Sede Salto, Universidad de la República,

Uruguay.

[3]Programa Nacional de Investigación en Producción Citrícola, Instituto Nacional de Investigación Agropecuaria (INIA), Uruguay.

Abstract: Citrus are the most economically important fruit crop worldwide [1]. In Uruguay, the citrus industry has a strong socio-economic impact and exports are constantly growing [2]. Nevertheless, citrus species are susceptible to several diseases caused by different types of micro-organisms which directly cause a decrease in production leading to economic losses [1]. Although citrus-producing areas in Uruguay are confined to around 15,000 hectares, no information about the sanitary status of these citrus plants regarding viruses and viroids was available. In order to know the prevalence of these pathogens in the Uruguayan citrus industry, 1175 samples from Valencia and Navel oranges, 'Afourer' and 'Clementine' mandarins, and lemons were collected from seven citrus producing areas. Additionally, an age range was established clustering the samples into four groups. Using fast, specific, sensible and low-cost molecular biology methodos, the presence of eight different viruses and viroids was analysed, including *Citrus tristeza virus* (CTV), *Citrus psorosis virus*, *Citrus Satsuma dwarf virus* (SDV), *Citrus exocortis viroid*, *Hop stunt viroid*, *Citrus dwarfing viroid*, *Citrus bark cracking viroid* (CBCVd), and *Citrus bent leaf viroid* (CBLVd). Only 6 % of the plants were pathogen-free, while 93% of the plants were CTV positive. SDV, CBLVd and CBCVd were not detected. Co-infections were very frequent. Plants were detected with as many as five pathogens, CTV being present in all co- infected plants. An in-depth molecular analysis including CTV-p25-gene amplification of infected samples revealed that five of the seven extant CTV genotypes were present, with a huge prevalence of NC- genotype (87%) and the concerning presence of RB-genotype (4%) because *P. trifoliata* is the main rootstock. To our knowledge, this is the first report of the sanitary condition of Uruguayan citrus.

Non-technical summary: As a growing economic good, the citrus industry needs to be monitored regarding different pathogens affecting them. In the present study we report the state-of-the-art of these crops in Uruguay generating important information to be used by the sanitary authorities.

Citations:

Moreno et al., 2008 Ministerio de Ganadería, Agricultura y Pesca, 2017

IOCV-P8-31

Serological-and-molecular-based detection of graft-transmissible pathogens associated with citrus from non-core areas of Pakistan

Syed Atif Hasan NAQVI[1], Sagheer ATTA[2], Ummad-ud-Din UMAR[1] and Huawei LIU[4]

[1]Department of Plant Pathology, Bahauddin Zakariya University, Multan. Pakistan; [2]Department of Plant Protection, Ghazi University, Dera Ghazi Khan, Pakistan; [3]China Agriculture University, Department of Plant Pathology

Abstract: To assess the prevalence of virus and virus-like diseases of citrus in the Southern Punjab, Pakistan, surveys were conducted in citrus groves during 2013-15. Based on serological and PCR detection methods, citrus orchards and nurseries were tested for prevalence of *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), Citrus variegated chlorosis (CVC) caused by *Xyellela fastidiosa*, huanglongbing (HLB, citrus greening) associated with '*Candidatus* Liberibacter asiaticus', and citrus stubborn disease (CSD) caused by *Spiroplasma citri*. Citrus leaves and twigs samples of 'Kinnow' and 'Feutrell's Early' mandarins (*Citrus reticulata* Blanco), 'Mosambi' sweet oranges (*Citrus sinensis* L.), 'Shamber' grapefruit (*Citrus x paradisi*) were

collected from five districts of Southern Punjab viz. Multan, Khanewal, Lodhran, Layyah, and Bhakkar. Direct antibody sandwich enzyme linked immuno sorbent assay (DAS-ELISA) was employed to detect CTV, CPsV, CVC, and CSD, whereas HLB was detected by PCR using specific primers from two regions of the 16S rDNA intergenic region and ribosomal protein gene of rplKAJL-rpoBCoperon (β- operon) of '*C.* Liberibacter asiaticus'. Sequence of 16S rDNA revealed nucleotide identity level up to 98% among different isolates. Disease incidence of CTV, CVC, HLB, and CSD was 36.7 %, 10 %, 40 %, and 36.7 %, respectively, whereas CPsV was not detected. All "citrus species and relatives" viz., 'Kinnow', sweet oranges, 'Feutrell's early', and 'Shamber' were susceptible these diseases. To our understanding this is first investigation of virus and virus-like diseases in citrus from non-core areas of Punjab, Pakistan.

Non-Technical summary: The Southern Punjab was surveyed for citrus diseases *Citrus tristeza virus* (CTV), *Citrus psorosis virus* (CPsV), Citrus variegated chlorosis (CVC) caused by *Xyellela fastidiosa*, huanglongbing (HLB, citrus greening) associated with '*Candidatus* Liberibacter asiaticus', and citrus stubborn disease (CSD) caused by *Spiroplasma citri*. Disease incidence of CTV, CVC, HLB, and CSD was 36.7 %, 10 %, 40 %, and 36.7 %, respectively, whereas CPsV was not detected.

General Sessions IRCHLB Invited Speaker Abstracts

The current Citrus Huanglongbing status in the P.R.China

Changyong ZHOU

Citrus Research Institute of Chinese Academy of Agricultural Sciences / Southwest University, Beibei, Chongqing 400715, P.R.China

Abstract: The occurrence of Citrus Huanglongbing (HLB) dates back at least a hundred years in China, and has since spread around most citrus areas in the southern part of China. Since 2012, HLB problem increased in severity in some fast-growing citrus provinces such as Guangdong, Jiangxi, Hunan and Guangxi, for example, over 45 million citrus trees have been cut off due to HLB damage in Ganzhou municipality, south Jiangxi within recent six years. So, a series of state actions have been focused on the control of HLB. This talking will briefly introduce the State Collaborative Innovation Alliance for Integrated Prevention and Control of HLB, the State Financial Funds for HLB Disaster Relief, the State Key R&D Project for Integrated Prevention and Control of HLB etc. Although a few progresses have been made: 1) its putative phage particles were observed in sweet orange; 2) the whole genome of several Chinese isolates were sequenced, bio-information accumulates quickly, focusing on the prophage genomic region with high genetic variation and recombination events, two HLB origin centers were proposed in the P.R.China; 3) transgenic citrus lines against HLB have been being addressed to field trials for safety evaluation; 4) a few effective combinations of pesticides, air sprays, and shoots-killing chemicals are addressed to control citrus psyllids (*Diaphorina citri*); 5) screened enclosures, including windbreaks, are also investigated as a means to prevent psyllid infestations with somewhat success, other than antibiotics showed somewhat in effect, no chemical therapy has really been accessible to cure the infected trees, mainly due to no progress on *in vitro* cultivation of HLB pathogen. Due to the increasing threat of HLB to the newly established superior citrus belts, two blocking valleys for citrus psyllids and three classified types for prevention and control on HLB in the P.R.China will be presented.

Non-Technical summary: Although Citrus Huanglongbing has occurred in China for more than a century, it has still been causing huge losses to citrus industry. Due to the difficulty of *in vitro* cultivation of HLB pathogen encountered for more than half century, no chemical therapy has really been approached to cure the infected trees. The progresses on its bio-information and control practices in China within recent years have been reviewed.

On the frontline: preparing for the arrival of HLB in Australia

Nerida DONOVAN[1], Tim HERRMANN[2], Nathan HANCOCK[3]

[1]NSW Department of Primary Industries, Menangle Australia, [2]Auscitrus, Dareton Australia, [3]Citrus Australia, Mildura Australia

Abstract: Huanglongbing (HLB) has not been found in Australia but the disease is known to occur close to our northern border and has been detected in airport interceptions. Current activities aim to reduce the likelihood of HLB, or potential vectors, arriving in Australia and the impact of an incursion. This includes Australia's strict quarantine protocols for incoming passengers and goods, and the Auscitrus propagation scheme supplying health-tested material to industry from screened source trees. There is also a strong focus on building biosecurity awareness, maintaining diagnostic capability and surveillance. A first detector network (FDN) of agronomists, crop scouts and growers is coordinated by Citrus Australia with funding from industry and government, and the Federal government Northern Australian Quarantine Strategy (NAQS) conducts surveillance in high risk areas of Australia and off-shore. Simulation exercises, and learning from those in HLB affected countries, have helped stakeholders to identify ways to strengthen our defence against HLB. Industry is now working towards certification of plants produced using Auscitrus material; mandatory use of health-tested propagation material and screening of citrus production nurseries. Nursery registration would enhance tracing of citrus and Murraya in the event of an incursion. Continued awareness activities and coordinated surveillance programs in urban and peri-urban areas will assist with early detection.

Non-Technical Summary: Citrus is one of Australia's most important horticultural export crops and HLB is considered to be the biggest threat to the Australian citrus industry. HLB, or its insect vectors, have not been found in Australia. Stakeholders have been proactive to prepare for the disease or its vectors arriving on our shores to minimise the impact to industry.

Current Management Strategies of Citrus Huanglongbing in China

Zheng ZHENG[1], Meirong XU[1], Xiaoling DENG[1]

[1]South China Agricultural University, Guangzhou, China

Abstract: In China, citrus growing acreage has reached to 2.6 M hectares with the production tonnage over 3.8 M tons as of 2017. The primary market is fresh fruits. Huanglongbing (HLB, yellow shoot disease) is a historical problem and has been endemic in southern China such as Guangdong Province for over a hundred years. Up today, HLB have been found in 11 out of the 19 citrus growing provinces. Researchers and citrus growers have been fighting with HLB through vigorous research and have exercised various control practices with successes and failures. The "three key measures" targeting the infectious nature of HLB pathogen, proposed by Prof. Lin Kong-Hsiang over 50-years ago, outlines the principles in HLB control. These are: (i) use of clean nursery stocks with regional quarantine, (ii) scouting and removal of diseased trees immediately, and (iii) control of insect vectors. In practical exercises, new knowledge from HLB research is incorporated; The changing social structure that governs citrus productions is considered; Education to citrus growers and general publics from experts is emphasized; And the involvement of central and

local governments for control measure implementations is getting more and more important. Most of citrus production area north of Yangzi River such as Hubei, Chongqing and Sichuan remain free of HLB. Strict quarantine measures are in effect. For HLB endemic regions, strict use of HLB free nursery stocks, large area-wide control of Asian citrus psyllids (ACP), and mandatory removal of diseased trees are common issues of HLB control practices. Meanwhile, new citrus culture models targeting HLB control based on recent research information with high profit potential are being developed and introduced to growers. These include appropriate selection of production sites, use of HLB-free 2-years old nursery stocks/seedlings for replanting, orchard-wide quick suppression of ACP with pesticides, and high-density planting. All these contribute to the steady citrus production and counteract the damage from HLB in China.

Non-Technical summary: Citrus HLB has been epidemic in southern China for over a hundred years. For HLB control, the "three key measures" strategy targeting the infectious nature of HLB pathogen remains in effect and practiced. In addition to active scientific research that brought in new models of citrus cultivation minimizing HLB damage, education to citrus growers and general publics and government involvements for control measures implementations are important.

Assessing the Economic Impact of HLB on Citrus

Bruce BABCOCK

School of Public Policy, UC Riverside

Abstract: Models designed to optimize ACP/HLB management decisions require a payoff function if decisions are to be made to maximize the net benefit of citrus. Epidemiological models can be used to assess the risk and associated time path of HLB infection in commercial groves under different management scenarios. The cost of implementing the different scenarios can be estimated using standard accounting practices. The benefit of a particular management scenario that slows or halts the spread of HLB into commercial groves equals the expected net present value of citrus under a particular management scenario minus the net present value under an alternative scenario, which could be a "no-management" scenario. The standard economic measurement of the value of a good is the sum of producer and consumer surplus. As the first step in a new project to assess the economic benefit of alternative HLB management scenarios in California, the factors that determine the magnitude of producer and consumer surplus for citrus are identified and their impact on value estimated. Substitutability of alternative crops in determining producer surplus from citrus production and substitutability of alternative food products in determining consumer surplus from citrus consumption are the key factors that determine the economic value of citrus. Estimates of crop and product substitution for California and Florida citrus are compared to illustrate their importance and differences between California production that is primarily destined for fresh market sales versus Florida production primarily destined for the juice market.

Non-Technical summary: To help ensure cost-effective use of grower and public funds to prevent the spread of HLB into commercial groves requires estimation of the economic payoff from the funded efforts. Both epidemiological models to estimate the efficacy of control efforts and economic models to estimate the value of citrus production and consumption are needed to estimate the net benefit of HLB control measures. The key roles that availability of substitute crops and substitute foods play in determining economic value of citrus in California and Florida are explored.

IRCHLB-01: Pathogen Genome Analysis Oral Presentations

IRCHLB-O1a-01

Genomic and phylogenetic analyses of *Candidatus* Liberibacter asiaticus

Shree P. THAPA[1], Nian WANG[2], Veronica ANCONA[3], Wenbo MA[4], **Gitta COAKER[1]**

[1]Department of Plant Pathology, University of California, Davis, CA, 95616

[2]Citrus Research and Education Center, Department of Microbiology and Cell Science, University of Florida, Lake Alfred, FL, 33850

[3] Citrus Center, Department of Agriculture, Agribusiness and Environmental Sciences, Texas A&M University-Kingsville, Weslaco, TX 78599

[4]Department of Microbiology and Plant Pathology, University of California, Riverside, CA, 92521

Abstract: Huanglongbing (HLB) is currently the most devastating disease of citrus. *Candidatus* Liberibacter asiaticus (CLas) is a Gram-negative HLB-associated bacterium. In the United States, HLB has been detected in Florida, Texas, and California. CLas is psyllid transmitted and resides in the phloem. In order to gain greater insight into CLas biology and genetic diversity, we have initiated genome sequencing and comparative analyses of CLas isolates from different geographical regions. CLas isolates from Texas, Florida, California, Asia, and Mexico were sequenced. We identified 584 core orthologous genes, which were used to analyze CLas phylogenetic relationships. Our results reveal diversity among different CLas isolates in different states. Our phylogenetic analyses indicates two different introduction events in California. Texas isolates were closely to Florida strain Psy62, while Asian isolates exhibited the most genetic variation. We have also identified and experimentally verified conserved SEC-dependent effectors presents in all isolates. The identity, conservation, and expression of specific SEC-dependent effectors will be presented. Taken together, these data have enabled the identification of biologically relevant CLas isolates that can be used to study the biology of this important pathogen.

Non-Technical summary: In the United States, HLB has been detected in Florida, Texas, and California. We have sequenced and compared the relatedness of CLas isolates from different geographical regions in the United States, revealing significant differences among isolates present in different regions. This information can be used to track CLas progression, identify representative strains for biological investigations, and identify conserved bacterial proteins that could be targeted for disease control.

IRCHLB-O1a-02

SureSelect targeted enrichment: an effective enrichment method for whole genome sequencing of '*Candidatus* Liberibacter asiaticus'

Weili CAI[1], John RASCOE[1], Michael J STULBERG[1]

[1]USDA-APHIS-PPQ-S&T-CPHST,
Beltsville, MD, USA

Abstract: Huanglongbing (HLB) is a deadly citrus disease associated with the phloem-limited bacteria '*Candidatus* Liberibacter asiaticus' (CLas) vectored by Asian citrus psyllids. In the US, CLas associated HLB was first found in Florida in early September 2005. Now the disease is found in multiple states. In order to effectively manage this disease, it is crucial to understand the relationship among the bacterial isolates from different geographical locations. Whole genome sequencing approaches will provide more precise molecular characterization of the diversity among populations. Due to the lack of *in vitro* culture, the whole genome sequence of CLas can only be

obtained using metagenomic sequencing. However, only a low percentage of reads are associated with CLas during sequencing analyses due to low pathogen copy number (especially in new infections) and large genome size difference between pathogen and host. Hundreds of millions of sequencing reads are needed to get good coverage of CLas from a positive citrus sample, meaning that whole genome sequencing of CLas is still difficult and costly. In order to overcome this limitation, we present here a new method, Agilent SureSelect XT HS target enrichment, which can specifically enrich CLas from a metagenomic sample while greatly reducing cost and increasing whole genome coverage of the pathogen. In this study, the pathogen reads to total reads ratio of a sequencing analysis increased from less than 0.1% to an average of 47.5%. The CLas genome was successfully sequenced with 99.3% genome coverage and over 72X sequencing coverage from low titer tissue samples (equivalent to 28.52 Cq using Li 16S qPCR), and this method may be suitable for even lower titer samples and CLas-infected root tissue. More importantly, this method also effectively captures regions of diversity in the CLas genome, which could provide precise molecular characterization of different isolates.

Non-technical summary: Here we present a SureSelect target enrichment method for whole genome sequencing of CLas from citrus tissue with relatively low bacterial titers with much higher coverage and lower cost than previously described methods, which may provide more useful molecular information for disease management.

IRCHLB-O1a-03

“*Candidatus* Liberibacter asiaticus” strains from multiple locations in southern California are different

Zehan DAI[1,2], Fengnian WU[2], Zheng ZHENG[2], Ray YOKOMI[1], Lucita KUMAGAI[3], Weili CAI[4], John RASCOE[4], MaryLou POLEK[5], **Jianchi CHEN[1]**, Xiaoling DENG[2]

[1]USDA-ARS, Parlier, CA, USA; [2]S. China Agr. Univ., China; [3]Plant Pest Diagnostic Center, CDFA, Sacramento, CA; [4]USDA- APHIS-PPQ, Beltsville, MD; [5]USDA-ARS, Riverside, CA

Abstract: “*Candidatus* Liberibacter asiaticus” (CLas) was first found in Florida in 2005 and is now endemic there. In California, CLas was first detected in Hacienda Heights in Los Angeles County in 2012 and has now been detected in multiple urban locations in southern California. Knowledge of CLas strain diversity in California is important for HLB management. In this study, genomic diversity among ten CLas strains from six California locations were analyzed using next generation sequencing (Illumina MiSeq and HiSeq) approach. Draft genome sequences of CLas strains were assembled. Sequences of 16S rRNA gene and *nrdB* confirmed CLas identity. Prophages were detected in all CLas strains. The California strains formed four prophage typing groups (PTGs): PTG1 with Type 1 prophage only (strains from Anaheim, San Gabriel, and Riverside); PTG2 with Type 2 prophage only (strains from Hacienda Heights); PTG1-3 with both Type 1 and 3 prophages (a strain from Cerritos); and PTG1- 2 with both Type 1 and Type 2 prophages (a strain from La Habra). Analyses of *terL* sequence showed that all California CLas strains were more related to strains in Asia, rather than those from Florida. Miniature inverted-repeat transposable elements (MITEs) were found in all CLas strains. Altogether, the NGS approach in this study revealed diversity of CLas strains in California which indicated independent introductions at multiple times.

Non-Technical summary: Citrus HLB was first diagnosed in Hacienda Heights in Los Angeles

County in 2012 and now in multiple urban locations in southern California. Knowledge of the California HLB pathogen is limited. This study analyzed the genomes of ten HLB pathogen strains from six California locations. Four different genomic groups were found, suggesting multiple introductions. The information is important for formulation of HLB management strategies.

IRCHLB-O1a-04

Development of routine multi-locus genotyping methods to characterize Huanglongbing (HLB) findings in California

Zonghe YAN[1], Beth KASIBORSKI[1,3], Lucita B. KUMAGAI[2], Mark K. NAKHLA[1], John RASCOE[1]

[1]USDA-APHIS-PPQ-CPHST Beltsville Laboratory, Beltsville, USA, [2]California Department of Food and Agriculture (CDFA), Sacramento, USA, [3]NCSU Department of Entomology & Plant Pathology, Raleigh, USA,

Abstract: Routine multi-locus genotyping methods were developed to differentiate HLB findings in California, making it possible to conduct large population studies of *Candidatus* Liberibacter asiaticus (*C*Las) strains to gain epidemiological knowledge necessary for HLB management in the state. These PCR product direct-sequencing methods are sensitive, cloning-free, and based on literature-validated Tandem Repeat Number (TRN) variations, namely four genomic loci A/B/C/D found in *C*Las. In our pilot study, 33 California *C*Las isolates from 13 cities in Los Angeles (LA), Orange and Riverside counties exhibited less than half of the TRN alleles found among 437 global HLB isolates reported in the literature. The haplotypes of the samples sorted into five major types implying different origins of introduction into CA, with possible divergence into sub-types from individual types from local HLB infections. The 1st type of 15 samples was found in all three counties; the 2nd type of 13 samples were only in San Gabriel and Rosemead of LA County; and the other three types were represented by isolated occurrences (one or two samples) from respective locations. Furthermore, preliminary prophage typing of 23 CA samples showed all 18 samples of the 1st and 2nd TRN type were prophage type1; prophage type2, type1&2, and type1&3 linked to corresponding unique TRN haplotypes represented by one or a few respective CA samples. All 25 CA samples tested by double-locus SNP analysis were A-term. We also tested some greenhouse samples, the three HLB grafts from Florida matched one of the two Florida haplotypes reported in 2013, and they differed from the CA types. Continued efforts to improve typing resolution and to genotype more CA *C*Las isolates will help with disease management and may open new avenues to protect the California citrus industry from HLB.

Non-Technical summary: 928 residential trees in California tested positive for *C*Las as of October 2018. To determine the number of *C*Las types (haplotypes) present in California and understand how they are spreading, routine genotyping methods were developed in a pilot study using 33 California *C*Las samples from 13 cities in Los Angeles, Orange and Riverside counties. The 33 CA HLB samples preliminarily grouped into five major types by “barcodes” denoting numeric variations on four HLB genotyping markers. One type of 15 samples was found in all the three Counties, another type of 13 samples was only in San Gabriel and Rosemead of LA County; other three types were represented by random one or few samples from respective locations. By testing a large collection of samples for a sufficient number of bacterial markers, we can “fingerprint” important HLB findings, track their origins, and gain a better understanding on how HLB is spreading in California. This information is critical in managing HLB in California.

IRCHLB-O1a-05

Genome Sequencing of psyllid-associated *Candidatus* L. caribbeanus and *Ca*. L. asiaticus from Colombia, South America.

Manjunath KEREMANE[1], Chandrika RAMADUGU[2], Surya SAHA[3], Stephanie HOYT[3], Adriana CASTAÑEDA[4], Marjorie SANTAMARIA[4], Jorge ÁNGEL[4], Emilio ARÉVALO[4], Lukas A. MUELLER[3], Jorge PALACINO[4], Mikeal ROOSE[2], and **Richard LEE[1*]**

[1*] USDA-ARS
[2] University of California, Riverside, CA, USA
[3] Boyce Thompson Institute, Ithaca, NY, USA
[4] Instituto Colombiano Agropecuario, Bogota, Colombia

Abstract: We described a new species of Liberibacter, *Ca*. L. caribbeanus (CLca) from Cordoba province of Colombia, South America in 2014, and an isolate of *Ca*. L.asiaticus (CLas_Colombia) from La Guajira province in 2016. Both the bacteria have now spread through psyllids to many surrounding provinces in Colombia. Citrus huanglongbing-associated symptoms were observed on plant hosts only with CLas infection, but not with CLca. While CLca reaches very high titers in psyllids (Ct up to 15), low titers of bacteria were recorded in plant hosts (Ct of >36). For the last five years, HLB-like symptoms have not been found in citrus plants positive for CLca. To generate genome sequence data, CLca+ psyllids were used to construct sequencing libraries for PacBio and Illumina platforms. CLas+ psyllid samples were used to generate libraries for Illumina sequencing only. After removing genomic sequences of the psyllid *Diaphorina citri*, and several commonly found endosymbionts, the remaining sequences were utilized for *de novo* assembly. We have a high quality draft assembly of CLas, and the *de novo* assembly of CLca genome is in progress. Comparative analysis of preliminary CLca scaffolds with CLas psy62, *Ca*. L. americanus, *Ca*. L. africanus, *Ca*. L. solanacearum, and *L. crescens* showed several blocks of microsyntenous and orthologous genes in CLca. Many genomic regions in CLca appear to have multiple sites of recombination. Phylogenetic analysis of several conserved genes suggest a close relationship with *L. crescens*. Studying the genome structure and biology of a psyllid-associated Liberibacter like CLca with a novel host-associated phenotype will aid in better understanding of the pathosystem.

Non-technical summary: We have studied a Liberibacter from Colombia that is present in high titers in psyllids but does not cause symptoms in citrus. Studying the genome and biology of this pathogen that does not cause HLB symptoms could provide valuable insights to help us understand the interactions between the psyllid vector/plant host and the HLB pathogen which may lead to development of novel solutions for the HLB management.

IRCHLB-O1a-06

Host-dependent chromosomal deletion mutations in the mosaic island of *Candidatus* Liberibacter asiaticus genome

Yongping DUAN, Cheryl M. ARMSTRONG, Lijuan ZHOU, Victoria ALLEN, Weiqi LUO, and David HALL

U.S. Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL, USA

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is the prominent species of Liberibacter associated with huanglongbing (HLB) of citrus. Previously, we published the first genome sequence of CLas obtained from single psyllid DNA via metagenomics. In an effort to verify the accuracy of the unculturable CLas bacterial

genome while not being subject to the identical limitations encountered with the original sequencing method, a BAC library, consisting of 61,440 clones, was constructed from the DNA of psyllids feeding upon HLB-affected citrus. Twenty-seven clones specific for CLas were sequenced in their entirety and used to reconstruct the genome. During the reassembly, a novel ~8.3 kb DNA fragment containing 9 putative ORFs was revealed. Comparisons with the other genome sequences determined this region to be a mosaic island that is associated with genome plasticity (instability). Using primers specific to this region, we were able to detect and differentiate CLas populations with (wild- type) and/or without the 8.3 kb fragment (mutant). Genotyping of infected samples demonstrated the presence of the wild-type CLas alone in psyllids, albeit at a low level (~11.0%), while the wild-type and mutants coexisted in most, if not all, infected plants. In contrast, the CLas mutant was detected either alone or mixture with the wild-type both in infected plants and psyllids. These results indicate that the deletion or excision of this mosaic island is critical for CLas fitness in planta. The dynamics between these two major CLas populations varied by host (variety-dependent) and geographical distribution with the highest ratio of mutant/wild-type reported in grapefruit, one of the most susceptible hosts. The functions of the nine putative ORFs and the excision mechanism of the 8.3 kb fragment remain to be elucidated.

Non-Technical summary: The HLB-associated bacteria, *Candidatus* Liberibacter asiaticus (CLas) exist as mixed populations both in insect and plant hosts. Beside the CLas variations caused by their endogenous prophages/phages, we identified a mosaic island in the chromosome of CLas genome. The deletion mutation or excision of this mosaic island was associated with the host tolerance and susceptibility. The mutation ratio of CLas populations may be applied for the prediction and selection for citrus HLB tolerance.

IRCHLB-01: Pathogen Genome Analysis Poster Presentations

IRCHLB-P1-01

A new genomic organization for prophages SC1 and SC2-like in *Candidatus* Liberibacter asiaticus from Brazil

Priscila A. SILVA, Camila G. FASSINI, Leandro PEÑA, Nelson A. WULFF.

Fundo de Defesa da Citricultura – Fundecitrus, Av. Dr. Adhemar Pereira de Barros, 201, Vila Melhado, Araraquara - SP, Brasil.

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is the predominant Liberibacter associated with Huanglongbing (HLB) worldwide. HLB was first detected in Brazil in 2004, with the presence of CLas and *Ca*. L. americanus (CLam). Today, CLas is the predominant species found in affected trees and HLB has spread unevenly in citrus orchards in Sao Paulo and Minas Gerais States, the major growing regions in Brazil. Methods to characterize bacterial strains are important for epidemiological analyzes and to elucidate the population genetic structure. Prophages are present in most sequenced Liberibacter genomes known so far. Two CLas prophages have been described, SC1 (Type 1) and SC2 (Type 2), related to lytic and the lysogenic cycle, respectively (Zhang et al., 2011). The characterization of the prophage occurrence and variability was assessed in a CLas population collected in 2015 in Brazil and reference strains from greenhouse. CLas strains were analyzed for the presence of prophages SC1 and SC2 (Zheng et al., 2016; Deng et al., 2014) using PCR markers (SC1-1, SC1-4, SC2-1 and SC2-8; Term-A or Term-G). CLas strains from Brazil were of the subunit phage terminase type Term-A. CLas in Brazil is very homogeneous for prophage markers, with 96.7% of strains carrying two prophages, SC1 similar to UF506 (SC1-type) and SC-2 only partially found (SC2-like). Prophage SC2-like was identified with an

inversion locus coupled with partial deletion in early genes of SC2. A 6.1 kbp PCR product was obtained with primers for SC2_SC1 prophage junction (SC2-8F and SC1-1R), instead of a 10 kbp described for UF506. This is indicative that prophage Type 2 of CLas strains in Brazil are divergent from other fully sequenced genomes, from the predominant type found in Florida, and a new genomic organization is suggested.

Non-Technical Summary: The occurrence and variability of the prophages in *Ca*. L. asiaticus strain from Sao Paulo indicates a highly homogeneous population, different from Florida (USA) in respect to prophage SC2 organization and corroborating a probable single introduction in Brazil.

Citations:

Deng, X., S. Lopes, X. Wang, X. Sun, D. Jones, M. Irey. 2014. Characterization of "*Candidatus* Liberibacter asiaticus" populations by double-locus analyses. Curr Microbiol. 69:554-56.

Zhang, S., Z. Flores-Cruz, L. Zhou. 2011. '*Ca*. Liberibacter asiaticus' carries an excision plasmid prophage and a chromosomally integrated prophage that becomes lytic in plant infections. Molecular Plant-Microbe Interactions 24:458-468.

Zheng, Z., M. Bao, F. Wu, J. Chen, X. Deng. 2016. Predominance of single prophage carrying a CRISPR/*cas* system in "*Candidatus* Liberibacter asiaticus" strains in southern China. PLoS One 11(1):e0146422. doi:10.1371/journal.pone.0146422.

IRCHLB-P1- 02

C2-like repressor may be involved in the *Candidatus* Liberibacter asiaticus SC1 bacteriophage regulation

Alejandra MUNOZ-BODNAR, Mukesh JAIN and Dean W. GABRIEL

[1]University of Florida, Department of Plant Pathology, Gainesville, FL, USA.

Abstract: Most *Candidatus* Liberibacter asiaticus (CLas) genomes sequenced to date harbor bacteriophages similar to SC1 and replicative prophage SC2 from Florida strain UF506. The SC1 bacteriophage lytic cycle is activated *in planta* and strongly repressed in psyllids, but the regulatory mechanism(s) are unknown. A C2-like repressor (LC2) is annotated on both bacteriophages. Bioinformatics analyses of the bidirectional LC2 promoter region predicted several potential DNA transcriptional regulation binding sites, one of which was also found in another bidirectional promoter between SC1gp-175 and SC1gp-185 (hypothetical proteins) and to a limited extend in the LC2 promoter region of SC2. These specific DNA regions were synthesized and used for DNA mobility shift assays. Commercially synthesized SC1-LC2 protein was found to bind to the fragment from SC1 but not to the similar promoter region on SC2. Surprisingly, sequencing from this binding region from infected psyllids DNA suggests that the LC2 binding region exhibits host dependent variability. The SC1-LC2 promoter region was fused in both directions with a GFP reporter and the constructs were transformed into *E.coli* and *Liberibacter crescens* (Lcr). The SC1-LC2 direction promoter exhibited significantly higher activity than any other Las promoter tested in Lcr. Moreover, a plasmid expressing SC1-LC2 in Lcr caused repression of the SC1-LC2 promoter in the LC2 direction. The expression levels of genes flanking these bidirectional SC1 promoter regions were examined by qRT-PCR of RNA extracted from Las-infected citrus, periwinkle and Asian citrus psyllids (ACP). Late direction genes (SC1-LC2 and SC1gp-175) were completely repressed in ACP and citrus, while in the same organisms little repression was observed for the early genes. No repression of any of these phage genes was observed in periwinkle, where SC1 phage particles readily form. Aqueous extracts of periwinkle and citrus

failed to interfere with LC2 repressionin these assays.

Non-technical summary: *Candidatus* Liberibacter asiaticus strains harbor active bacteriophages in their genomes. SC1 has the ability to lyse the bacterial cells in planta, representing a potential tool to control HLB, but its regulatory mechanisms are largely unknown. In this work, the SC1-LC2 repressor is demonstrated to be functionally active, regulate its own expression and have a binding site in a late gene promoter region known to be differentially regulated in psyllid and plant hosts.

Citations:

Zhang, Shujian, et al. "'Ca. Liberibacter asiaticus' carries an excision plasmid prophage and a chromosomally integrated prophage that becomes lytic in plant infections." *Molecular Plant-Microbe Interactions* 24.4 (2011): 458-468.

IRCHLB-P1- 03

Contextualization of CLas Expression Data using Metabolic Models to Obtain a Deeper Understanding of HLB Pathology

Beth B. PEACOCK[1], Cristal ZUNIGA[2], Paul M. RUEGGER[1], Greg MCCOLLUM[3], Karsten ZENGLER[2], and James BORNEMAN[1]

[1]University of California, Riverside, USA, [2]Univeristy of California, San Diego, USA, [3]U.S. Horticultural Research Laboratory, Agricultural Research Service, USDA, USA

Abstract: Huanglongbing (HLB) is a citrus disease associated with the putative bacterial pathogen *Candidatus* Liberibacter asiaticus (CLas), and vectored by the Asian citrus psyllid (ACP) (*Diaphorina citri*). CLas has not yet been grown in *in vitro* culture, limiting research into its pathogenicity. One way to deepen our knowledge of CLas that does not require *in vitro* cultivation is to examine CLas RNA-Seq data in the context of metabolic modeling. Metabolic models are functional mathematical representations of all the metabolic pathways and reactions in a cell. Contextualization of RNA-Seq data with CLas metabolic models enables sample- specific prediction of phenotypic states. This type of analysis greatly extends the information in the RNA-Seq data to include the activity of all of the reactions and pathways in the cell. To deepen our knowledge about CLas and HLB, our group created (reconstructed) metabolic models of six CLas stains (manuscript in preparation). Taking advantage of this valuable resource, RNA-Seq data from citrus and ACP at several time points from ACP egg-laying on citrus flush through ACP maturation to adults will be analyzed in the context of the CLas metabolic methods

Non-Technical Summary: Modern experimental approaches often endeavor to understand complex living systems by creating a list of their parts. This is akin to trying to understand how an airplane flies based on its parts list. In contrast, metabolic modeling determines how all of these individual parts function and interact to create a living organism. We are utilizing a newly constructed metabolic model of the putative HLB pathogen to obtain a much deeper understanding of this pathogen and of HLB pathology.

IRCHLB-02: Cultural Control Oral Presentations

IRCHLB-O1b-01

Citrus Under Protected Screen for grapefruit HLB management in Florida's East Coast

Rhuanito FERRAREZI[1] and Jawwad QURESHI[1]

[1]University of Florida, Fort Pierce, FL, USA

Abstract: Citrus production under protected environments can reduce huanglongbing (HLB) disease incidence and damage by excluding the Asian Citrus Psyllid vector. Our objectives were assess the ability of covered structures to reduce HLB incidence and determine the yield of soil and container- grown 'Ray Ruby' grapefruit at super-high planting densities relative to in-ground open- air trees. We tested two production systems (screenhouse and open-air), two planting systems (in-ground and potted) and two rootstocks (Sour orange and US897). The experimental design was a RCBD split-split- plot with four replications. Trees were planted in Sept/2013 on a density of 1,957 trees/ha (total 896 trees/0.46 ha). Irrigation was performed on-demand using two 7.6- LPH drip emitters per tree, and fertigation was applied three times/week using 15N- 2.6P-22.4K water soluble fertilizer at 180 kg N/ha. Psyllids were detected inside the screenhouses post Hurricane Irma, which damaged the screen structures in Sept/2017, leaving openings until repairs were completed in Apr/2018. HLB diagnosis indicated no disease in the screenhouses and fast disease progression in the open-air, with 100% infection in all outside treatments in Mar/2017. The 2017/18 season yield was greater inside the screenhouses due to the absence of HLB (0.33 boxes/tree; 263 boxes/acre) compared to the outside blocks (0.02 boxes/tree; 18 boxes/acre). Potted trees on sour orange and in-ground trees on US- 897 resulted in higher yield compared to other treatments. Potted grapefruit trees cultivated inside CUPS had the highest soluble solids content. The screenhouses provide for disease exclusion, increased yield, and quality. The cost of the technology is still under evaluation along with structural modifications needed to deal with environmental challenges such as hurricanes in Florida. IRREC CUPS screenhouses are 5- years old, and the screen is experiencing degradation from UV, rainfall, and wind, resulting in screen rupture at several points in the roof, requiring replacement in the near future.

Non-Technical Summary: Citrus production under screenhouses can reduce huanglongbing (HLB) disease incidence and damage by excluding the Asian Citrus Psyllid vector. However, long-term studies evaluation the changes in environmental data, tree size, fruit yield and quality, and economics are still necessary. Our goals were to assess the ability of covered structures to reduce HLB incidence and determine the yield of soil and container- grown 'Ray Ruby' grapefruit at high planting densities relative to in-ground open- air trees. We tested two production systems (screenhouse and open-air), two planting systems (in-ground and potted) and two rootstocks (Sour orange and US897).

IRCHLB-O1b-02

Hydroponically-grown grapefuit Maximize HLB free fresh fruit production in CUPS

Arnold SCHUMANN[1], Laura WALDO[1], William HOLMES[1], Timothy EBERT[1], Napoleon MARINER[1] and Gary TEST[1]

[1]University of Florida, Lake Alfred, FL, USA

Abstract: Loss of grapefruit production due to Huanglongbing (HLB) disease in Florida has been more severe than with round oranges, and the general consensus is that the favorite red and white varieties in commercial production are highly susceptible to HLB. Grapefruit production of both red and white varieties has plummeted in Florida during the past eight years. Production in 2009/10 was 20 million boxes, and in 2017/18 it was 3.88 million boxes, constituting a five times reduction. Growing citrus trees in screen houses ("Citrus Under Protective Screen", CUPS) excludes the Asian citrus psyllid vector and therefore completely blocks transmission of *Candidatus* Liberibacter asiaticus (*C*Las) pathogen. The CUPS production system has for four years completely

avoided psyllid infestations and thereby eliminated HLB from grapefruit trees grown for fresh market fruit. That ensured sustained high yields of greater than 800 boxes per acre per year with the use of hydroponics and high planting densities. Pack-out of near 100% and larger, more lucrative fruit sizes that were common before HLB became widespread in Florida, are now possible with this intensive production system.

Non-Technical summary: A novel high-yielding fresh fruit production system to grow HLB-free grapefruit was developed for Florida citrus growers using the Citrus Under Protective Screen (CUPS) system. Fruit pack- out and yields were boosted by hydroponics and high planting densities to higher levels than were possible before HLB was detected in Florida.

IRCHLB-O1b-03

Individual Protective Covers (IPCs) prevent young citrus trees from psyllids and infection with CLas, and promote vegetative growth

Fernando ALFEREZ[1], Ute ALBRECHT[1], Ozgur BATUMAN[1], James H. GRAHAM[2]
[1]University of Florida, Immokalee, USA, [2]University of Florida, Lake Alfred, USA

Abstract: Controlling HLB by suppressing its psyllid vector is critical especially in young citrus trees. Reducing HLB incidence in young trees is essential for survival and productivity under current endemic conditions in Florida. In addition, there are concerns about increased use of pesticides and the negative effects they pose to human health and the environment. The situation is further exacerbated by increasing levels of insecticide-resistance in psyllids, resulting in even more aggressive pesticide applications. In this scenario, new solutions are urgently needed. Psyllid exclusion is a promising strategy. Although growing citrus under protective screens is effective, many growers cannot afford the cost, and it is economically not feasible for large-acreage juice-orange production. Here, we show that Individual Protective Covers (IPCs) made of a polyethylene screen on young Valencia trees, provide an effective barrier against the insect vector and reduces incidence of HLB infection. In addition, some phenological effects including earlier flushing, more chlorophyll accumulation in leaves, and faster trunk diameter growth were observed when compared to trees planted without IPCs. Vapor pressure deficit was monitored hourly for 9 months and was consistently lower in trees with IPC than non-IPC trees during spring and early summer. Furthermore, photosynthesis was improved, probably as a consequence of prolonged stomata opening. Currently, we are studying if transition from vegetative to reproductive stage will be affected in trees with IPC and if the system is feasible for fresh market varieties as well. Finally, we are assessing the most efficient IPC layouts in the grove, because infection and distribution of HLB are irregular and higher psyllid densities and flush shoot infestation levels are usually observed in trees located at the outer edges of groves ("edge effect").

Non-technical summary: Psyllid exclusion from young citrus trees by using Individual Protective Covers (IPCs) is an efficient way to avoid HLB infection. In addition, IPCs improve phenology of young trees and result in faster tree growth. We are investigating if IPCs affect the transition from vegetative to reproductive stage of the trees and which IPC layout is most effective in hindering disease transmission in a grove environment with irregular vector distribution.

IRCHLB-O1b-04

New approach for enhance the defense against diseases in citrus plants.

Eduardo CANALES[1], Ingrid HERNÁNDEZ[1], Roxana PORTIELES[1], Mayra RODRÍGUEZ[1], Yunior LÓPEZ[1], Osmani GUIROLA[1], Luis

Javier GONZÁLEZ[1], Orlando BORRÁS-HIDALGO[1] and **Meilyn RODRÍGUEZ**[1].

1 Center for Genetic Engineering and Biotechnology (CIGB), Havana, Cuba

Abstract:
Citrus production is one of the most important in the world. However, these plants are very liable to infestation by aphids, whiteflies and scale insects, as well as by viral infections. Today, one of the most recent and devastating diseases is Huanglongbing (HLB) or citrus greening yet no established efficient management measures exist for it. In our previous work, as a possible control strategy for HLB, epibrassinolide (eBL) was applied to as a foliar spray to citrus plants infected with the associated agent of HLB, '*Candidatus* Liberibacter asiaticus'. The bacterial titers were reduced under both greenhouse and field conditions but were stronger in the greenhouse. The application of eBL holds promise to improve the innate immune response to this pathogen and might provide a useful tool within an integrated management program. Taking into account this, we selected novel synthetic compounds using the Computer Assisted Drug Design (CADD) method to develop a strategy for stimulating the defense and induction of the plant disease natural resistance. The results obtained from this study allowed to select a group of synthetic compounds that activate various plants signaling pathways related to immune response, such as, salicylic acid, ethylene and jasmonic acid. Also, the novel synthetic compounds stimulate the systemic acquired response and give the plant a higher level of resistance against subsequent infection by the same pathogen. Finally, we suggested that new synthetic compounds could be used to control '*Candidatus* Liberibacter asiaticus' or other citrus diseases.

Non-Technical summary:
Previously, we demonstrated that the application of epibrassinolide to HLB infected citrus plants reduces the titers of '*Candidatus* Liberibacter asiaticus'. In this study we suggest that the application of synthetic compounds holds promise to improve the innate immune response to this pathogen and might provide a useful tool within an integrated management program.

IRCHLB-O1b-05

Root damage on HLB-affected trees: Consequences and mitigation with soil acidification

Jim GRAHAM[1] and Kelly MORGAN[2]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA, [2]Southwest Florida Research and Education Center, Immokalee, USA

Abstract: *Candidatus* Liberibacter asiaticus moves downward in the phloem and infects roots soon after transmission into shoots. Before canopy symptoms appear, 30-50% of the roots are lost. Root lifespan is decreased from 9-12 to 4 months. What causes HLB root loss is not well understood but is probably due to faster root death rather than fewer new roots. Las infection actually stimulates new root growth in symptomatic trees. Short term gains in root density do not sustain canopy and fruit growth in declining trees due to increasing root growth costs of carbohydrate that would normally be allocated to fruit. Without aggressive management to reduce abiotic and biotic stress, root loss increases to 70-80%. In response to such root loss, the canopy thins due to premature leaf drop and twig dieback. An extensive survey of HLB- affected groves indicated that greater decline in fibrous root health and expression of HLB symptoms is observed where irrigation water is high in bicarbonates (>100 ppm) and soil pH > 6.5. HLB symptom expression of trees on different rootstocks follows the known intolerance to bicarbonate (Swingle citrumelo > Carrizo citrange > sour orange > Cleopatra

mandarin). Over three seasons of survey, acidification of irrigation water in central ridge and south central flatwoods sustained root density as an index of root heath. Irrigation water acidified for 36 months to establish a soil pH range from 4.0 to 7.0 increased leaf Ca, Mg, Mn, and Zn in response to gradual reduction of soil pH in young and mature groves on Swingle with starting soil pH of 7.3. Acidification by soil amendment with elemental sulfur and/or water treatment with sulfuric acid increased yield as soil pH decreased.

Non-Technical Summary: Soil pH affects the ability of HLB damaged roots to extract nutrients under prevalent conditions of high well water and soil bicarbonates in Florida groves. Acidification to lower soil pH in groves with bicarbonate stress increases solubility and uptake of Ca, Mg, Mn, Zn and reverses yield decline, especially for trees on intolerant Swingle rootstock.

IRCHLB-O1b-06

Can additional supply of Ca and/or Mg reduce the progress of HLB incidence, severity and crop loss?

Renato Beozzo BASSANEZI[1], José Antonio QUAGGIO[2], Rodrigo Marcelli BOARETTO[3], Joseph M. BOVÉ (*in memoriam*), Juliano Antonio AYRES[1], Dirceu MATTOS-JR.[3]

[1]Fundecitrus, Araraquara, Brazil, [2]Instituto Agronômico, Centro de Solos e Recursos Ambientais, Campinas, Brazil, [3]Instituto Agronômico, Centro de Citricultura Sylvio Moreira, Cordeirópolis, Brazil.

Abstract: Nutrient supply to improve carbon metabolism in citrus, such as synthesis and transport of sucrose by the phloem, could be used as a management practice to minimize plant damages caused by HLB. This work evaluated the progression of the incidence and severity of HLB, as well fruit yield of citrus trees fertilized with different nitrogen sources and additional supply of Mg. The experiment was carried out in a commercial orchard of Hamlin sweet orange grafted onto Rangpur lime, planted on Dec/02, without irrigation, from Nov/12 to Nov/18. A factorial 2x2 (Source of N x Addition of Mg) was set up in a randomized block design, with four replications. Treatments were: T1 = NPK (doses according to Quaggio et al. (2010), with N as ammonium nitrate); T2 = NPK (according to T1, with N as calcium nitrate); T3 = T1 + Mg (4 applications via foliar and/or soil as sulfate); T4 = T2 + Mg (according to T3). All treatments were supplied with micronutrients. Incidence of HLB- symptomatic plants was evaluated every six months and disease severity was evaluated bimonthly in 50 plants/plot (30 marked in Feb/13 and 20 marked in Mar/14). Fruit yield of diseased and healthy plants was measured. Treatments did not prevent infection and expression of HLB symptoms in new plants. The mean disease incidence in the orchard increased from 18.7% to 56.9%, with no difference between treatments. Similarly, treatments did not prevent reduction the area of plant canopy with disease symptoms and symptoms from progressing to other parts of the plant canopy. Fruit yield of healthy plants varied accordingly to climatic conditions in every harvesting season. Diseased plants produced less fruits than healthy plants year after year, accounting for 61% less in 2018. Project supported by FAPESP (2015/13572- 8).

Non-Technical Summary: This research evaluated whether the supply of Ca (as calcium nitrate) and/or Mg (as magnesium sulfate) could reduce the progression of the incidence and severity of HLB and the losses of production in a 10-year-old commercial Hamlin/Rangpur lime orchard. After six years, none of tested treatments prevented infection and expression of disease symptoms in plants initially asymptomatic and did not prevent or reduce the progression of the disease symptoms on canopy of symptomatic trees. Fruit yield of diseased plants was always lower than that of healthy plants, which reduced year after year (from 28% in the first year to 65% lower in the last year), regardless of the treatment.

Citations:

QUAGGIO, J.A.; MATTOS Jr., D.; BOARETTO, R.M. Citros. In: Prochnow, L.I.; Casarin, V.; Stipp, S.R. (Org.). Boas práticas para uso eficiente de fertilizantes. Piracicaba: Internation Plant Nutrition Institute, 2010, v.3, p.373-412.

IRCHLB-02: Cultural Control Poster Presentations IRCHLB-P2-04

Anaerobic soil disinfestation impacts the soil microbiome and growth of citrus trees infected with *Candidatus* Liberibacter asiaticus

Greg MCCOLLUM, Jason HONG, Randy NIEDZ, and Erin ROSSKOPF
USDA, ARS, Fort Pierce FL, USA

Abstract: Numerous management strategies are currently being evaluated in an effort to maintain citrus productivity in trees affected by HLB. Anaerobic soil disinfestation (ASD) is a treatment that has been shown to improve productivity of vegetable crops. ASD involves amending the soil with a carbon source and an organic nitrogen source, covering the soil with gas-impermeable plastic mulch followed by irrigation to saturation. During treatment, the soil microbiome is shifted to facultative and obligate anaerobes. An experiment was designed to determine ASD effects on newly-planted citrus. The experiment compared soil -/+ ASD as the main treatment and rootstock as the subtreatment. Following ASD, Valencia orange scions grafted onto each of three rootstocks (Swingle citrumelo, Sour orange, or Kuharske citrange) were planted. Two trees on each rootstock were planted in four replicates per treatment. The experiment was conducted twice with one planting established in 2013 and the second in 2014 at the USDA research farm in St. Lucie Co., FL, an area with intense ACP pressure and high incidence of HLB. Within two years after each planting, 100% of the trees in each planting were infected with *Candidatus* Liberibacter asciaticus (CLas) and expressed HLB symptoms. There were no differences in incidence of HLB between ASD treatments or rootstocks. However, both ASD and rootstock had significant effects on the soil microbial community. Rootstock had no impact on tree growth or productivity, but tree growth, as measured by stem and canopy diameter was greater in ASD treated plots vs non ASD- treated plots. In addition, there was ca. a 17% greater fruit yield in response to ASD treatment at the first harvest. ASD treatment is beneficial for citrus even when declining as a result of CLas infection.

Non-technical summary: Anaerobic soil disinfestation (ASD), a treatment that alters the soil microbial community has beneficial effect on citrus affected by HLB. Although the treatment does not impact incidence of CLas infection, trees growing in ASD-treated plots grew and produced more fruit than those in non-treated plots. ASD may be of value for establishing productive citrus trees even with inevitable infection with CLas.

IRCHLB-P2-05

Assessing CLas viability to determine the efficacy of steam-generated thermotherapy.

Naweena THAPA[1]**,** Evan G. JOHNSON[1] and Megan M. DEWDNEY[1]

[1] University of Florida, Citrus Research and Education Center, Lake Alfred, Florida, USA.

Abstract: Florida's citrus production has declined by over 70% in recent years, mostly due to Huanglongbing (HLB). Thermotherapy on HLB-affected trees was proposed to be a short-term management solution to maintain field

productivity. Our hypothesis for this study was that specific time-temperature combinations could eliminate HLB from the affected branches. The objectives of this study were to show which time-temperature combinations eliminated CLas from small diameter woody tissues. Just hardening, rounded Valencia twigs were collected from HLB-affected field trees. A steam chamber was used to treat branches at different time- temperature combinations (50°C-60s, 55°C- 0s, 55°C-30s, 55°C-60s, 55°C-90s, 55°C-120s, 60°C-30s, and an untreated control (UTC)). Three independent repetitions of 13 branches/treatment were used. Bark samples from each branch were collected before and after steam treatment and stored at -80°C for RNA/DNA extraction and viability assay. Remaining branches were grafted the next day, onto healthy rootstocks in the greenhouse, and tested for CLas detection after 6, 9 and 12 months; however, not all grafts survived, particularly at more extreme time-temperature treatments. For RNA-based viability assay, several genes (*16S rRNA*, *rpoB*, *rpl*, *dnaK*, *groEL*, *nrdB*, and *SDE1*) were targeted to analyze the gene expression patterns. CLas *16S* was highly stable and not suitable for the viability assay, whereas low transcriptional activity of other genes did not allow detection of the differential transcription. There was significantly lower qPCR detection of CLas DNA in the surviving grafted plants after 9-months of treatment with 55°C-90s (2/9) and 55°C-120s (0/11) ($\chi^2 = 34.36$, $P < 0.0001$). Treatment 60°C-30s was lethal to the branches. For the UTC, 50°C-60s and 55°C-0s, over 75% of the plants remained CLas-infected. Further monitoring is necessary to confirm reliable CLas elimination from branches with the minimum time-temperature of 55°C-90s and 55°C-120s. Thicker branches may need more treatment, limiting efficacy of field thermotherapy treatment.

Non-technical Summary: Steam heat treatment of HLB-affected plants has an impact on the CLas population, harbored in the phloem. With the objective to determine the optimum time-temperature combination to eliminate CLas with minimal effect to the plant, we explored several bacterial genes to develop a viability assay and determine the efficacy of the treatments. Additionally, branches that were grafted as bud sticks, treated at different time-temperature combinations, were tested for the presence of the bacterium after 6, 9 and 12 months of grafting. Only the most severe treatments eliminated bacteria from small young branches, which may be useful in propagation of infected scions, if not in field application.

IRCHLB-P2-06

Benefits of biochar on HLB-affected citrus in sandy soils

Andrea NUZZO[1], Arnold SCHUMANN[2], Davie KADYAMPAKENI[2], and **Sarah L STRAUSS**[1]

[1]University of Florida, Immokalee, FL, USA,
[2]University of Florida, Lake Alfred, FL, USA.

Abstract: Citrus greening (or Huanglongbing, HLB) is an endemic disease affecting citrus trees in Florida and causing severe impacts on production. Affected trees show an overall decrease in water and nutrient uptake, caused by root damage. Such issues are amplified by the conditions of South Florida’s soil, often showing low activity of resident microbiota due to the lack of micronutrients, low water retention, and high pH. This greenhouse project aimed to increase micronutrient availability to roots of affected trees by increasing the soil microbiota (Bacteria and Fungi) diversity and activity through the application of biochar or organic matter (compost) and testing different irrigation rates (100% and 75% evapotranspiration rates). Preliminary analysis after 6 months of treatment showed that diversity of soil microbiomes sampled in proximity of affected trees increased

to the same values found in proximity of healthy trees, particularly for the 100% irrigation rate. While treatment eigenvectors explain only 19% of the differences among samples, application of biochar had the greatest impact on the soil microbiome, especially of healthy trees ($p < 0.05$). This could be due to higher concentrations of Ca and Mg in those soils compared to no-treatment controls. Concomitantly, and irrespective of tree disease status, biochar soils showed greater abundances of *Xanthomonadales, Corynebacteriales, Sphingobacteriales, Rhizobiales* orders and *Ascomycota* class compared to controls. This might indicate an increase of potentially beneficial species in the biochar-amended soils. Biochar appears thus to have several beneficial impacts on HLB-affected citrus trees in sandy soils, including micronutrient availability and enrichment of potentially beneficial microbes as well as providing a suitable matrix for microbial growth. Further studies are in progress to confirm these findings after 18 months and to predict the impact of these treatments on the metabolic diversity of the soil microbiome.

Non-Technical summary: HLB-affected citrus can have reduced water and nutrient uptake, which in South Florida, is exacerbated by soils with low microbial activity due to low micronutrient availability, low water retention, and high pH. In a greenhouse experiment, we examined whether adding biochar or compost along with different irrigation rates would improve micronutrient availability and microbial activity. After 6 months, biochar improved both soil micronutrient concentrations and microbial diversity compared to non-treated soils. In addition, there were more potentially beneficial microbes in the biochar-amended soils compared to the controls.

IRCHLB-P2-07

Can negative effects of HLB be mitigated by calcium and magnesium fertilizations in citrus trees?

Camila de Oliveira MILANI, Veronica Lorena DOVIS[1], Franz Walter Rieger HIPPLER[2], José Antonio QUAGGIO[2], Rodrigo Marcelli BOARETTO[1], Helvécio Della COLETTA-FILHO[1], **Dirceu MATTOS-JR**[1]

[1]Instituto Agronômico, Centro de Citricultura Sylvio Moreira, Cordeirópolis, Brazil, [2] Yara Brasil Fertilizantes SA, Sumaré, Brazil, [3]Instituto Agronômico, Centro de Solos e Recursos Ambientais, Campinas, Brazil,

Abstract: We considered the interactive effects of Ca and Mg supplies on a variety of important physiological and growth traits in citrus trees infected with *Ca.* Liberibacter asiaticus' (CLas). Healthy (-) and CLas infected (+) 'Valência' orange trees grafted onto 'Swingle' citrumelo were exposed to one of four nutrient treatments for 210 days: (1) Control: plants not fertilized with Ca and Mg;
(2) Ca: plants substrate fertilized with Ca(NO3)2.5H2O (14.2 g of Ca); (3) Mg: plants foliar (0.4 g of Mg) and substrate (3.3 g of Mg) fertilized with MgSO4.7H2O; and (4) Ca+Mg: plants fertilized with Ca(NO3)2.5H2O and MgSO4.7H2O at the same amounts described for Ca and Mg treatments. Moreover, all plants were equally supplied with 10.0 g of N,
0.9 g of P, 8.0 g of K. Cycle threshold values (CT) were determined by qPCR[1] and used to verify the titer of CLas in laves of infected plants (CT $\approx 18.36^{\pm 0.50}$). Impairment of tree growth was observed in (+) plants, regardless of nutrient treatments. However, greater net photosynthetic rates and stomatal conductance were observed in (+) Ca in relation to (+) control. Likewise, (+) Ca and (+) Ca+Mg presented higher electron transport rates (ETR) than (+) control. HLB infection did not damage or inactivate PSII reaction centers since all treatments presented maximum quantum yield of primary photochemistry (Fv/Fm) higher than 0.79. Thereby, no signs of photoinhibition were

observed and hence, no excess of energy was available to increase reactive oxygen species production in (+) plants. Thus, no clear trends were observed in both H2O2 and MDA concentrations in response to treatments. Furthermore, higher photosynthetic capacity of (+) Ca plants increased starch transport from shoots to roots. Thus, Mg and specially Ca fertilizations alleviated HLB negative effects on photosynthetic capacity and starch metabolism in citrus trees.

Non-technical summary: HLB is mostly associated to the presence of a phloem-limited bacterium named as CLas. Typical symptoms of HLB are corked central vein and flushes with yellowish/blotchy leaves. Such symptoms are believed to be caused by phloem plugging, which prevents sugar transport from shoots to roots, causing starch accumulation in leaves and root decline. We found that supply of Mg and Ca as a complement to current fertilizations for citrus trees likely reduce the negative effects of CLas on key physiological traits of citrus trees.

Citations:

Coletta-Filho et al., *Phytopathology* **104**, 416-421 (2014).

IRCHLB-P2-08

Citrus nutrient uptake, biomass accumulation and root density patterns as influenced by citrus greening and regulated deficit irrigation under greenhouse conditions

Davie KADYAMPAKENI[1]

[1]University of Florida, Lake Alfred, USA.

Abstract: Florida citrus production accounts for $8 billion of economic activity and represents about 49% of production in the US. Potential for improving citrus tree performance lies in the ability to develop appropriate water management strategies, good nutrient management options, and the restoration of soil and root health to optimize water and nutrient uptake. Current citrus production is at a historical low due to the devastating impact of citrus greening (aka Huanglongbing, HLB) which triggers tree defoliation, root loss, fruit drop and canopy decline. A study was conducted in the greenhouse on 2-year old Hamlin oranges to: 1) determine citrus nutrient and biomass accumulation of HLB-affected and non- affected trees; and 2) determine the effect of irrigation rate on root density of HLB-affected trees. A total of 72 trees were healthy and 72 were HLB-positive by graft-inoculation of the *Candidatus* Liberibacter asiaticus (CLas) associated pathogen. Nitrogen fertilization rates included UF Institute of Food and Agricultural Sciences (IFAS) recommended rate, and 75% and 125% of IFAS recommended rates. Irrigation rates were applied at 75% and 100% evapotranspiration (ET). Results showed that nutrient accumulation and biomass was severely limited for HLB-affected and non-HLB affected trees receiving 75% of the recommended IFAS rate. Tree performance with respect to canopy size, leaf area and trunk cross-sectional area showed limited growth for HLB-affected trees compared with non- HLB affected trees receiving 75% ET irrigation rate. No differences in root density were observed between irrigation and fertilization rates, but lower root densities were noted on HLB positive trees, as expected. These results suggest that it critical to maintain macronutrient supply at optimal level or 25% above current recommendations while maintaining irrigation at 100% ET using a daily schedule to avoid limiting tree performance in HLB-affected trees.

Non-Technical Summary: Tree growth for HLB-affected trees was limited compared with non-HLB affected trees receiving reduced amount of water for irrigation. No differences in root density were observed between irrigation and fertilization rates, but lower root densities were noted on HLB positive trees, as expected. These results suggest that it critical to maintain nutrient supply at optimal level or slightly above current recommendations while maintaining irrigation at recommended rate using a daily schedule to avoid limiting tree performance in HLB-affected trees.

IRCHLB-P2-09

Development of a predictive screening process including field assessment for identification of potential commercial therapies for HLB

Denise MANKER[1], Jean BROADHVEST[1], Thomas KNOBLOCH[2], Bryce FALK[3], Sandra VU[3], Ozgur BATUMAN[4]

[1]Bayer Crop Science, Biologics, West Sacramento, CA USA, [2]Bayer Crop Science, Biochemistry and Biotechnology, Lyon, France, [3]University of California, Davis, USA, [4]University of Florida, Immokalee USA

Abstract: The collaboration between Bayer and the Citrus Research and Development Foundation (CRDF) involves a systematic approach to develop a validated, predictive screening cascade leading to a process for high-throughput in vitro discovery followed by greenhouse testing to allow for selection of promising candidates for field-testing. Important goals include determining the best predictive in vitro model for HLB and optimizing in planta assays with Liberibacter on both tomatoes and citrus to help predict eventual field activity. Leads funneled into the screening cascade come from two main sources: 1) microorganisms to target reduction of *Candidatus* Liberibacter asiaticus (CLas) and 2) synthetic compounds to boost the immunity of citrus trees to minimize bacterial infection. Bayer has expertise with both approaches for different crops and diseases. Initial field investigations of promising leads will begin in 2019 and the design and approach of these replicated studies will be discussed.

Non-Technical Summary: Progress on an industrial approach to developing a predictive screening process that allows for identifying promising treatments for HLB from lab bench to field will be presented. Potential leads selected from naturally occurring microbes that can decrease HLB infections and synthetic compounds that turn on the citrus trees' immune system to fight off the disease will be evaluated in field trials to determine their commercial potential for eventual use with other technologies such as resistant varieties and insecticide regimes.

IRCHLB-P2-10

Droplet deposition and control efficacy of pyriproxyfen sprayed with an unmanned aerial vehicle against citrus psyllid *Diaphorina citri*

Li CUI[a], Peng HAN[a], Qinqin WANG[a], Ming WANG[a], Saichao GAO[a], Huizhu YUAN[a]

[a] Key Laboratory of Integrated Pest Management in Crops, Ministry of Agriculture, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, 100193, China

Abstract: The small unmanned aerial vehicle (UAV) that can spray pesticides with high efficiency and with no damage to crops is required for the timely and effective spraying of small fields and/or those in hilly mountains. This study aimed to illuminate the droplet density and droplet deposition of the UAV on citrus canopy and control efficacy against citrus psyllid. Droplet deposition of pyriproxyfen (at a dose of 150 g a.i. ha^{-1}, spray volume of approximately 60 L ha^{-1}) was collected using water-sensitive paper. When the spraying height was 2 m, the droplet density on the upper external, upper internal, lower external and lower internal were 97.2, 78.0, 87.9 and 8.6 droplet cm^{-2}, with the variation coefficient of 16.0%, 21.5%, 15.3% and 11.0%, respectively. And the insecticide

deposition on the upper part and lower part of citrus was 2.39 and 1.23 μg cm^{-2}, with the variation coefficient of 37.5% and 57.3%. However, the insecticide deposition on the upper part and lower part of citrus was 0.98 and 1.74 μg cm^{-2} for the manual knapsack sprayer. The results showed that UAV spraying could reach a high droplet density and deposition. Therefore, the control efficacy of pyriproxyfen sprayed by UAV against the nymph of citrus psyllid was 64.0% 3 days after treatment (DAT). And the efficacy was up to 81.7% 20 days after treatment. The control efficacies of pyriproxyfen sprayed by manual knapsack sprayer were 61.4% and 89.3% 3 DAT and 20 DAT, respectively. We can conclude that UVA is suitable for the control of citrus psyllid.

Non-Technical summary: UAV is suitable for working in rough terrain and small plots with high efficiency, high automaticity, less flight crew and low labor intensity. Our results indicated that the use of UAV provides a useful operating platform for controlling against citrus psyllid compared with traditional insecticides application.

IRCHLB-P2-11

Effect of soil and irrigation water pH on physiology of Huanglongbing-affected sweet oranges

Tripti VASHISTH and Lushan GHIMIRE

Citrus Research Education Center, University of Florida, Lake Alfred, FL 33850

Abstract: Field observations in Florida have shown that Huanglongbing (HLB)-affected trees becomes heavily symptomatic when grown in high soil pH groves or irrigated with high pH water. Irrigation water with higher than 100 ppm bicarbonates concentration exacerbates the productivity of HLB-affected trees and results in significant pre-harvest fruit drop. Therefore, this study was conducted to understand the interaction between soil/irrigation water pH and HLB-affected plants. In this greenhouse study, 1.5 year old ‘Midsweet’ on ‘Kuharske’ healthy and HLB-affected plants were irrigated with water at pH 5.8, 7, and 8 for 2 months. At the end of the study, 38% of HLB-affected plants irrigated with pH 8 water were dead; this treatment plants also suffered highest leaf loss among all the treatment combination. HLB-affected plants irrigated with water at pH 7 and 8 suffered significantly more leaf loss as compared to their complementary healthy plants. HLB-affected trees irrigated with pH 5.8 water showed significantly lower leaf drop than healthy trees. The leaf chlorophyll content significantly decreased with increase in irrigation water in both HLB and healthy plants. Both healthy and HLB-affected plants irrigated with water at pH 5.8 showed lower electrical conductivity in soil as compared to pH 7 and 8 treatments suggesting that nutrient uptake in former plants was higher. HLB- affected plants irrigated with water at pH 8 showed highest concentration of reactive oxygen species in roots indicating a higher level of stress on the plant. Interestingly, no significant difference was observed in leaf nutrient analysis among all the 3 pH treatment and two health conditions, suggesting that soil pH influences physiological traits other than nutrient availability. A transcriptomic analysis is underway to understand this interaction. Overall, this study supports the field observation; HLB-affected plants do not perform better under high pH soil conditions, HLB plant performs best around pH 6.

Non-Technical Summary: HLB-affected plants irrigated with high pH water do not perform well. High mortality and leaf drop can be observed in plants that are irrigated or grown under high pH conditions. HLB- affected plants perform better when irriated or grown at soil pH 6. The effect of high irrigation water pH is more dramatic in HLB- affected plants as compared to healthy plants. Therefore, growers are suggested to maintain their soil pH around 6.

IRCHLB-P2-12

Effects of Huanglongbing Disease on Mature Fruit Detachment Force and Expression of Genes Related to Fruit Abscission in 'Valencia' Sweet Orange

Lisa TANG and **Tripti VASHISTH**

Citrus Research and Education Center, University of Florida, IFAS, Lake Alfred, FL

Abstract: Huanglongbing (HLB)-associated pre-harvest fruit drop can result in significant yield reduction. The underlying mechanism of HLB-associated pre-harvest fruit drop is still unknown. In this experiment, field-grown 'Valencia' sweet orange (*Citrus sinensis*) trees displaying severe symptoms of HLB (S trees) had 74% fruit drop prior to harvest, whereas the drop rate for less symptomatic ones (L trees) was 45%. For mature fruit (3 weeks before harvest) still attached on the tree, 60% of those from S trees were "loose fruit" (fruit detachment force [FDF] < 6 KgF). In contrast, only 13% of attached fruit from L trees were loose. Irrespective of the symptom levels of trees, the concentration of glucose, fructose, and glucose in juice was greater in loose fruit than "tight fruit" (FDF > 6 KgF), suggesting the shortage in carbohydrates is not the dominant cause of HLB-associated preharvest fruit drop. Expression levels of cell wall remodeling genes encoding cellulase (endo-1,4-β-glucanase), polygalacturonase, and pectate lyase were greater in calyx abscission zones (AZ-C) of loose fruit than tight fruit, indicating cell separation was in process in the former at the time of collection. No difference in expression levels of genes encoding the ethylene biosynthesis enzymes, including 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO), and *ETHYLENE RESPONSE FACTOR1* (*ERF1*) were observed in leaves of loose versus tight fruit. Interestingly, *ACS* and *ACO*, and *EFR1* expression was lower in AZ- C and leaves of S trees compared to those of L trees, respectively. These results suggest a HLB-dependent reduction in ethylene biosynthesis and/or signaling, but the role of ethylene in HLB-associated preharvest fruit drop remains to be determined. The results leave open the possibility of early ethylene action prior to the action of fruit abscission.

Non-Technical Summary: HLB-associated pre-harvest fruit drop can significantly reduce the yield. This fruit drop increases with the severity of HLB; trees showing higher HLB symptoms undergo a significantly higher fruit drop. We found that carbohydrate shortage to fruit is not the sole cause of fruit drop.

IRCHLB-P2-13

Field evaluation of integrated management for mitigating citrus huanglongbing in Florida

Muqing ZHANG[1,2,3], Chuanyu YANG[1,2], **Charles A. POWELL**[2], Pasco B. AVERY[2], Jihua WANG[1,2], Youzong HUANG[1,2], Yongping DUAN[3*]

[1] Guangxi University, Nanning, Guangxi 530005, China; [2] University of Florida, Fort Pierce, FL 34945, USA; [3] USHRL, U.S. Department of Agriculture, Fort Pierce, FL 34945, USA

Abstract: We implemented an integrated strategy that includes chemotherapy, thermotherapy, and additional nutrition treatment in three field trials over three years. In these trials, only trees already showing HLB symptoms with Ct values ranging from 25.1 to 27.7 were selected for treatments. Several methods were used for evaluating the effectiveness of integrated management, including the slopes (b) of the Ct increase (dy/dt), the pathogenic index (PI) and the decline index (DI) from Ct value and tree scores, and the antimicrobial efficacies from PI and DI. This comprehensive analysis showed that most of the

tested chemicals were effective to some degree in killing or suppressing the Las bacterium, with higher efficacies seen for Grove B, where citrus trees were severely affected by HLB, and they had a higher number of psyllids, relative to Grove E and P in the first two years. Trunk-injected penicillin G potassium (PEN) was the most effective chemical treatment in all groves, followed by Oxytetracycline Calcium Complex (OXY), and Silver Nitrate (SN) delivered as foliar sprays. Although mobile steam heat treatment and additional nutrition did not eliminate or suppress Las bacteria over the long term, these treatments did positively affect tree growth and recovery in the short term.

Non-Technical Summary:
Citrus huanglongbing (HLB) is extremely difficult to control since the Psyllid-transmitted bacterial pathogen resides inside the citrus phloem and the disease is systemic. In Florida, the nine billion dollar citrus industry has been significantly impacted by severe HLB epidemics. Integrated management of chemotherapy coupled with thermotherapy and additional nutrition represents a realistic, economical, short-term and possibly long- term approach to combat HLB. Therefore, our results provide new insights into HLB control methods and strategies for integrated management for HLB epidemic plantations.

IRCHLB-P2-14

Flush Phenology Manipulation by Naphthalene Acetic Acid and Gibberellic Acid Application in Sweet Orange (*Citrus sinensis L.* Osbeck).

Sheng-yang LI, Christopher VINCENT, and Kirsten PELZ-STELINSKI

University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL 33850

Abstract: New flush is central to the spread of HLB, and manipulation of flush phenology could lead to more effective coordination of vector management. We initiated a series of experiments to ascertain the effects of Plant Growth Regulators (PGRs) on flush phenology. In the first greenhouse experiment, Naphthalene Acetic Acid (NAA) and Gibberellic Acid 3 (GA3) were applied at 5 rates: 0.1x, 0.3x, 1x, 3x, 9x, where x is the typical tissue concentration of each compound. All plants treated with foliar applications of 21.6 ppm of GA3 produced new flushes by 5 weeks after application. For NAA, the highest rate (11.7 ppm) was effective in delaying flush and reducing flush number. The highest rate of NAA was 11.7 ppm in the first study, so the second study was initiated to assess the effects of higher rates of NAA (0, 12, 60, 300, 1000 ppm) to either soil or canopy. Foliar NAA at 60 and 300 ppm and soil-applied NAA at 300 and 1000 ppm delayed flush by approximately 2 weeks. In both greenhouse studies, a pulsing pattern of new flush emergence was apparent, indicating that the age of the previous flush might play a role in the induction of new flush. We implemented a field study testing the effects of GA3 on subsequent flushing when applied 4, 6, 8, or 10 weeks after an initial flush. GA3 only induced a new flush at 8 weeks. Flushing percentage in 8-week-shoots was significantly higher than in control shoots, but only achieved a 10.1% re-flushing rate. Meanwhile, we observed that more flushes emerged from basal buds or previously dormant buds in woody tissue, but not from the previous flush. We conclude that flush induction likely results from whole plant signals, rather than individual bud maturity. Overall conclusions are that traditional PGRs can be used to delay or hasten flushing patterns, with promise for better-coordinated pest management.

Non-Technical Summary: Naphthalene Acetic Acid (NAA) and Gibberellic Acid 3 (GA3) can delay or induce new flush, respectively, which means that we can predict the flushing time more precisely. The flush emergence pattern occurs in pulses, but the critical flush age in response to GA3 is still unclear and needs further study. The better understanding of flush modulation may contribute to psyllid control work in the field.

Citations:

Krajewski, A.J. and E. Rabe. 1995. Bud age affects sprouting and flowering in clementine mandarin (Citrus reticulata Blanco). HortSci. 48:920-928.

IRCHLB-P2-15

Fruit quality of orange trees, cultivar Valencia, infected with HLB submitted to different nutritional treatments.

Andre Luis Teixeira CRESTE[1], José Eduardo CRESTE[2], Guilherme BARONI[1].

[1]Companhia Agrícola São Jose, Brazil, [2]Unoeste University, Brazil.

Abstract: Valencia orange trees, infected with HLB, present fruits with low fruit quality, with changes in Brix, Ratio, Vitamin C and their minerals. In this light, fruits of sick plants at different stages of HLB infection, classified with grades 1 to 3, were submitted to four nutritional treatments containing different levels of Potassium and Calcium and compared to fruits of healthy plants. The plants were treated during a whole productive cycle, from blooming to harvesting, pulverized monthly with these elements in the form of nitrate. Plants with infection levels of 1 to 2, submitted to treatments with high levels of potassium and calcium did not have the quality of the affected fruit. Plants with infection level 3 presented decreased values of the evaluated parameters when submitted to different treatments, but with much higher values when compared to the plants that received no treatment. The treatments showed that the contribution of potassium and calcium during the period of formation and maturation of the fruit significantly interferes in the quality gain and maintenance of the nutritional levels of the fruit. The vitamin C content did not change in any of the samples evaluated.

Non-Technical summary: Plants infected with HLB present low fruit quality. Some nutrients have a fundamental role in the formation of fruits and also in the quality of their juice. Our objective was to evaluate the influence of Calcium and Potassium on fruit formation and its quality parameters.

Citations:

Aubert, B. (1990). Integratedactivities for thecontrolofhuanglungbin-greeningand its vector DiaphorinacitriKuwayama in Asia. In B. Aubert, S. Tontyaporn& S. D. Buangsuwon (Eds.), RehabilitationofCitrusIndustry in theAsia Pacific Region (pp. 133–144). Rome: UNDP-FAO.

Bassanezi, R. B., Montesino, L. H., Busato, L. A., &Stuchi, E. S. (2006b). Damagescausedbyhuanglongbingonsweetorangeyieldandquality in São Paulo. In ProceedingsoftheHuanglongbing – GreeningInternational Workshop, Ribeirão Preto, 2006 (p.39). Fundecitrus, Araraquara.

Di Giorgi, F., Ide, B. Y., Dib, K., Triboni, H. R., Marchi, R. J., & Wagner, R. L. (1993). Qualidade de laranja para industrialização. Laranja, 14, 97–118.

IRCHLB-P2-16

Getting Out of the HLB Jar of Pickles – A Texas Model

Mani SKARIA, PhD[1], Ron SKARIA, MD, MBA[2], and Cheri ABRAHAM, PhD[3]

1. Founder and CEO of US Citrus company and Professor Emeriti at Texas A&M University-Kingsville.
2. Marketing Director, US Citrus.
3. Operations Manager and Entomologist, US Citrus.

Abstract: A 2018 study reported by the National Academy of Sciences (NAS) called for a master plan for HLB management. It stated that multi-million dollars were spent on research effort of

many scientists since 2005. Research effort produced a substantial volume of scientific knowledge on the pathogen and vector, but no real solutions for growers. In Texas, the first HLB-infected orchard ranks among the top fruit producers in Hidalgo County, in the Rio Grande Valley. After the HLB find, the citrus blocks received special care in plant nutrition and controlling psyllids. TAMUK Citrus Center and the Texas citrus industry efforts, along with grower practices reportedly brought an area-wide psyllid control. Reduced fecundity of the vector and higher heat index in the Rio Grande Valley seem to be helping towards limiting severity of vector populations, disease spread, and tree decline. In addition to the Valley-wide common factors, we at the US Citrus company has established several innovations. We have established the following five advantages over conventional practices. 1) From decision making to planting a new block of citrus in the field takes only 1/6th of waiting time compared to the conventional 18 months. 2) Plant cost savings of up to 50% allows higher density planting.

3) Inherent quality of precociousness in micro-budded plants bring commercial fruit harvest 40-50% faster 4) Higher-density plants bring higher yield per acre. 5) Economic stabilization of the orchard happens in 7 years compared to 15 years for conventional plantings. Our experience with multiple varieties of citrus in more than a decade indicates hope for a profitable citrus production even under HLB conditions. Our lead in this area has attracted young professionals into citrus production, even under an HLB scare. Elpis would pleasantly smile at growers with applied strategies to bring immediate and practical HLB management for profitability.

Non-technical summary: Multi-pronged grower adaptations via higher density planting, using innate values of micro-budded citrus has proven to bring quicker and higher profitability in citrus even under HLB conditions in Texas.

IRCHLB-P2-17

HLB differential response of ‘Valencia' sweet orange grafted on several citrus rootstocks in a endemic area

Jardel RODRIGUES[1], **Eduardo STUCHI[2, 3]**, Eduardo GIRARDI[3], Alécio MOREIRA[3], Everton CARVALHO[1], Francisco LARANJEIRA[3], Renato BASSANEZI[4]

[1]Unesp – Campus de Jaboticabal, Jaboticabal, Brazil, [2]Estação Experimental de Citricultura, Bebedouro, Brazil, [3]Embrapa Mandioca e Fruticultura, Cruz das Almas, Brazil, [4]Fundecitrus, Araraquara, Brasil

Abstract: Huanglongbing (HLB) is the most critical and devastating citrus disease worldwide. So far, there are no effective curative measures, and all commercial citrus varieties are susceptible to disease. In this study, we evaluated the performance of 'Valencia' orange trees grafted on 16 citrus rootstocks in an HLB endemic area under rain-fed conditions. The experiment was carried out for six years (2011-2017) in Bebedouro, Sao Paulo State, Brazil, in spacing 6.0 m (between rows) x 2.5 m (between trees) in a completely randomized experimental design using 30 replicates for each rootstock. The canopy volume, flushing shoots per canopy surface area (m^2), and HLB incidence were monthly evaluated. Results showed lower HLB incidence in scion trees grafted onto Flying Dragon trifoliate orange (FD) rootstock (10.3%) than Rangpur lime (RL) (45.2%) and Swingle citrumelo (SC) ones (20.0%), the most planted in São Paulo citrus belt. Scion varieties grafted on FD rootstock showed the highest cumulative proportion of asymptomatic trees after 73 months of evaluation, even though equivalent to those grafted on SC. Also, the FD trees showed the lower canopy volume trees (1.6 m^3) comparing to RL (7.4 m^3) and SC (6.1 m^3), and lower flushing shoots per canopy surface area. Our results suggest that scion varieties grafted on low vigor inducing rootstock are less exposed to *Diaphorina citri*, the psyllid vector of the bacteria associated with HLB, and have potential

use for HLB endemic areas.

Non-Technical Summary: Huanglongbing (HLB) incidence is closely related to the occurrence of citrus tree flushing shoots and *Diaphorina citri*, the vector of the bacteria associated with the disease. The combination of different rootstock and scion citrus varieties induces trees with different canopy volume and flushing shoot intensity, which affect the *D. citri* population and the exposure for bacteria inoculation. Here, we are investigating if scion citrus varieties grafted on low vigor inducing rootstocks are less exposed to *D. citri*, thereafter that impact on HLB incidence in endemic areas. Findings may be useful for HLB management in those areas.

IRCHLB-P2-18

Huanglongbing severity and AUDPC in mandarin genotypes grafted onto three rootstocks and grown under field conditions in Isabela, Puerto Rico

Diana Intriago MENDOZA[1], **Consuelo Estévez de JENSEN[1]**, Ricardo GOENAGA[2]

[1] University of Puerto Rico, Agro Environmental Sciences Department
[2] Tropical Agriculture Research Station, United States Department of Agriculture

Abstract: Huanglongbing (HLB) associated with '*Candidatus* Liberibacter asiaticus' is widespread in Puerto Rico and has affected the citrus industry in the island (Estevez de Jensen, et al, 2010). Based on disease severity data, the response to HLB of eight mandarin genotypes (M-IS-1 to M-IS-8) grafted onto three rootstocks were evaluated. The rootstocks C-35 Citrange (hybrid of Ruby Blood orange and *Poncirus trifoliata*), Cleopatra mandarin and US-942 originated from a cross of Sunki mandarin (*Citrus reticulata*) x Flying Dragon Trifoliate Orange (*Poncirus trifoliata*) from USDA, ARS, USHRL, Florida were included. The disease severity was assessed using a visual scale from 1 to 7, where 1= no symptoms apparent of HLB and 7= plant dead. A total of five evaluations were performed at 15, 19, 23, 31 and 35 months after transplanting (MAT). Statistical differences were found in the last three disease severity evaluations, six of the eight genotypes (excepting M-IS-4 and M-IS-5) grafted onto the hybrid US-942 had the lowest disease severity (between 3.22 and 3.63) respectively. The genotypes grafted on US-942 rootstock showed less disease severity in all the evaluations. The eight genotypes grafted on Cleopatra mandarin showed lower disease severity than the genotypes grafted onto C-35 Citrange rootstock. The Area Under the Disease Progress Curve (AUDPC) values increased from 554.30 at the first evaluation (15 MAT) to 2263.91 at 35 MAT in the last evaluation. In this analysis treatments with the lowest AUDPC values corresponded to genotypes M-IS-1, M-IS-2, M-IS-3, M-IS-6, M-IS-7, M-IS-8 grafted onto the US-942 and Cleopatra mandarin rootstocks which showed AUDPC values between 1140.3 and 1545.2; these did not differ statistically. The M-IS-1 genotype showed low severity values in all rootstocks evaluated. This field study confirms that US-942 is an outstanding rootstock.

Non-Technical Summary: The response to HLB of eight mandarin genotypes (M-IS-1 to M-IS-8) grafted onto rootstocks C-35 Citrange, Cleopatra mandarin and US-942 were evaluated. The genotypes grafted on US-942 rootstock showed less disease severity in all the evaluations. The M-IS-1 genotype showed low severity values in all rootstocks evaluated. This field study confirms that US-942 is an outstanding rootstock.

Citations:

Estévez de Jensen, C., Vitoreli, A. & Román, F. (2010). Citrus greening in commercial orchards in Puerto Rico. *Phytopathology*, APS Annual meeting, 100: S34.

IRCHLB-P2-19

Molecular Therapy Targets for

Huanglongbing

Hong LIN[1], Yongping DUAN[2] and Xianyang SHI[1]

[1]USDA-ARS, Crop Diseases, Pests and Genetics Research Unit. San Joaquin Valley Agricultural Sciences Center, Parlier, CA 93648, USA [2]USDA-ARS, Horticultural Research Laboratory, Fort Pierce, Florida 34945, USA

Abstract: The goal of this research was to develop a short-term practical approach to mitigating huanglongbing (HLB). We employed an orthologous gene replacement technique to functionally characterize key virulence genes/factors of the bacterium, '*Candidatus* Liberibacter asiaticus' (CLas), an associated agent of HLB. We then developed a molecular-based strategy to deliver anti-virulence molecules into host plants that block the activity of the target of virulence traits and reduce the virulence of CLas. To achieve that, we screened a chemical library and successfully identified potential anti-virulence compounds through *in vitro* bioassays in which putative anti-virulence compounds effectively suppressed several virulence traits associated with biofilm formation, cell-cell aggregation and cell twitching motility. Next, we validated the efficacy of selected anti-virulence molecules through greenhouse experiments in which HLB-affected citrus and CLas-infected periwinkle plants were foliar-sprayed with various concentrations of the selected anti-virulence compounds once a week for eight weeks. Plants treated with anti-virulence molecules showed alleviated symptoms and promoted new growth. CLas cell titers also were reduced compared to the untreated plants determined by quantitative polymerase chain reaction (qPCR). Anti-virulence therapy aims suppression of virulence traits rather than directly killing pathogens. Thus, this strategy unlikely imposes survival pressure on pathogen populations and therefore less likely induces resistance strains, which are frequently observed in antibiotic applications. HLB management based on the use of anti-CLas virulence compounds is a short-term, practical approach to reduce symptom severity in HLB-diseased citrus. Considering the current situation of HLB in the U.S, a pest and disease management system balanced with short-term and long-term goals is urgently needed. We envision this strategy as a novel practical approach to supplement current HLB management programs.

Non-Technical Summary: The goal of this research was to develop a short-term practical approach to mitigating huanglongbing (HLB). A novel strategy based on targeting key virulence genes of HLB associated bacterium, *Candidatus* Liberibacter asiaticus' (CLas) by delivering anti-virulence chemicals into HLB-affected trees has been experimentally evaluated. The results from *in vitro* and *in planta* studies suggested the potential application of anti-virulence chemical treatment to alleviate the effect of HLB.

IRCHLB-P2-20

Nutrition programs featuring soil-applied controlled release fertilizer (CRF) containing enhanced micronutrient packages can reduce CLas populations and improve the health, growth and productivity of HLB-infected trees

GROSSER, J.W., F. ZAMBON, D. KADYAMPAKENI, and M. DUTT.

University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, FL USA

Abstract: Our previously reported field and greenhouse data shows that secondary and micronutrient deficiencies are much greater in roots than in leaves of HLB-impacted trees. Not only is there severe feeder root loss, but the ability of the remaining roots to mine and translocate essential nutrients is compromised. Restoring root health is imperative for keeping trees productive in an HLB endemic environment. In an effort to improve tree health by focusing on the roots, we have been experimenting with polymer coated and TigerSul nutrients in the greenhouse and field. In a greenhouse experiment comparing various CRF formulas containing elevated levels of micronutrients on HLB+ Valencia trees on multiple rootstocks, many trees showed reduced *Candidatus* Liberibacter asiaticus (CLas) populations and good health after 1-1.5 years. Some trees showed no active infection (ct values >32), especially trees on experimental WGFT+50-7 rootstock grown on the Harrell's 12-3-9 St. Helena CRF mix. In a field experiment comparing CRF treatments with overdoses of manganese and/or boron on 9-year old Vernia sweet orange/rough lemon trees, trees treated with elevated levels of manganese for 2 years showed significantly reduced CLas titers and improved yields. 7 of 12 trees receiving the 4x TigerSul manganese treatment showed no active infection (ct values > 32). No differences were observed in fruit quality. With a combined 4x overdose of boron and manganese, the high boron level was found to be antagonistic to the positive manganese effect, resulting in poor tree performance. These results suggest that optimized combinations of controlled release micronutrients can reduce CLas populations and improve tree health and yield. Data also suggests that each scion/rootstock combination has a different micronutrient threshold that must be reached to achieve optimum tree health and productivity.

Non-Technical Summary: In an effort to improve the health and productivity of HLB-infected trees, we have been experimenting with polymer coated and TigerSul micronutrients, testing slow release mixes containing elevated levels of micronutrients on HLB+ Valencia trees on various rootstocks in the greenhouse and field. Greenhouse trees on different rootstocks under some treatments showed good health with reduced CLas titers. Infections became inactive in some trees, especially trees on experimental WGFT+50-7 rootstock receiving the Harrell's 12-3-9 St. Helena mix. In the field, mature trees of Vernia/rough lemon receiving high doses of manganese showed significantly reduced CLas titers and improved yields. Additional fine-tuning of fertilizer composition, type and delivery method should lead to affordable, sustainable and profitable citriculture where HLB is endemic.

IRCHLB-P2-21

Phenology driven management: a useful tool for citrus management in the tropics.

Andres CASTILLO [1], Juan DELGADO[1], Esteban BLANCO[2]

[1]LIFE-RID Amvac/Agricenter, [2]TicoFrut, Costa Rica/Nicaragua.

Abstract: Tropical agro-production systems are challenged by several such as plant pests and diseases, heavy rain intensity, high humidity, steady high temperatures, between others. Climatic change is triggering most of this stress factors, making them more intense and frequent. One of the most affected factor is rainfall distribution and intensity. Field crops are exposed to these environmental changes where most of the management practices (soil preparation, xenobiotic applications, harvest, etc.) are established according to the rainfall patterns. Plant phenology monitoring stands as a tool to adjust the timing of cultural practices and xenobiotic spraying, instead of chronological programed tasks. Citrus has been a model crop for the application of this approach in Costa Rica. Phenological data of sweet orange (*Citrus sinensis* L. Osbeck cv: *Valencia*) grafted onto Swingle citrumelo (*C. paradisi* x *Poncirus trifoliata*) or Flying Dragon (*Poncirus trifoliata* L. Raft cv. *Monstruosa*) were annotated from December 2015 to October 2018, using a semiquantitative estimation for each phenological phases (BBCH) based on Fournier (1974). Phenograms were obtain and differences between rootstocks were evidenced. The implications of tree phenology and weather

information on the dynamics of pests and diseases are described. Issues regarding large scale data acquisition and report emission for the grower are mentioned. To overcome this difficulty, the development of a smartphone application is proposed, where georeferenced sampling points are summarized by plot or farm (crop management preferences) and a statistical report is emitted automatically to the grower regarding crop phenological event happening in the field. Being plants the primary producers in the agroecosystem, biotic stress agents can be predicted because they need the correct phenological stage to thrive. Technological revolution have dabbled into agriculture for the last two decades and this kind of applications are being developed for extensive crops in other latitudes, but tropical crops are far behind.

Non-technical summary: Agriculture around the world is challenged by climatic change and stress factors (biotic and abiotic). To stand and overcome this uncertainty and difficulties technology applied to the crop systems have become a tool for monitoring field activities and adequate resource management. In this document we propose the development of an smartphone application to register crop phenology and biotic stresses in order to enhance the timing and efficiency of agrochemical applications. Sweet orange is presented as a model crop for this approach in tropical crops.

IRCHLB-P2-22

Physiological effects of oak bioactive compounds on Florida citrus when applied to contain and suppress HLB disease

Lorenzo ROSSI[1], **Marco PITINO[2]**, Christina DORADO[3], Robert G. SHATTERS, Jr.[3], Liliana M. CANO[2]

[1] Department of Horticultural Sciences, University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, Fort Pierce, FL, 34945

[2] Department of Plant Pathology, University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, Fort Pierce, FL, 34945

[3] Horticultural Research Laboratory, U.S. Department of Agriculture, Fort Pierce, FL 34945

Abstract: Huanglongbing (HLB) is rapidly destroying the Florida Citrus industry. To date, plant treatments and attempts to develop sustainable management practices have only provided modest improvements in controlling the disease impact on plant health and yield. Consequently, there is an urgent demand for innovative methods/strategies to look for HLB cures. Plants are considered new resources for producing agents that could act as alternatives to antibiotics in the treatment of the associated bacterium (*Candidatus* Liberibacter asiaticus, CLas). Plants produce an array of secondary metabolites, many of which are used in defense against potential bacterial pathogens. These compounds can form new sources of antimicrobial molecules to use against CLas. The present study was aimed at screening the antibacterial properties oak leaf (*Quercus germinata*) extracts. Nine sweet oranges (*Citrus* × *sinensis* ‘Valencia’) grafted on ‘Volkamericana’ rootstock were used. Six individual 1-year-old healthy plants were graft-inoculated via side-grafting with CLas-positive sweet orange bud sticks while 3 individuals were kept uninfected as control. Ct values corresponding to CLas titer from qRT-PCR were used to confirm that plants were either healthy or CLas-infected. After HLB infection was confirmed 3 individuals were chosen and treated with root drench and foliar intended spray applications (twice per week for 2 months) of oak leaf extract. This extract was prepared by macerating the oak leaves in distillated water and allowing to set over-night at 28 °C. After 6-months of treatment, a series of physiological parameters, as well as nutrient content and CLas bacterial titer, were measured via qRT-PCR. Additionally, a biochemical composition of the oak leaf extracts was performed. The oak-treated

plants had a reduction in titer and improved health as compared to untreated CLas-infected and demonstrated a restoration of physiological and nutritional levels. The untreated CLas-infected plants showed classic HLB symptoms and had impaired physiological processes and altered nutritional values.

Non-technical summary: Citrus greening (HLB) is one of the deadliest citrus disease and is rapidly destroying the Florida Citrus industry. Unfortunately, no compounds to date have been successful in controlling HLB, and no sustainable management practices have been established yet for citrus greening disease. Antimicrobial properties of various plant extracts against numerous bacteria have been demonstrated by a large number of researchers in different parts of the world with a great deal of work demonstrating antimicrobial properties in oak extracts. We therefore developed a research approach to evaluate the effects of oak leaf extracts on CLas when applied directly to citrus trees. We demonstrate that directly applying oak leaf extracts through a combination of foliar spraying and soil drenching resulted in a reduced CLas titer and improvements in the physiological and nutritional state of the trees. This work is presented as preliminary results suggesting that oak leaf extracts may have potential as a treatment for CLas infected trees.

IRCHLB-P2-23

Protecting the UCR Citrus Variety Collection from the citrus disease Huanglongbing.

Tracy KAHN[1], Toni SIEBERT-WOOLDRIDGE[1], Dawn STREICH[2], Andrew DAHNIER[3], Karene TRUNNELLE[1] and MaryLou POLEK[4]

[1]Department of Botany and Plant Sciences, University of California-Riverside, Riverside, California; [2]Givaudan Flavors Corp., 4705 US Highway 92 East, Lakeland FL-33801-3255, [3] Givaudan Flavors Corp., 1199 Edison Drive, Cincinnati OH 45216, [4]USDA ARS National Germplasm Repository, Riverside, CA

Abstract: The Citrus Variety Collection (CVC) was established in 1910 by the University of California as part of the Citrus Experiment Station in Riverside as a resource for research that benefits the citrus industry. Today, with approximately 4,500 trees of over 1048 cultivars and species of citrus and related taxa, the CVC is one of the most extensive collections of citrus diversity in the world. This collection includes both commercial and historic citrus cultivars as well as other genera and species in the Aurantioideae subfamily of the Rutaceae. The CVC is a resource for research, breeding, and characterization of citrus germplasm by scientists from UC and other academic institutions and companies. The discovery of three huanglongbing (HLB) infected trees in Riverside, CA 2.25 miles from the University of California, Riverside during the summer of 2017, increasing number of HLB infected trees and the pervasive spread of the vector Asian Citrus Psyllid in and beyond Southern California crystalized the need for further protection of the CVC. A “back-up” collection of two small trees per accession was established in 2008 in federally approved Asian citrus psyllid (ACP) exclusionary structures at the University of California and the USDA ARS facilities in Riverside. These provide a high level of protection, but field evaluation of horticultural traits, especially of fruit is severely limited. Research was conducted to determine the best design to house an additional tree of each accession as field trees in a “CUPS” (Citrus Under Protective Screen) structure. The design of this screened structure focuses on sustainable growth, flowering and fruit set of trees under southern CA climate to allow for continued breeding, research and to protect this valuable germplasm collection as a resource for the long-term sustainability of the industry and the public.

Non-Technical Summary: The University of California Riverside (UCR) Citrus Variety

Collection is a key resource for research, breeding, and characterization of citrus germplasm by scientists from UC and other academic institutions and companies. Discovery of three huanglongbing (HLB) infected trees in Riverside, CA 2.25 miles from UCR during the summer of 2017, increasing number of HLB infected trees and the pervasive spread of the vector Asian Citrus Psyllid necessitated further protection of the CVC for continued research and breeding efforts in the event of further spread of HLB . Research was conducted to determine the best design for a screened structure to assure sustainable growth, flowering and fruit set.

IRCHLB-P2-24

The effects of N-acetylcysteine on oxidative stress of sweet orange plants infected with *Candidatus* Liberibacter asiaticus

Henrique A. S. BERGAMO[1,2], Alessandra A. de SOUZA[1,2] and Helvecio D. COLETTA-FILHO[3]

[1]IB - UNICAMP, Campinas, BRAZIL, [2]Centro de Citricultura Sylvio Moreira/ Instituto Agronômico, BRAZIL,

Abstract: The *Candidatus* Liberibacter asiaticus (CLas) bacteria is the associated agent of Asiatic HLB in citrus. At an ultracellular level, infected plant cells show chloroplasts grana structure disorganization and rupture of its internal membranes. Lipid peroxidation and oxidative stress caused by the infection may aid the development of these ultracellular symptoms. N-acetylcysteine (NAC) is a cysteine analogue with antioxidant properties. The hypothesis of this work is that the oxidative stresses caused by CLas infection could be reduced by NAC treatment. To test this hypothesis sweet orange plants were infected with CLas by grafting using buds from HLB symptomatic plants. The plants were treated with NAC adsorbed in an organic fertilizer and in solution delivered by roots and by spray, respectively. After 4 and 8 months of treatment, leaves were sampled to quantify hydrogen peroxide, ascorbate peroxidase (APX) activity and the expression of genes that encode chloroplastidial ascorbate (APXchl) and glutathione peroxidase (GPXchl), the main enzymes responsible for reactive oxygen species scavenging in the chloroplast. In general diseased plants treated with NAC reduced 38% of hydrogen peroxide levels compared to plants without NAC and the activity of APX enhanced 2.33 fold in NAC treated plants. In addition, the expression of APXchl and GPXchl were enhanced 1.32 and 2.34 fold respectively, in plants treated with NAC. These results indicate that NAC decreased the oxidative stress, including in chloroplasts, the most affected organelle in this pathosystem. Although preliminary, these data open the possibility of using anti-oxidant molecules on treatment of HLB disease tress.

Non-Technical summary: HLB diseased trees show strong disorganization at the chloroplast level as a consequence of rupture of the internal membranes resulting from oxidative stress. We tested the hypothesis that antioxidant molecules such as N-acetyl cysteine (NAC) could reduce oxidative stress in HLB disease plants. In general NAC-treated plants resulted in increase of antioxidant enzymes and reduction of hydrogen peroxide, as well as an increase of expression of anti-oxidant genes at the chloroplast level. Anti-oxidant compounds may be one more measure for management of HLB infected trees.

IRCHLB-P2-25

TsnRNA-IIIb reduces citrus apical shoot growth and overall canopy size. Can it help citrus growers in the HLB management?

Irene LAVAGI, Aleyda Acosta RANGEL, Roya CAMPOS, Rock CHRISTIANO, Carol LOVATT, Louis SANTIAGO, Georgios VIDALAKIS

University of California Riverside, Riverside, CA, U.S.A.

Abstract: 'Transmissible small nuclear Ribonucleic acids' (TsnRNAs) are viroid RNA species that modify tree performance in specific citrus hosts without inducing disease. TsnRNA-IIIb can reduce the canopy volume of navel orange trees on trifoliate rootstock (approx. 50%), increase the yield per canopy volume, and concentrate fruit in the optimum canopy zone for harvest without affecting fruit quality. To understand how TsnRNA-IIIb reduces canopy volume, a survey of navel orange tree growth was initiated in an experimental block planted in 1998 at the Lindcove Research and Extension Center (LREC). We also monitored vegetative apical shoot growth over two seasons and found that the net growth of the apical shoots of TsnRNAIIIb-treated trees was reduced by almost 30% compared to the non-treated controls. Dwarfed trees are fundamental for high-density plantings, which will be critical to meet challenges posed by water shortages, disease spread, farmland reduction, and increasing labor costs. Dwarfed trees also have the potential to play an important role in the management of Huanglongbing (HLB). To assess the commercial potential of this technology we began a comprehensive fruit quality analysis and a study to determine nitrogen fertilizer requirements, nutrient uptake efficiency and water-use efficiency. Findings from these assessments will be presented and their implications discussed.

Non-technical summary:

High-density plantings of dwarfed citrus trees with reduced size apical shoots and small canopies will be advantageous for different elements of HLB management programs. Recent studies indicated that citrus flushes play an important role in HLB epidemiology by affecting psyllid colonization, life cycle, and bacteria transmission. Canopy geometry and density are affecting spray application efficiency and efficacy as well as HLB surveys and tree sampling for laboratory testing. Finally, high-density plantings of dwarfed citrus trees are excellent candidates for citrus production under protective structures.

IRCHLB-P2-26

Use of Engineered Solar Energy Thermotherapy to Treat HLB Infected Citrus Trees

AIKEN[1], Geoff, KAINZ[2], James, TRUETT[3], John, GOTTWALD[4], Tim, and POOLE[4], Gavin

[1]ARA Albuquerque, NM [2]ARA Raleigh, NC [3]ARA Ft. Pierce, FL [4]USDA ARS Ft. Pierce, FL

Abstract: HLB has devastated the citrus industry in Florida and still has no known cure. While not a cure, thermotherapy treatments have shown increased fruit production and extended tree life in numerous independent studies. Despite these positive results, thermotherapy is still not deployed at grove levels and uncertainty exists about set points for in situ treatments. In this paper, we will discuss thermotherapy results from small and field-scale applications on citrus trees. A comprehensive study of set points from 5°C to 65°C was induced on HLB affected trees to explore the best treatments (Development of the Hydro-Solar Thermotherapy (HSTT) for treatment of HLB infected citrus Aiken et al. 2017). Best results, as tracked throughout the previous test series, were achieved by parameters obtainable by HSTT. Two additional trailers were constructed that operate only on solar energy. Using these new HSTT trailers, we provided thermotherapy to a statistically significant number of Valencia orange trees in a commercial grove. Tree markers as well as fruit quantity and quality were tracked throughout the test series and positive results were recorded. Data results imply HSTT as an effective method to maintain citrus tree viability (fruit production and tree health) despite HLB prevalence.

Non-Technical Summary:

Use of thermotherapy treatment on HLB infected citrus trees holds the promise of extending tree life and increasing fruit production. Numerous studies show the positive effects of thermotherapy treatments. We conducted research on thermotherapy applications to determine ease of use, cost, and scale (on a grove level.) We discuss thermotherapy results from small and field-scale applications on citrus trees using a novel, engineered solar-based heating unit. Preliminary results show positive treatment effectiveness.

Citations:

2017 HLB Conference, Orlando, FL – **Development of the Hydro-Solar Thermotherapy (HSTT) for treatment of HLB infected citrus** – G AIKEN1, TR Gottwald2, J Kainz1, GH Poole2, and J Truett1 (1 Applied Research Associates, Inc., Raleigh, NC, USA; 2USDA-ARS, Fort Pierce, FL, USA.)

IRCHLB-03: Vector Function Oral Presentations

IRCHLB-O2a-01

The Quest for a Non-Vector Psyllid: Heritable Variation in Acquisition and Transmission Efficiency of '*Candidatus* Liberibacter asiaticus' by *Diaphorina citri*

MichelleHECK[1,2,3],
Saeed HOSSEINZADEH[1,2],
El-Desouky AMMAR[4],
and David G. HALL[4]

1 Boyce Thompson Institute for Plant Research, Ithaca, NY
2 Department of Plant Pathology and Plant Microbe Biology, Cornell University, Ithaca, NY
3 USDA Agricultural Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, NY
4 USDA Agricultural Research Service, US Horticulture Research Laboratory, Fort Pierce, FL

Abstract: Insect genetic variability is valuable to study vector competence determinants and to select non-vector populations that may help reduce the spread of vector-borne pathogens. We collected and tested vector competency of 15 isofemale lines of Asian citrus psyllid, *Diaphorina citri*, vector of '*Candidatus* Liberibacter asiaticus' (CLas). CLas is associated with huanglongbing, the most serious citrus disease worldwide. *D. citri* adults were collected from orange jasmine (*Murraya paniculata*) hedges in Florida, and individual pairs (females and males) were caged on healthy *Murraya* plants for egg laying. The progeny from each pair that tested CLas-negative by qPCR were maintained on *Murraya* plants and considered an isofemale line. Six acquisition tests on *D. citri* adults that were reared as nymphs on CLas-infected citrus, from various generations of each line (5-27 generations), were conducted to assess their acquisition rates (percentage of qPCR-positive adults). Three lines with mean acquisition rates of 28 to 32 %, were classified as 'good' acquirers and three other lines were classified as 'poor' acquirers, with only 5 to 8 % acquisition rates. Lines were further tested for their ability to inoculate CLas by confining CLas-exposed psyllids for one week onto healthy citrus leaves (6-10 adults/leaf/week), and testing the leaves for CLas by qPCR. Mean inoculation rates were 19 to 28% for the three good acquirer lines and 0 to 3 % for the three poor acquirer lines. Statistical analyses indicated positive correlations between CLas acquisition and inoculation rates, as well as between CLas titer in the psyllids and CLas acquisition or inoculation rates. Phenotypic and molecular characterization of the good and the poor acquirer lines revealed differences between them in color morphs and hemocyanin expression, but not the composition of bacterial endosymbionts. Understanding the genetic architecture of CLas transmission will enable the development of new tools for combating huanglongbing.

Non-technical summary: Unravelling the interactions between *D. citri* and CLas is a critical area of HLB research, and may lead to the development of acquisition and/or transmission blocking strategies. Our work showed that some *D. citri* transmit CLas very efficiently and others not at all. We showed that the ability or inability to transmit the pathogen is passed on from parents to offspring for several generations and have established isofemale lines that differ in their transmission abilities. Our studies provide evidence that psyllid genes contribute to the regulation of CLas transmission. Future work will focus on identification of the psyllid genes involved in transmission.

Citation:

Ammar, E.D., et al., The quest for a non- vector psyllid: Natural variation in acquisition and transmission of the huanglongbing pathogen 'Candidatus Liberibacter asiaticus' by Asian citrus psyllid isofemale lines. PLoS One, 2018. **13**(4): p. e0195804.

IRCHLB-O2a-02

Asian citrus psyllid adults are more efficient than nymphs in inoculating the huanglongbing bacterium into citrus if both stages of the vector were reared on infected plants

El-Desouky AMMAR[1], Justin GEORGE[1,2], David G. HALL[1], Stephen LAPOINTE[1], Robert G. SHATTERS[1]

[1]USDA-ARS, US Horticultural Research Laboratory, Fort Pierce, FL, USA

[2]University of Florida, IFAS, Lake Alfred, FL, USA

Abstract: The Asian citrus psyllid (ACP, *Diaphorina citri*) transmits the Asiatic huanglongbing (citrus greening) pathogen '*Candidatus* Liberibacter asiaticus' (CLas) in a persistent, propagative manner. Previous investigations have shown that ACP nymphs are more efficient than adults in acquiring CLas from infected plants. In this study, we compared the efficiency of ACP nymphs and adults to inoculate CLas into healthy citron leaves, when both stages of the vector have been reared on infected citron. CLas-exposed adults (<2 wk. old) or 4th instar nymphs were caged on excised young healthy citron leaves for 4 days (5 insects/leaf, 30-60 leaves/treatment), and these leaves were analyzed by qPCR one week later. None of the leaves inoculated by nymphs became qPCR- positive for CLas, whereas 50% of those inoculated by adults became infected (mean Ct value = 28.1). EPG recordings showed that ACP nymphs salivate and ingest into/from citrus phloem (E1 and E2 waveforms, respectively) longer than the adults. We hypothesized that for ACP to inoculate CLas, this pathogen has to reach the salivary glands in a high enough titer and that in nymphs it may not have enough time to do so. Thus, we cut CLas-exposed nymphs and adults (previously reared on infected citron) into two parts each: the head-thorax part (which includes the salivary glands) and the abdomen. QPCR results indicated that 6.7-46.7 % of the head-thorax parts in nymphs were CLas infected (mean Ct=34.5-35.1, n=60), compared to 15-75% of comparable parts in adults (mean Ct=28.8-36.7, n=80). We are continuing this study, but these results support our hypothesis that ACP adults from nymphs that have been reared on infected citrus are more efficient than nymphs developing on infected citrus in inoculating CLas, because the time necessary for translocation and/or replication of CLas into the salivary glands may not have been completed during the nymphal stage.

Non-technical summary:

Previous investigations have shown that Asian citrus psyllid nymphs are more efficient than adults in acquiring huanglongbing (HLB) bacterium from infected plants. In this study, we found out that when both nymphs and adults have been reared on infected plants, adults are more efficient in transmitting HLB bacterium to citrus, probably because this bacterium does not have enough time during the nymphal stage to reach and/or multiply in the psyllids' salivary glands. Thus, although psyllid nymphs are more efficient in acquiring the HLB pathogen adults from these nymphs are more efficient in transmission of this pathogen. Although nymphs do not typically move among trees, and therefore would not be considered a vehicle of transmission to healthy trees, there has been speculation that psyllid feeding and re-inoculation of CLas in already infected trees could cause more rapid and severe development of disease symptoms. Our results suggest that if this is the case, adults and not nymphs are the primary source of CLas re-inoculation.

IRCHLB-O2a-03

Is the acquisition of HLB-associated bacteria by Psyllids reduced through Copper, Manganese and/or Zinc fertilizations in infected citrus trees?

Jefferson Rangel da SILVA[1], Fausto Veiga de ALVARENGA[1],
José Antonio QUAGGIO[2], Rodrigo Marcelli BOARETTO[1], João Roberto Spotti LOPES[3], Helvécio Della COLETTA-FILHO[1], Dirceu MATTOS-JR[1]

[1]Instituto Agronômico, Centro de Citricultura Sylvio Moreira, Cordeirópolis, Brazil,, [2]Instituto Agronômico, Centro de Solos e Recursos Ambientais, Campinas, Brazil, [3]Universidade de São Paulo, Escola Superior de Agricultura Luiz de Queiroz, Piracicaba, Brazil

Abstract: We investigated whether micronutrient fertilization in Pera sweet orange trees (*Citrus sinensis*) grafted onto Rangpur lime trees (*C. limonia*) mitigates the acquisition of '*Ca.* Liberibacter asiaticus' (CLas) by both nymphs and adults of *Diaphorina citri*. CLas infected plants were exposed to one of five nutrient treatments: 1- Control: plants not fertilized with Cu, Mn and Zn; 2-Zn: plants foliar (750 mg L^{-1} of Zn) and substrate (200 mg of Zn) fertilized with ZnSO4.H2O; 3-Mn: plants foliar (500 mg $L^{-1}$of Mn) and substrate (140 mg of Mn) fertilized with MnSO4.H2O; 4-Cu: plants foliar (800 mg L^{-1} of Cu) and substrate (240 mg of Cu) fertilized with Cu(OH)2; and 5-CPLT: plants foliar and substrate fertilized with ZnSO4.H2O, MnSO4.H2O and Cu(OH)2 at the same concentrations described for the Zn, Mn and Cu treatments. Fertilizations were conducted every 30 days for 9 months. Moreover, all plants were monthly fertilized with macro and micronutrients (other than Zn, Mn and Cu). Thereafter, uninfected nymphs and adults of *D. citri* were confined for an acquisition access period of seven days on new vegetative flushes of each treatment. Cycle threshold values (CT) were determined through qPCR[1] and used to verify the presence of CLas in tested flushes and psyllids (five nymphs and five adults per plant x three plants per treatment). Samples presenting CT values ≤36 were considered positive for the presence of CLas. Although flushes CT ranged from 26 to 32, 60% of adults, as well as 100% of nymphs did not acquire CLas from CPLT plants. However, 67, 100, 86.5 and 77% of psyllids (overall avg. of adults and nymphs) acquired CLas from Control, Mn, Zn, and Cu flushes, respectively. Thus, our results suggest that Mn, Zn and Cu can reduce the acquisition of CLas by both adults and nymphs of *D. citri*, when applied in combination.

Non-technical Summary: Huanglongbing (HLB) is mostly associated to the presence of Las in citrus trees. This bacterium is transmitted by the Asian Citrus psyllid *D. citri*. Both adult and nymph specimens of *D. citri* acquire Las from infected plants. Nonetheless, nymphs have higher probability of acquiring the bacteria and, in the future, the emerging adults will have higher capacity to infect new plants. We found that the use of micronutrients applied in combination (Mn, Zn and Cu) and as a complement to the fertilizations currently

recommended for citrus trees can reduce the acquisition of CLas by the psyllid, specially by nymph specimens. This may eventually be an additional tool to prevent HLB spread within and between citrus orchards.

Citations:

Coletta-Filho et al., *Phytopathology* **104**, 416-421 (2014).

IRCHLB-O2a-04

Psyllid-*Candidatus* Liberibacter interactions involved in the circulative, propagative transmission pathway: molecular and cellular interfaces

Judith K. BROWN[1], Timothy J. RAST[1], Tonja W. FISHER[1], Ruifeng HE[2], and David GANG[2]

[1]The University of Arizona, Tucson, AZ 85721 USA, [2]Institute of Biological Chemistry, Washington State University, Pullman, WA 99163

Abstract: '*Candidatus* Liberibacter asiaticus' (CLas) is the obligate, fastidious bacterial pathogen associated with citrus greening disease, or Huanglongbing. CLas is transmitted in a circulative, propagative manner by Asian citrus psyllid (ACP) *Diaphorina citri* Kuwayama adults. Using the surrogate study system involving the potato psyllid *Bactericera cockerelli* Sulc. and the analogous bacterial pathogen of tomato potato plants ' Candidatus plants,'*Candidatus*Liberibacter solanacearum' (CLso). The identification and functional characterization of effectors involved in invasion of the psyllid gut and blood, with presumed entry into the salivary glands was investigated using combined transcriptomic, proteomic, yeast-2 hybrid (Y2H) and co-immunoprecipitation (Co-IP) analyses. *In silico* annotation and differential expression analysis of contigs from ACP and/or PoP nymphs and adults, and adult midgut and salivary gland tissues identified transcripts and proteins with altered expression in response to CLas/CLso infection. Several differentially expressed transcripts were selected as bait for Y2H-detection in protein-protein interactions studies. Those positive by Y2H were subjected to verification by bait to prey co-transformation and Co-IP. Proteins positive in one or both assays were tested in 'knock down' experiments using dsRNA to induced RNA-interference (RNAi), with quantification by qPCR. Candidates of the greatest interest were those with a predicted role in entry, gut /salivary gland invasion, virulence, and systemic infection, that if disrupted by RNAi could abate Liberibacter accumulation in and exit from the gut, circulation in hemolymph, and acquisition in the salivary glands. Collectively, the results suggest a model for invasion in which Liberibacter- and prophage-encoded effectors that exploit psyllid cellular pathways, including membrane ruffling for entry into the gut, followed by cytoskeletal remodeling, and exocytosis leading to bacterial exit into the hemocoel. Following systemic invasion of the hemocoel and transport in the hemolymph (blood), CLas/CLso are predicted to use an endocytosis-like process to enter the salivary glands from where transmission to the plant host occurs post-acquisition.

Non-Technical Summary: To understand the interactions between the psyllid host-Liberibacter pathogen that systemically infects both the plant host and its psyllid vector, molecular and cellular pathways must be dissected using several combined approaches. Identification of protein-protein interactors critical to invasion and systemic infection of the psyllid host is expected to lead to deployment of dsRNAs to 'knock down, or silence' the expression of proteins found required for early events in the Liberibacter- psyllid transmission pathway. The use of dsRNA biopesticides is expected to provide an alternative therapy for HLB control, which presently relies primarily on conventional pesticides.

Citations:

Cicero, J. M., Fisher, T.W., and Brown, J.K. 2016. Localization of '*Ca.* Liberibacter solanacearum'and evidence for surface appendages in the potato psyllid vector. Phytopathology **106**:142-154.

Cicero, J.M., Fisher, T.W., Qureshi, J.A., Stansly, P.A., and Brown, J.K. 2017. Colonization and intrusive invasion of potato psyllid by '*Ca.* Liberibacter solanacearum'. Phytopathology **107**:36-49. Epub 2016 Oct 17 http://dx.doi.org/10.1094/ PHYTO-03-16-0149-R.

Fisher, T.W., M. Vyas, R. He, W. Nelson, J.M. Cicero, M. Willer, R. Kim, R. Kramer, G.A. May, J.A. Crow, C.A. Soderlund, D.R. Gang, and J.K. Brown. 2014. Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative Liberibacter transmission. Pathogens **3:** 875-907; doi: 10.3390/pathogens3040875.

IRCHLB-O2a-05

Diaphorina citri employs the metabolism of citrus host to fulfill its nutritional needs

Nabil KILLINY[1], Michael E. ROGERS[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: The citrus industry is currently threatened by huanglongbing (citrus greening disease). The bacterial pathogen, *Candidatus* Liberibacter asiaticus is phloem restricted and transmitted by the Asian citrus psyllid, *Diaphorina citri*. In addition to citrus varieties, this vector can colonize other species from the family Rutaceae such as orange jasmine (*Murraya paniculata*), and curry leaf tree (*Bergera koenigii*) which may act as reservoir hosts in the field. Using gas chromatography-mass spectrometry, we studied the effects of infestation by *D. citri* on the phloem sap composition of Valencia sweet orange (*Citrus sinensis*), orange jasmine, and curry leaf tree. Additionally, the honeydews secreted from the *D. citri* nymphs, which have the most feeding activity and higher transmission rate for the bacterium, were investigated. Our findings show that while the overall effect on phloem saps is similar, some specific varietal differences were observed. Amino acids and organic acids were the most affected compounds by the infestation with*D. citri*. The increase in γ-aminobutyric acid and L-proline indicated that infested plants were under stress, while the increase in glycine and L-serine indicated an increase in photorespiration. The increase in citric acid and succinic acid may indicate an induction of the tricarboxylic acid cycle in *D. citri*-infested plants. In addition, the honeydew profiles were similar among the three hosts with slight differences, which could be attributed to the differences in the phloem saps. The trace amounts of amino acids in the honeydew indicated that amino acids were the growth- limiting nutrients for *D. citri* and their increase in the phloem sap of their host plants upon infestation may enhance the development and reproduction of *D. citri*. Understanding the specific interactions between the vector and the host plants will help in finding a weak link of the chain that we can target to better control vector-borne diseases.

Non-technical summary: Understanding the nutritional requirement the vector, *Diaphorina citri*, will provide important information leading to effective control of Huanglongbing by interfering with psyllid feeding on the citrus host.

IRCHLB-O2a-06

Sex-dependent effects of CLas exposure on *Diaphorina citri*.

Laurynne COATES[1], Jaclyn MAHONEY[2,3], John RAMSEY[2,3], EricaRose WARWICK[4], Richard JOHNSON[5], Michael MACCOSS[5], Stuart KRASNOFF[3], Kevin HOWE[3], Kathy MOULTON[6], Robert SHATTERS[6], David HALL[4], Michelle HECK[2,3,7] and Carolyn SLUPSKY[1]

[1]Department of Food Science and Technology, University of California, Davis, California, U.S.A.; [2]Boyce Thompson Institute for Plant Research, Ithaca, New York, U.S.A.; [3]Robert W. Holley Center for Agriculture and Health, Emerging Pests and Pathogens Research Unit, USDA Agricultural Research Service, Ithaca, New York, U.S.A.; [4]Plant Pathology, University of Florida Citrus Research and Education Center, Lake Alfred, Florida, U.S.A.; [5]Department of Genome Sciences, University of Washington, Seattle, Washington, U.S.A.; [6]U.S. Horticultural Research Laboratory, Unit of Subtropical Insects and Horticulture, USDA Agricultural Research Service, Fort Pierce, Florida, U.S.A.; [7]Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, New York, U.S.A.

Abstract: ‘*Candidatus* Liberibacter asiaticus’ (CLas) putatively causes huanglongbing (HLB) in all commercial citrus varieties, and is transmitted by Asian citrus pysllids (ACP, *Diaphorina citri*). Interrupting ACP transmission of CLas could prevent HLB spread and consequently avoid HLB-related fruit loss and tree death. Determining the best targets for CLas transmission intervention in ACP requires a thorough understanding of ACP-CLas interactions. The extent to which male and female ACP respond differently to CLas is not well understood; therefore, adult male and female ACP were studied separately with regard to their responses to CLas using a combination of transcriptomics, proteomics and metabolomics analyses, combined with 16S rRNA gene sequence and qPCR analyses of the endosymbiotic microbial community. Compared to males, females had more extensive alterations in gene expression and protein and metabolite abundances in response to CLas. Although CLas similarly impacted energy metabolism in males and females, it differentially impacted markers of defense, oxidative stress, and neurobiology. Furthermore, CLas differentially impacted the endosymbionts according to insect sex, with the male microbial composition more signficantly altered than that of females. ACP and endosymbiont responses to CLas that are shared between males and females are being investigated for their importance in CLas transmission and potential development of targets for CLas transmission intervention. As a whole, these findings suggest that insect sex be considered in the development and assessment of transmission interventions.

Non-technical summary: As adults, male and female ACP have sex-specific responses to the pathogen CLas, which is considered the causative agent of HLB. The set of responses to CLas that is shared between male and female ACP suggest interactions that are critical to CLas survival in ACP. These results are critical in the quest to find molecules that may inhibit transmission or impact colonization of CLas in its insect vector host.

IRCHLB-03: Vector Function Poster Presentations

IRCHLB-P3-27

Citrus hosts drive the genetic differentiation of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) in China

Zhen-yu ZHANG [1], **Zhichao YAO** [1], Lixue MENG [1], Umar Anwar AWAN [1], Hongyu ZHANG[1]

[1] Key Laboratory of Horticultural Plant Biology (MOE), State Key Laboratory of Agricultural Microbiology, Institute of Urban and Horticultural Entomology, College of Plant

Science and Technology, Huazhong Agricultural University, Wuhan, 430070, China.

Abstract: As one of the most destructive pests of citrus in China, the Asian citrus psyllid, *Diaphorina citri* Kuwayama, has been reported widely across eastern and southern China. However, the genetic diversity and population structure of *D. citri* was still largely unknown, which was one of the obstacles in controlling this pest. Here, nine specifically developed short simple repeats (SSR) markers were screened and applied to the population genetic structure of *D. citri* within 225 samples collected from 7 different regions in China. The results showed that *D. citri* was highly diverse with a mean observed heterozygosity of 0.50, and all populations fell into three subgroups by host plants, including: (1) Shatangju, NF mandarin and Ponkan (2) Murraya paniculata and lemon (3) Citrus unshiu, Bingtangcheng, Summer orange and navel. Besides the SSR data, three known mtDNA genes (*COI*, *Cytb* and *ND5*) were also used for the analysis of unimodal distribution of paired differences, the negative and significant Tajima's D and Fu's F S parameters, which suggested a recent demographic expansion of *D. citri* population. Taken together, as a widely distributed citrus pest in China, *D. citri* shows high genetic diversity, which is putatively influenced by the citrus hosts and may occur after a recent event for the demographic expansion.

Non-Technical Summary: In China, the Asian citrus psyllid, *Diaphorina citri* Kuwayama has been found widely distributed across eastern and southern China, which were considered as one of the key factors for the outbreak of the devastating citrus disease, huanglongbing (HLB) in these areas. Here, we found that *D. citri* possessed high genetic diversity, which was influenced by the citrus host. As well, a recent event for the demographic expansion of *D. citri* was putatively happened, which could explain the widely distribution of *D. citri* in eastern and southern China.

IRCHLB-P3-28

DcitOBP3 may work as a potential essential olfactory protein in the volatile recognition system of psyllid-citrus

Runxian YAO[1], Feng ZHANG[1], Dan TANG[1], Mingming ZHAO[1], Ronghua LI[2], Dazhi LI[1], Suming DAI[1], Xianfeng MA[1], Ziniu DENG[1]

[1]National Center for Citrus Improvement and Horticulture and Landscape College, Hunan Agricultural University, Changsha, Hunan 410128, P.R. China.

[2]Department of Agriculture, Yizhang County, Hunan, P.R. China

Abstract: Huanglongbing (HLB), associated with *Candidatus* Liberibacter asiaticus (CLas), is a devastating bacterial disease of citrus and its spreading mainly because of the insect vector Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera, Liviidae). ACP always has the tendency of oviposition on growing young leaves in the groves. In hence, we conducted GC-MS to characterize the differences of volatile substances between young and matured leaves of 'Bingtang' sweet orange (*Citrus. sinensis*). In the young leaves, 12 specific volatile substances were detected. One of these volatiles (or complex) seemed to induce citrus psyllid oviposition on young leaves via insect olfactory system. Odorant-binding proteins (OBP) and chemosensory proteins (CSP) are core factors for sensing the volatiles. Here, we examined five OBPs and two CSPs for their binding ability with 11 citrus volatiles. DcitOBP1 and DcitOBP3 could be bound to all 11 volatile substances, but DcitOBP3 showed stronger binding capacity for all 11 volatile substances than DcitOBP1, and especially the binding ability with beta-Caryophyllene was ~7 times higher than DcitOBP1 did. Using molecular docking simulation to speculate the amino acid binding

sites of DcitOBP3, there are eight amino acid binding sites with all 11 volatile substances and two specific binding sites with beta-Caryophyllene. Then, we performed PCR-based mutagenesis to mutate the binding sites of DcitOBP3, and the binding capacity of DcitOBP3$^{\text{Mutant1-10}}$ (all 10 binding sites knocked out) decreased 89.4% comparing with DcitOBP3$^{\text{WT}}$, it indicated that one or multiple binding sites are essential for binding capacity of DcitOBP3. Furthermore, we mutated binding sites one by one, intriguingly, the binding capacity of DcitOBP3$^{\text{L51V}}$ and DcitOBP3$^{\text{K671}}$ decreased 67.3% and 77.0%, respectively, being the weakest binding capacity. These demonstrated that L51 and K67 are the core citrus volatile binding sites of DcitOBP3.

Non-technical summary: Asian citrus psyllid is the insect vector of HLB-associated pathogen. Controlling of citrus psyllid is one of the most effective measures to restrict the destroyed influence of HLB. In this study, volatile substances in citrus mature leaves and young leaves were different. Asian citrus psyllid could sense volatiles with odorant binding proteins (OBPs) and chemosensory proteins (CSPs). DcitOBP3 might be an essential proteins in the volatile recognition system of psyllid-citrus.

IRCHLB-P3-29

Differential expression analysis and validation of lncRNA between Asian citrus psyllid and *Candidatus* Liberibacter interaction

CHEN, Mengyao[1,2], CAO, Zhenghong[2], HE, Kang[2], MEI, Yang[2], WEI, Shengfei[2], **XIAO, Huamei**[1]

[1]College of Life Sciences and Resource Environment / Key Laboratory of Crop Growth and Development Regulation, Yichun University, China, [2]Ministry of Agriculture Key Lab of Molecular Biology of Crop Pathogens and Insects / Institute of Insect Sciences, Zhejiang University, China

Abstract: Asian citrus psyllid (*Diaphorina citri* Kuwayama) is the only natural vector of Huanglongbing (HLB) and controlling the population of Asian citrus psyllid can stop the spread of the HLB associated pathogen (*Candidatus* Liberibacter asiaticus, CLas). Long non-coding RNA (lncRNA) widely participate in inhibiting replication of bacteria and viruses *in vivo*, but it is unknown whether lncRNA regulates CLas infection and replication. To study this, a computational pipeline was used to predict 10,192 lncRNA genes corresponding to 15,747 lncRNA transcripts from 13 Asian citrus psyllid RNA- Seq databases. According to characteristics analysis, Asian citrus psyllid lncRNA genes have fewer exons and shorter transcripts compared to protein-coding genes. Ten lncRNA genes were randomly selected for RT-PCR validation. The results showed seven lncRNAs expressed in both the CLas-free Guangzhou strain and the CLas-free Ganzhou strain while one lncRNA only was expressed in the Ganzhou strain. The differential expression analysis of lncRNA in CLas– infected and CLas–free adults was compared by bioinformatics and it predicted two lncRNAs: TCONS_00096118 and TCONS_00234564 would express differentially. QPCR was used to validate the prediction result: TCONS_00234564 matches the result that highly expressed in CLas- infected adults. In summary, lncRNA is involved in the interaction of the pathogen CLas and its host Asian citrus psyllid. This study provides theoretical support for the development of environmental and safe management of Asian citrus psyllid and HLB disease in agriculture.

Non-technical summary: A bioinformatics pipeline was constructed to the lncRNAs in Asian citrus psyllid, and most of the lncRNAs were found to have similar structural characteristics compared with other insects. One lncRNA only was expressed in the Ganzhou strain, two lncRNAs expressed differentially in CLas–infected and CLas–free adults. LncRNA may be involved in the interaction of the pathogen CLas and its host Asian citrus psyllid.

IRCHLB-P3-30

Effect of host switch between *Murraya* and citrus plants on acquisition and transmission of huanglongbing (citrus greening) bacterium by the Asian citrus psyllid *Diaphorina citri*

El-Desouky AMMAR[1], John RAMSEY[2,3], Jackie MAHONEY[2,3], David G. HALL[1], Michelle HECK[2,3]

[1] USDA-ARS, US Horticultural Research Laboratory, Fort Pierce, FL, USA
[2] Boyce Thompson Institute, Ithaca, NY USA
[3] USDA-ARS, Ithaca, NY, USA

Abstract: Huanglongbing (citrus greening), the most devastating citrus disease worldwide, is associated with *Candidatus* Liberibacter asiaticus (CLas) transmitted by the Asian citrus psyllid (ACP, *Diaphorina citri*, Hemiptera: Liviidae). The citrus relatives *Murraya paniculata* (orange jasmine) and *Murraya koenigii* (curry leaf) plants are good hosts of ACP, but are more resistant to CLas compared to all tested citrus genotypes. We are studying the effects of host-plant switching of ACP between citrus and *M. paniculata* plants on the acquisition and transmission of CLas. We started our experiments with ACP that has been reared on CLas-infected citron (*Citrus medica*) for many generations. QPCR tests showed that 93-97% of these adults were CLas-infected (mean CT value = 27.6). In the first three generations after transferring adults to healthy *M. paniculata*, percentage of CLas-infected psyllids went down to 16.5, 3.9 and 2.5% respectively (mean CT values =36.2-37.8). After being reared on *M. paniculata* for 6 months, psyllids were switched back to infected citron. After 1-2 generations, percentage of infected psyllids was 47.7- 70.5%, with mean CT value of 33.1. These values are significantly different from those mentioned above for ACP reared longer on infected citron. In separate experiments, we discovered that ACP reared on *M. paniculata* express an ACP antibacterial immunity protein at higher levels than ACP reared on *Citrus macrophylla*. We are investigating the role of this protein in the ACP immune response, and the effects of rearing ACP on *M. paniculata* and/or *M. koenigii* plants for longer periods on both acquisition and transmission of CLas. This study elucidates further the complex interactions affecting ACP vector competence and may lead to devising novel control measures for the devastating huanglongbing disease.

Non-technical summary: Orange jasmine plants are good hosts of the Asian citrus psyllid, vector of the huanglongbing (HLB) bacterium, but are more resistant to this pathogen compared to all tested citrus genotypes. Our study indicates that feeding highly infected psyllids on orange jasmine for 6 months reduced their infectivity levels considerably and also their ability to newly acquire HLB bacterium from infected citron. This study elucidates the complex interactions affecting the psyllids' ability to acquire and transmit HLB and may lead to devising novel control measures for this devastating citrus disease.

IRCHLB-P3-31

Hot Spot Cluster Analysis of Asian Citrus Psyllid Samples in Texas and California

David BARTELS[1]

[1]USDA APHIS Plant Protection and Quarantine, Fort Collins, CO, USA

Abstract: Since 2008, regulatory agencies in Texas and California have been conducting

extensive surveys for detecting Asian citrus psyllids and Huanglongbing (HLB) disease in citrus trees. The surveys collect and test both plant and psyllid samples for the disease-associated bacteria using quantitative polymerase chain reaction (qPCR) diagnostic methods. Quantitative PCR reactions that indicate a Ct-value above the set thresholds have been difficult to confirm as positive using conventional PCR bands and DNA sequence data. Thus, samples above the threshold and below the 40-cycle cutoff were classified as CLas inconclusive. In order to gain information from these inconclusive samples, hot spot cluster analyses were conducted to determine if a spatial pattern exists. Based on our analyses of psyllid Ct- values in both Texas and California, there appears to be a biological process underlying inconclusive Ct-values. Our analyses indicate significant clustering of inconclusive and positive Ct-values of psyllid samples. Subsequent positive HLB tree detections were proximate to hot spot clusters in both Texas and California. Further improvements in the last few years to qPCR methodology have also shown the threshold for indicating the presence of CLas is higher than the original threshold level of 32. In California, the San Gabriel area positive trees were found after intensive survey around an inconclusive psyllid sample. The Riverside, California area positive trees were found within 1km of inconclusive psyllid samples 3 years prior. These analyses have served as early warning indicators for predicting locations with HLB positive trees and the analyses have been updated as new survey data becomes available.

Non-Technical Summary: Since 2008, regulatory agencies in Texas and California have been conducting extensive surveys for detecting Asian citrus psyllids and Huanglongbing (HLB) disease in citrus trees. We continue to analyze the spatial pattern of the diagnostic values from psyllid samples. Our analyses indicate clustering of inconclusive and positive psyllid samples. Subsequent positive HLB tree detections were found close to hot spot clusters in both Texas and California. Our goal with this effort is to focus the survey effort for HLB infected citrus trees.

IRCHLB-P3-32

Impact of *Candidatus* Liberibacter asiaticus on Adaptability of Asian Citrus Psyllid (*Diaphorina citri* Kuwayana)

Chun RAN, Ren LUO, Haoqiang LIU, Lin CONG, Shijiang YU, Qi PAN and Cuilun WANG
Citrus Research Institute, Southwest University/Chinese Academy of Agricultural Sciences, National Engineering Research Center for Citrus, Chongqing, China.

Abstract: The Asian citrus psyllid, *Diaphorina citri* Kuwayana (Hemiptera, Liviidae) is a vector of Citrus Huanglongbing, which is a destructive disease for the citrus industry. It is very important to clarify the impact of pesticides and the interaction with *Candidatus* Liberibacter asiaticus (CLas) (the associated pathogen of Huanglongbing) on the survival of *D. citri*, since the insect vector functions as a critical factor in disease control. Five pesticides were tested against *D. citri* (C*Las*-infected VS uninfected) psyllids and high throughput sequencing was applied to obtain the expression profile analysis. Imidacloprid: the indicators of LC50 against 3rd, 4th, 5th instar and adult of *Las*-infected were 3.32 mg/L, 6.19 mg/L, 12.75 mg/L and 7.34 mg/L, respectively. And the indicators of *Las*-uninfected were 5.45 mg/L, 11.20 mg/L, 20.14 mg/L and 9.62 mg/L , respectively. Thiamethoxam: the indicators of LC50 against 3rd, 4th, 5th instar and adult of *Las*-infected were5.76 mg/L, 7.81 mg/L, 13.85 mg/L and 8.96 mg/L, respectively. And the indicators of *Las*-uninfected were 7.26 mg/L, 9.82 mg/L, 17.92 mg/L and 9.24 mg/L. Acetamiprid: the indicators of LC50 against 3rd, 4th, 5th instar and adult of *Las*-infected were 4.86mg/L, 16.31 mg/L, 35.45 mg/L and 11.37 mg/L, respectively. And the

indicators of *Las*- uninfected were 8.20 mg/L, 22.88 mg/L, 38.77 mg/L and 12.63 mg/L. Emamectin benzoate: the indicators of LC50 against 3rd, 4th, 5th instar and adult of *Las*-infected were 1.80 mg/L, 3.50 mg/L, 6.03 mg/L and 3.17 mg/L, respectively. And the indicators of *Las*- uninfected were 1.47 mg/L, 3.92 mg/L, 6.21 mg/L and 3.59 mg/L. Buprofezin: the indicators of LC50 against 3rd, 4th, 5th instar and adult of *Las*-infected were 70.15 mg/L, 104.45 mg/L, 228.40 mg/L and 99.33 mg/L, respectively. And the indicators of *Las*- uninfected were 89.74 mg/L, 124.04 mg/L, 244.55 mg/L and 118.90 mg/L. As a whole, C*Las*-infected *D. citri* showed increased sensitivity against these pesticides compared to *Las*-uninfected *D. citri*; 1640 differentially expressed genes were found in the expression profile analysis of C*Las*- infected VS C*Las*-uninfected *D. citri*'s sample. Some detoxifying enzymes were down-regulated when *D.citri* against disease, such as *CYP450 4C1*, *P450 49a1* and glutathione-s-transferase. We found 8 Unigenes which annotated ATP-binding cassette transporter in infected *D.citri* and 7 of them were down-regulated.

Non-Technical Summary: *Candidatus* Liberibacter asiaticus had a significant impact on adaptability of *D. citri*, the C*Las*-infected *D. citri* showed increased sentitivity against common pesticides. The data indicated that *Candidatus* Liberibacter asiaticus may have a potential capacity to compel its hosts to change their strategy on energy distribution to help itself to spread more quickly and effectively. Some detoxifying enzymes have differential expression in infected *D.citri*, there low abundance expression may explain the pest's sensitivity improvement against pesticides. The research on the expression profile analysis will deepen our understanding on molecular mechanism of adaptability evolution between *Candidatus* Liberibacter asiaticus and its vector, and provide potential targets on pest control.

IRCHLB-P3-33

Interactive digital video animation promotes accessibility of complex ACP anatomy to cytological and molecular audiences in pursuit of solving the citrus greening problem

Joseph M. CICERO[1], Javier ALBA-TERCEDOR[2], Wayne B. HUNTER[3], Liliana M. CANO[4], Surya SAHA[5], Lukas A. MUELLER[5], Susan J. BROWN[6]

[1]Entomology and Nematology Dept., 1881 Natural Area Dr., Steinmetz Hall, University of Florida, Gainesville, 32611. [2]Dept. de Zoologia, Facultad de Ciencias, Universidad de Granada 18071, Spain. [3]USDA-ARS, Horticultural Research Lab, 2001 S. Rock Rd., Fort Pierce, FL 34945. [4]IRREC, University of Florida, 2199 S. Rock Rd., Ft. Pierce, FL 34945. [5]Boyce Thompson Institute, 533 Tower Rd., Ithaca, NY 14853. [6]Dept. Biology, Ackert Hall, Kansas State University, Manhattan, KS 66506.

Abstract: We used premier 2D interactive digital video animation of the ACP alimentary canal and stylet biogenesis at http://citrusgreening.org as a solution to the lack of synthesis between organ level and cell/molecular level studies. Prodigious data sets and analyses of proteomes, transcriptomes, metabolomes, biochemical pathways, and cell-HLB pathogen interactomes have been generated in the collective efforts to solve the citrus greening problem. However, studies on their direct correlation to anatomy at the organ, tissue, and cell-field levels are relatively few. One reason for this is the difficulty in exposition by authors, and assimilation by readership, of ACP organ systems, owing to their 3- dimensionality, multifunctionality, ontogenetic differences, and in many cases their dynamic nature. Other reasons include the limitations of specimen processing techniques and interpretation of results, the static nature of cross-sectional micrographs, and their infinitesimal representation of the total organ

from which they came. Our platforms target broad audiences and are designed to be educational and to supplement, rather than replace, the traditional monographic style. They are novel in that their text flows with the frames, and micrographs appear when the animation calls for them. Viewers can page through the animation and hop to any micrograph in any direction by clicking buttons. Detailed study of organ components is simplified so that readers can assimilate organ complexity much more easily in association with their high- density monograph equivalents. Dynamics, such as the food stream, waste stream, seminal fluid stream, midgut transposition, and mobilization of the stylet biogenesis apparatus can be animated in lieu of available technology to visualize them in vivo. When combined with 3D computed tomography, this approach has the potential to revolutionize how anatomy is published.

Non-Technical summary: Insect organs are 3-dimensional, and may be multifunctional, dynamic, and have developmental differences between instars. These criteria alone are enough to make the traditional, monographic approach to elucidating insect anatomy very difficult for the author to compose and very difficult for the reader to study and assimilate. Our emergent solution is to design 2D animated tutorials (see citrusgreening.org) that cover the basics of organs and organ systems to facilitate reading the suite of monographs published on the subject.

IRCHLB-P3-34

Localization of *Candidatus* Liberibacter asiaticus in *Diaphorina citri* at the ultrastructural level

Diann ACHOR[1], El-Desouky AMMAR[2] and **Amit LEVY[1,3]**

[1] Citrus Research and Education Center, University of Florida, Lake Alfred, USA, [2] ARS-USDA, Fort Pierce, USA, [3] Department of Plant Pathology, University of Florida, Gainesville, USA

Abstract: Huanglongbing (HLB also known as citrus greening), the most destructive disease to citrus plants, is associated with the phloem-restricted gram-negative bacterium '*Candidatus* Liberibacter asiaticus' (CLas) and is vectored by the Asian citrus psyllid (ACP, *Diaphorina citri*). Current management options for HLB are limited, and heavily rely on the application of chemical insecticides for limiting ACP populations. However, these strategies are problematic and have not been effective so far. Understanding the complex interactions between ACP and CLas may open new avenues for psyllid or bacterial control. It has been strongly suggested that CLas is transmitted by ACP in a circulative- propagative manner since the bacterium was shown to accumulate/propagate in infected ACP using qPCR, and it was also detected in various psyllid organs by qPCR and fluorescence *in situ* hybridization (FISH). However, since these methods could not provide ultrastructural details, we used transmission electron microscopy (TEM) and immunogold labeling to study the localization and ultrastructure of CLas inside various organs and tissues of infected ACP. Our results show that CLas is abundantly found in filamentous and quasispherical forms in many different ACP organs including the salivary glands, midgut, filter chamber, bacteriome and other tissues. In most cases, CLas cells were localized inside intracellular membranous vacuoles, and some appeared to be dividing. Our results further point to the close association between CLas and its psyllid vector, and strongly support a circulative-propagative mode of CLas transmission by ACP.

Non-technical summary: The control of citrus greening disease (HLB) relies heavily on using chemical insecticides for controlling the insect vector populations, and therefore the development of sustainable control measures is needed. Better understanding of the interactions that take place between the greening bacterium and its vector may enable the development of an efficient control mechanism for this pathogen. Here we used electron microscopy to show that CLas is abundantly found, and probably replicating, in most psyllid organs, supporting a circulative- propagative transmission mode of this bacterium in its psyllid vector.

IRCHLB-P3-35

Lower reproduction rates of *Diaphorina citri* on ‘Tahiti’ acid lime than ‘Valencia’ sweet orange

Jeane PULICI[1,2], Juan CIFUENTES-ARENAS[2], **Silvio LOPES**[2]

[1]São Paulo State University (UNESP), Jaboticabal, [2]Fund for Citrus Protection (FUNDECITRUS), Araraquara, Brazil

Abstract: The Asian citrus psyllid *Diaphorina citri* (ACP) vectors the bacterium associated with Huanglongbing (HLB), ‘*Candidatus* Liberibacter asiaticus’ (CLas), which may infect all commercial citrus types. Despite the great commercial importance of acid limes, little is known about their suitability for ACP reproduction. Two greenhouse and/or acclimatized room experiments were carried out using two-year- old plants of ‘Tahiti’ acid lime and ‘Valencia’ sweet orange, both grafted on ‘Cravo’ Rangpur lime. In the first experiment, two unmated couples of 15-day-old adult ACPs were confined on a single newly developed young shoot per plant. After 48 hours from the onset of oviposition, the insects were removed and the number of eggs and hatched nymphs were counted. In the second experiment, 30 newly hatched individual nymphs were transferred to the newly developed individual shoots and the time needed for them to become adults was evaluated. In both experiments, the duration of nymph stage was longer (19.8±0.88 and 28.3±2.14 days) and the percentage of nymphs that became adults (31.2±3.51 and 21.7±4.79 %) was lower on ‘Tahiti’ than on ‘Valencia’ (14.0±0.17 and 20.1±0.64 days, 64.3±3.22 and 65.0±2.90 %).

In addition, the period for the adults to emerge was higher and irregular on ‘Tahiti’. On this host 80% of the individuals appeared in 13 and 19 days of confinement against six and ten days on ‘Valencia’.

Non-Technical summary: In Brazil, orchards of ‘Tahiti’ acid lime are commonly found near those of sweet oranges. ‘Tahiti’ and sweet oranges host ACP and are susceptible to HLB. Under controlled environment ACP reproduced two times slower on ‘Tahiti’ than on ‘Valencia’ sweet orange. The lower ability of ACP to reproduce on ‘Tahiti’ may have some impact on CLas dissemination in this host, which needs to be confirmed under field conditions.

IRCHLB-P3-36

Peptidomics-driven insights into *Diaphorina citri* physiology and response to ‘*Candidatus* Liberibacter asiaticus’.

Laura A. FLEITES[1,2,3] Angela R. KRUSE[2], Richard JOHNSON[4], John S. RAMSEY[3], Michael MACCOSS[4], Ronald J. NACHMAN[5], David G. HALL[6], and Michelle HECK[1,2,3]

[1]Boyce Thompson Institute for Plant Research, Ithaca, NY, [2] Department of Plant Pathology and Plant Microbe Biology, Cornell University, Ithaca, NY, [3] USDA Agricultural Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, NY, [4] Department of Genome Sciences, University of Washington, Seattle, WA, [5] USDA Agricultural Research Service, Insect Control and Cotton Disease Research Unit, College Station, TX, [6] USDA Agricultural Research Service, US Horticulture Research Laboratory, Fort Pierce, FL

Abstract: The importance of the psyllid in the

'*Candidatus* Liberibacter asiaticus' (CLas) disease cycle has driven multiple 'omics-based studies; however, the psyllid peptidome, a dynamic set of small polypeptides continuously produced by proteolysis and other cellular processes, has not yet been described. To characterize the psyllid peptidome, we tailored conventional proteomics methodology for the isolation and identification of native peptides in adult psyllids reared on healthy or CLas-infected citrus. Briefly, proteins were extracted from four replicates of ~700 adult psyllids reared on CLas-infected or uninfected citrus, native peptides separated by centrifugal ultrafiltration, and the extracts were analyzed by high resolution mass spectrometry. Peptides with strong sequence and structural homology to neuropeptides were identified, and remarkably, 10 of the 13 neuropeptide precursors identified were downregulated in CLas-infected insects. These neuropeptides are compelling candidates for the development of interdiction strategies in the psyllid, and functional validation is in progress. In addition, a series of candidate peptide biomarkers that predict the infection state of psyllids were identified. Many of these candidate biomarkers, which were found consistently upregulated in or unique to CLas-infected insects, resemble known antimicrobial peptides (AMPs). These candidate AMPs have been synthesized and will be tested for activity against a panel of bacteria, including the only culturable species of Liberibacter, *L. crescens*. Taken together, the information obtained in this work not only yields new insights into psyllid physiology and multitrophic interactions, but may also lead to the development of novel diagnostic and management tools for HLB.

Non-technical summary: Unravelling the interactions between the Asian citrus psyllid and CLas is a critical area of HLB research, and may lead to the development of acquisition and/or transmission blocking strategies. In this work describing the collection of small proteins found in the psyllid (the peptidome), we have found evidence for multiple classes of bioactive molecules that are responsive to the presence of the bacteria that cause HLB. Enhancement or attenuation of the production of these small proteins, which may play a key role in the ability of the psyllid to spread CLas, may successfully interrupt the disease cycle and enable growers to halt the spread of HLB in groves and to potentially eradicate CLas.

IRCHLB-P3-37

Phylogeography of *Diaphorina citri* and its primary endosymbiont, '*Candidatus* Carsonella ruddii' : an evolutionary approach to host-endosymbiont interaction

Yanjing WANG[1, 2], Jinming LU[1, 2], G. Andrew C. BEATTIE[3], Md. Rashidul ISLAM[4], Paul HOLFORD[3], Yurong HE[1], **Yijing CEN[1]**

[1]South China Agricultural University, Guangzhou, China, [2]Zhejiang A&F University, Hangzhou, China, [3]Western Sydney University, Penrith, Australia, [4]Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

Abstract: In insects, little is known about the coevolution between their primary endosymbionts and hosts at the intraspecific level, and this study has examined codiversification between the notorious agricultural pest *Diaphorina citri* and its P-endosymbiont, '*Candidatus* Carsonella ruddii' at the population level. To test whether *D. citri* and its P-endosymbiont have parallel evolution and to trace spread of the psyllid from its assumed origin in South Asia, five mitochondrial fragments from *D. citri* and three housekeeping genes from *C. ruddii* were used to reconstruct phylogenies, and then measured the congruence between the phylogenetic trees derived from these two species. Maximum likelihood, haplotype network, principal components and Bayesian clustering identified three lineages for *D. citri* and its P-endosymbiont: a Western clade containing individuals from Pakistan, Bhutan (Phuentsholing), Vietnam (Son La), USA, Myanmar and China (Ruili, Yunnan); a Central

clade, with accessions originating from Southwest China, Bhutan (Tsirang) and Bangladesh; and an Eastern clade containing individuals from Southeast Asian countries, and East and South China. A more diverse genetic structure was apparent in the host mtDNA compared to their P-endosymbionts; however, the two sets of data were strongly congruent. This study provides evidence for the codiversification of *D. citri* and its P-endosymbiont during the invasion process from South Asia to East and Southeast Asia. We also suggested that the P-endosymbiont may facilitate investigations into the genealogy and migration history of the host. The biogeography of *D. citri* and its P-endosymbiont indicated that *D. citri* colonized and underwent a secondary dispersal from South Asia to East and Southeast Asia.

Non-Technical summary: Symbiosis-based approaches have been recognized as a potential strategy for insect pest control, one of them is the disruption of bacterial symbionts required by insect pests. Understanding the co-divergence will be informative both in advancing our understanding of the association of *D. citri* with its endosymbionts and developing specific molecular targets in the endosymbiont for biological control.

IRCHLB-P3-38

Effect of agriculturally common metals on Asian citrus psyllid sheath morphology and transmission of *Candidatus* Liberibacter asiaticus

Angela KRUSE[1], Chris HOSFORD[1], Joshua CHAPPIE[1], Robert SHATTERS[2], **Michelle HECK[1,3]**

[1]Cornell University, Ithaca, USA
[2]US Department of Agriculture, Fort Pierce, USA
[3]US Department of Agriculture, Ithaca, USA

Abstract: Huanglongbing, also known as citrus greening, is the most economically significant disease of citrus worldwide. It is associated with plant infection by the phloem-limited bacterium "*Candidatus* Liberibacter asiaticus" (CLas), which is transmitted between hosts by its hemipteran insect vector, *Diaphorina citri*. The transmission cycle begins with psyllid feeding, during which a protective sheath derived from salivary secretions forms around the insect's stylet mouthparts. Required for psyllid feeding, sheath formation is dependent on metal ions, and sheaths can form highly branched structures during feeding. This structure is made from carbohydrates with a small amount of proteins. We used high-resolution mass spectrometry to identify three copper oxidase proteins in the sheath. These proteins are hypothesized to oxidatively cross-link carbohydrates to form the sheath structure. We determined that these proteins bind and oxidize copper when expressed in both bacterial and insect cell lines. We then tested whether copper sulfate, which is a component of many common fungicides, has a direct effect on stylet sheath morphology when delivered to insects in artificial diets. These experiments showed that copper is required for higher-order sheath branching *in vitro*. In light of this striking finding, we are currently testing the effects of each agriculturally significant metal ion on sheath morphology and whether delivery of copper and other metal ions to plants changes the ability of the insect to transmit CLas.

Non-Technical Summary: The Asian citrus psyllid is the insect vector of the HLB bacterium. To effectively feed on citrus trees and transmit bacteria, the psyllid must form a sheath around its stylet mouthparts which is made of carbohydrates and proteins and forms branches during feeding. We identified three copper-binding proteins in this sheath which evidence supports to be important for sheath formation. We also found that copper is required for sheath branching and may also be required for insect transmission of the HLB bacterium.

IRCHLB-04: Pathogen Detection and Culturing Oral Presentations

IRCHLB-O2b-01

Culturing "*Candidatus* Liberibacter asiaticus", the Associated Agent of Citrus Greening Disease (Huanglongbing, HLB)

David R. GANG[1], Haluk BEYENAL[2], Anders OMSLAND[3], Nabil KILLINY[4], Judith K. BROWN[5], Steven FUTCH[4], Elham ATTARAN[3], Anna BERIM[1], Phuc Thi HA[2], Ruifeng HE[1], Banafsheh MOLKI[2], Mark WILLIS[1]

[1]Laboratory for Cellular Metabolism and Engineering, Institute of Biological Chemistry, Washington State University, Pullman, WA 99164, USA; [2]The Gene and Linda Voiland of ChemicalEngineering and Bioengineering, Washington State University, Pullman, WA 99164, USA; [3]Paul G. Allen School of Global Animal Health, Washington State University, Pullman, WA 99164, USA; [4]Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850, USA; [5]School of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

Abstract: Over the past several years, infection rates of Huanglongbing (HLB, citrus greening disease) in citrus groves have increased dramatically. Development of methods to fight the disease, associated with *"Candidatus* Liberibacter asiaticus" (*C*Las), has been hampered by lack of ability to culture *C*Las in the lab, which precludes the testing of gene functions that may be involved in *C*Las viability or virulence. Identification of such gene functions will have an enormous impact on the ability to control *C*Las and eventually allow development of integrated strategies to stop HLB spread by the Asian citrus psyllid (ACP) and the resulting economic devastation to citrus agriculture. Development of the ability to culture *C*Las in vitro will open up new avenues of research that are guaranteed to provide a game change in knowledge of *C*Las biology and transmission, including a culturing platform necessary for development of an amenable genetic system. Recent progress by our team includes the development of methods to grow *C*Las within eukaryotic tissues and, equally exciting, growth of *C*Las within biofilms that can be repeatedly subcultured or reinitiated. Sequential biofilm cultures have been maintained for going on 2 years now, with more than a dozen subculture transfers having been performed. Microbial community analysis of the biofilms, physicochemical data from infected ACP, and results from single- insect metabolomics analysis will be presented as well. **Non-Technical Summary**: As infection rates of Huanglongbing (HLB, citrus greening disease) rise worldwide, the need for tools to fight this devastating disease becomes increasingly clear. Until now, the lack of a reliable, reproducible, and extensible culturing method for *"Candidatus* Liberibacter asiaticus" (*C*Las) have hampered these efforts. Our method to culture CLas within biofilms has led to continuous culture of this important pathogen for 2 years and now opens the door to development of new strategies to fight this important pathogen.

IRCHLB-O2b-02

Liberibacter crescens rapid death phase in rich medium is due to medium alkalinization via ammonia evolution

Marta,SENA-VELEZ[1],
Manu AGGARWAL[2], Sean HOLLAND[1], Nick COGAN[2], Mukesh JAIN[3], Dean W. GABRIEL[3], **Kathryn M. JONES**[1]

[1]Department of Biological Science, Florida State University, Tallahassee, FL 32306, USA [2]Department of Mathematics, Florida State University, Tallahassee, FL 32306, USA

[3]Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA

Abstract: As the only cultured species of *Liberibacter*[1], *Liberibacter crescens* (Lcr) has emerged as an important model organism for the study of *Candidatus* Liberibacter asiaticus (CLas) and other related plant pathogens. Understanding the constraints on Lcr growth and metabolism will provide a foundation for developing culture methods for the uncultured *Liberibacter* plant pathogens. Our goals are to optimize growth conditions for Lcr strain BT- 1 in rich medium and establish parameters for mathematical modeling of the rapid death phase of Lcr in batch culture. We have made several important determinations about Lcr BT-1 growth and metabolism. Under optimized growth conditions at 30^{o}C in ACES-buffered BM7 medium, Lcr rapidly alkalinizes the medium. Cultures reach a maximum pH of 8.3-8.5, leading to a death phase that is accelerated at higher temperatures. Increasing the ACES buffer concentration 2-fold prevents alkalinization above pH 7.5 and prolongs survival of Lcr cells in stationary-phase cultures. Medium alkalinization by aerobic chemoheterotrophs is usually due to the evolution of ammonia from the deamination of amino acids[2]. We have found that Lcr grown in BM7 evolves NH4 (or other amines detectable by the phenol nitroprusside/alkaline hypochlorite assay[3]). Prolonging culture survival by maintaining pH 7.5 with 2x ACES buffer allows the cultures to evolve a higher concentration of NH4, although they remain limited to a similar maximum culture density. Thus, medium alkalinization rather than accumulation of NH4 is the apparent cause of loss of viability. Growth of Lcr in NH4Cl as high as 12 mM in otherwise standard BM7 medium has no adverse effect on growth, slows alkalinization, and increases the recoverability of cells from late stationary-phase cultures by ≥100 fold. A possible explanation is that the high concentration of NH4$^+$ from NH4Cl inhibits deamination of amino acids by Lcr, and that under these conditions, other carbon sources are preferred.

Non-technical summary: *Liberibacter crescens* (Lcr) is the only culturable bacterium in the genus *Liberibacter*. Thus, it is a useful model system for the study of growth dynamics of this genus of bacteria, which includes CLas, the citrus-greening associated pathogen. We have found that in rich medium Lcr alkalinizes the culture, poisoning itself. The alkalinization appears to be caused by production of ammonia. The mechanism of this effect is currently under investigation. CLas and other *Liberibacter* plant pathogens must survive in their plant and insect hosts under very different sets of conditions based on the availability of nutrients and metabolites, and these findings may elucidate the mechanisms by which they do this.

Citations:

Leonard MT, Fagen JR, Davis-Richardson AG, Davis MJ and Triplett EW. 2012. Std. Gen. Sci. 7:271.

Robinson TP, Wimpenny JWT, Earnshaw RG. 1991. J. Gen. Microbiol. 137:2885.

Chaney AL, Marbach EP. 1962. Clin. Chem. 8:130.

IRCHLB-O2b-03

Galleria mellonella: A potential artificial host for Liberibacters

Alejandra MUNOZ-BODNAR, Lulu CAI, Mukesh JAIN and Dean W. GABRIEL
University of Florida, Department of Plant Pathology, Gainesville, FL, USA

Abstract: *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter africanus (CLaf) and *Candidatus* Liberibacter americanus (CLam) are the associated agents of HLB, currently the most serious disease affecting citrus worldwide. None of the pathogenic

Liberibacters have been sustainably cultured to date. Their fastidious nature makes them difficult subjects for functional genomics, endosymbiotic, pathogenicity or host range studies. The only Liberibacter that has been cultured is *Liberibacter crescens* (Lcr), originally isolated from Babaco papaya plants. Despite genomic similarities with the pathogenic species, Lcr appears to be nonpathogenic and has no known insect associations. Nevertheless, Lcr has been successfully used as a model for functional genomics to demonstrate activity of certain Las genes implicated in pathogenicity and symbiosis. The Greater wax moth (*Galleria mellonella*) is used effectively as a model host for several bacterial pathogens, including some plant pathogenic bacteria. Due to their reduced immune system and rich environment in the hemolymph, the larvae provide all the essential nutrients needed for many pathogenic bacteria to multiply. Here, we report marker integration and expression of genes encoding GFP and kanamycin resistance into Lcr strain BT-1. The resulting BT-1::pMJ029 strain was injected into wax moth larvae. Fluorescence microscopy and qPCR performed one and seven days post inoculation into the anterior hemocoel confirmed movement of the bacteria from the injection site to the Malpighian tubules, known to support the growth of several endosymbiotic bacteria. Moreover, GFP expression indicated growth. This is the first report of growth of Lcr in any host and provides a possible model host for any Liberibacter.

Non-technical summary:

Candidatus Liberibacter asiaticus, the bacteria associated with HLB, are not cultivable under laboratory conditions, which seriously inhibits experimental study. The closest cultured relative of Las bacteria is *Liberibacter crescens* (Lcr), but it has the serious limitation in having no known hosts. In this work, we demonstrated wax moth larvae can be a host for Lcr, constituting the first report of a possible model host for pathogenic Liberibacters.

Citations:

Killiny, Nabil. "Generous hosts: Why the larvae of greater wax moth, Galleria mellonella is a perfect infectious host model?." *Virulence* 9.1 (2018): 860-865.

Leonard, Michael T., et al. "Complete genome sequence of Liberibacter crescens BT-1." *Standards in genomic sciences* 7.2 (2012): 271.

IRCHLB-O2b-04

HLB associated bacteria in Brazil: qPCR multiplex diagnosis, occurrence and distribution

Elaine C. MARTINS, Diva do Carmo TEIXEIRA, Daniela A. B. COLETTI, **Nelson** A. WULFF**.**

Fundo de Defesa da Citricultura – Fundecitrus, Av. Dr. Adhemar Pereira de Barros, 201.Araraquara - SP, Brazil.

Abstract: The occurrence of *Candidatus* Liberibacter asiaticus, *Ca*. L. americanus, 16Sr group IX and 16Sr group III phytoplasmas in citrus samples, all associated with blotchy mottle symptoms, poses challenges to diagnose using molecular techniques. qPCR has several advantages in this regard, highlighted here by multiplexing and sensitivity. We developed a group of primers and probes that can be used either in single reactions or together, multiplexing the detection and identification of HLB- associated bacteria. The use of more conserved genes from the beta operon of ribosomal proteins for both Liberibacters circumvent specificity and cross-reactivity problems related with 16Sr DNA detection. On the other hand, primers and probes targeted to 16Sr DNA, allow a broad detection in relation to phytoplasmas, a more diverse group than Liberibacters. Targeted genes were evaluated for sequence diversity and multiplex detection was validated against published protocols. Our results allow a precise and specific detection of HLB associated bacteria in Brazil and can be

used worldwide for the detection of such bacteria. The predominance of *Ca*. L. asiaticus in citrus samples in Brazil was demonstrated with field samples diagnosed for the above-mentioned bacteria. Additionally, the distribution and occurrence of Liberibacters and Phytoplasmas is recorded in a state-wide scale, a necessary evaluation to understand the shift in prevalence along time and space for Huanglongbing associated bacteria.

Non-Technical summary: Diagnose of HLB symptoms with qPCR is a research tool and service for growers, requiring high throughput, sensitivity and specificity. Here we show results from the development of a multiplex qPCR system to detect HLB- associated bacteria.

IRCHLB-O2b-05

Detection of '*Candidatus* Liberibacter africanus subspecies' from commercial citrus orchards in South Africa.

Ronel ROBERTS[1,3]
and Gerhard PIETERSEN[2, 3]

[1]Plant Microbiology Division, Agricultural Research Council-Plant Protection Research, Private Bag X134, Pretoria 0001, South Africa
[2]Genetics Department, University of Stellenbosch, Stellenbosch 7600, South Africa,
[3]Biochemistry, genetics and Microbiology Department, University of Pretoria, Pretoria, 0002, South Africa.

Abstract: '*Candidatus* Liberibacter africanus subsp. clausenae' (LafCl) was shown to be associated with citrus trees displaying mottling symptoms, characteristic of African Citrus Greening (ACG) disease, in East Africa. Up until the description of LafCl from citrus, it was accepted that '*Ca.* L. africanus' (CLaf) is the sole agent causing African greening in the majority of African countries, and that the five known subspecies of Laf i.e. '*Ca.* L. africanus subsp. capensis' (LafC), '*Ca.* Liberibacter africanus subsp. tecleae'(LafT), '*Ca.* L. africanus subsp. vepridis' (LafV) and '*Ca.* L. africanus subsp. zanthoxyli' (LafZ) in addition to LafCl, were unlikely to play a role in the epidemiology of this disease. To determine whether any of these subspecies infected commercial citrus crops in South Africa, primer sets specific to the available outer-membrane protein (*omp*) sequences of each of these CLaf subspecies were designed. These primer sets were additionally assessed in multiplex reactions. Each primer set was shown to specifically amplify its intended target, with no cross-amplification of non- target sequences observed. All except LafC primer sets could be multiplexed in different combinations. Using these primer sets, 243 DNA extracts, sampled from commercial citrus farms across South Africa, were screened for the presence of CLaf-subspecies. The results from this study indicated that Laf is the only known Liberibacter species associated with citrus in South Africa, suggesting that the transmission of the CLaf-subspecies to citrus is not a common occurrence and may be reliant on various external factors.

Non-technical summary: Five subspecies of '*Candidatus* Liberibacter africanus' (CLaf), the associated agent of African Citrus Greening (ACG), are known from South Africa and are associated with indigenous citrus-family species. One of these subspecies was shown to infect commercial citrus is East Africa. To determine whether these subspecies infect citrus in South Africa, a CLaf-subspecies test was developed. The test confirmed that CLaf is the sole agent associated with ACG in South Africa.

IRCHLB-O2b-06

Performance comparison of main real-time PCR detection methods for the plant pathogen '*Candidatus* Liberibacter' spp. associated with the Huanglongbing disease on *Citrus* spp.

Gilles CELLIER[1], Géraldine ANTHOINE[2], Jaime CUBERO[3], Cristina REDONDO[3], Pilar SABUQUILLO[3], Monserrat ROSELLO[3], Eugénia de ANDRADE[4], Leonor CRUZ[4], Paula Sá PEREIRA[4], Elen INCE[5], H. Nilüfer YILDIZ[5], Pakize Gök GULER[5], Anna Maria D'ONGHIA[6], Thaer YASEEN[6], Christina DEVORSHAK[7] and Baldissera GIOVANI[8].

[1] Anses, National Plant Health Laboratory, Tropical Pests and Diseases unit, Reunion Island, France, [2] Anses- National Plant Health Laboratory, Coordination of Reference Activities unit, Angers, France, [3] National Institute for Agricultural and Food Research and Technology (INIA), Spain, [4] National Institute for Agricultural and Veterinarian Research (INIAV), Portugal, [5] Biological Control Research Institute (BMAE-BCRI), Turkey, [6] CIHEAM-Istituto Agronomico Mediterraneo of Bari, Italy, [7] U.S. Department of Agriculture, Animal and Plant Health Inspection Service (APHIS-USDA), United States of America, [8] European and Mediterranean Plant Protection Organization (EPPO) - Euphresco, Paris, France

Abstract: The disastrous Huanglongbing disease is found on the Rutaceae family and associated with three nonculturable species of recognized bacteria of the genus *Candidatus* Liberibacter: asiaticus (CLas), africanus (CLaf) and americanus (CLam). Considered as a quarantine pest in Europe, as well as in the United States of America, these three species pose a major risk for the citrus industry across the world. The visual inspection of symptomatic plants is a routine method for the surveillance of HLB disease, but it can be misinterpreted as symptoms can be confused with nutritional disorders or with other diseases. Furthermore, due to the low titer and uneven distribution of the bacterium within its host plant, conventional PCR detection can lead to false negative results. Seeking for a more adapted solution to detect this disease especially in symptomless plants, the three main diagnostic real-time PCR tests [1-3] recommended by both the EPPO and FAO were fully assessed for their performance and compared to a conventional PCR, within the framework of the EUPHRESCO project 2016-A-232. This comparative and assessment analysis produced both intra and inter-laboratory performance data following the EPPO validation protocol PM7/098.Full results of the performance validation of the Real Time PCR tests, along with data from a collaborative test performance study organized among eight international laboratories will be presented and compared to the reference conventional PCR test. Such validation data will beneficiate to national, European, and International policy makers, National Plant Protection, and citrus industry and other stakeholders. The thorough and comparable assessment framework guaranteed the reliability and homogeneity of the data obtained. Finally, this work built trust and expertise both in the research and the diagnostic fields for the management of the HLB disease along with the international scientific effort.

Non-Technical summary: The biology of the bacteria associated with the HLB disease makes its detection tricky: symptoms can be confused with nutritional disorders or with other diseases, is nonculturable and distributed unevenly in the host plant. Early detection tests based on a high sensitivity need to be employed to follow up the infestation of crops; nevertheless performance validation need to be produce in first place in order to ensure a robust framework and then guarantee the reliability and homogeneity of the data obtained. Here, we are comparing main high sensitivity detection tests to produce validation data to help decision-makers choosing the right tool for reliably detect the pathogen in the early stages of infection.

Citations:

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: 104-115.

Morgan JK, Zhou L, Li W, Shatters RG, Keremane M, Duan YP. 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. 2012; 26: 90-98.

Bertolini E, Felipe RTA, Sauer AV, Lopes SA, Arilla A, Vidal E, Mourão Filho FAA, Nunes WMC, Bové JM, López MM, Cambra M. Tissue-print and squash real-time PCR for direct detection of '*Candidatus* Liberibacter' species in citrus plants and psyllid vectors. Plant Pathol. 2014; 63: 1149-1158.

IRCHLB-04: Pathogen Detection and Culturing Poster Presentations

IRCHLB-P4-39

A new real-time PCR method for the detection of *Candidatus* Liberibacter africanus (*C*Laf) in citrus root tissue

John V. DA GRAÇA[a], Jong-Won PARK[a], Glynnis COOK[b], Eliezer S. LOUZADA[a], John E. RASCOE[c], and **Madhurababu KUNTA**[a]

[a]Texas A&M University-Kingsville Citrus Center, 312 N. International Blvd., Weslaco, TX 78599 [b]Citrus Research International, 2 Baker Street, Nelspruit 1200, South Africa [c]USDA APHIS PPQ CPHST, Beltsville Laboratory, Beltsville, MD 20705

Abstract: Huanglongbing (HLB), a great threat to citriculture worldwide, is associated with three '*Candidatus* Liberibacter' species, *Ca.* L. asiaticus (*C*Las), *Ca.* L. americanus (*C*Lam) and *Ca.* L. africanus (*C*Laf). Since there are no known HLB-resistant commercial cultivars available, early detection of those HLB-associated bacteria is critical to deploy a more efficient disease control strategy. Currently, the routine HLB diagnostic procedure relies on the visual inspection for the symptoms on the tree canopy followed by molecular biological test to confirm the presence of HLB-associated bacteria. However, slow symptom development and the uneven distribution of HLB-causing bacteria in the tree canopy can lead to a misdiagnosis, especially when no distinct HLB symptoms are present. Our previous study with *C*Las showed that *C*Las-positive tree exhibits more uniform distribution of *C*Las in the root system, which led us to the development of a root assay for *C*Las detection. The data obtained with *C*Las root assay indicated that earlier *C*Las detection can be achieved by the root assay. However, unlike *C*Las, there are no real-time PCR primer sets available for *C*Laf detection in the root tissue since the currently available *C*Laf primer set, HLBafpr, based on *C*Laf 16s rDNA, is not suitable for root assay due to its cross-reactivity with 16s rDNAs of non-target organisms present in the rhizosphere. In this study, we developed and evaluated a new set of real-time PCR primers/probe based on *C*Laf 16s rDNA (TXCC-Laf) for *C*Laf detection in the root tissue. The data showed that the *C*Laf- detection sensitivity of TXCC-Laf is comparable to that of HLBafpr and has minor cross-reactivity with *C*Las when it was tested against *C*Las-positive leaf tissue. The preliminary results obtained with *C*Laf- positive root tissue confirmed that the newly developed real-time PCR primers/probe, TXCC-Laf, can be applied for *C*Laf detection in root tissue for improved *C*Laf detection.

Non-technical summary: Huanglongbing (HLB), also known as citrus greening, continuously poses a great threat to the citrus cultivation worldwide. Since our ongoing project indicated that the root assay can improve the detection rate of HLB-causing bacteria and achieve earlier detection, the current study focused and developed a new detection method of another HLB associated bacteria, *Candidatus* Liberibacter africanus (*C*Laf) in the root tissue. The preliminary data obtained with *C*Laf positive root tissue indicated that the newly developed *C*Laf root assay can lead to improved *C*Laf

detection.

IRCHLB-P4-40

Antibody-based detection of Huanglongbing (HLB)-associated pathogen

Agustina DE FRANCESCO[1], Kelley CLARK[1], Kibaek LEE[2], Xin GE[2], Wenbo MA[1]

[1] Department of Microbiology and Plant Pathology, University of California, Riverside, USA
[2] Department of Chemical and Environmental Engineering, University of California, Riverside, USA

Abstract: HLB is associated with the phloem-colonizing bacterium *Candidatus* Liberibacter asiaticus (CLas) in the US. Direct detection of CLas is challenging due to sporadic distribution of the bacterial cells in infected trees and the long, variable asymptomatic phase. To reinforce the current qPCR-based diagnosis, we have been developing antibody- based diagnostic methods by targeting unique cell surface and secreted proteins of CLas. The secreted proteins could serve as promising detection markers because they are not restricted to the infection sites after being secreted from the CLas cells in the phloem. CLas possesses the Sec secretion system, through which a variety of Sec-delivered effectors (SDEs) could be secreted. Using CLas genome sequences, we predicted SDEs and analyzed their expression profiles in order to select suitable biomarker for detection. Antibodies have been generated to target selected cell surface or secreted proteins as antigens and used in different formats of enzyme-linked immunosorbent assay (ELISA). The ELISA protocols were evaluated *in vitro* and are currently tested using field samples. On-going efforts on the optimization of the ELISA protocols for HLB diagnosis will be discussed.

Non-Technical Summary: HLB is the most devastating disease threatening the citrus industry, and there is still no effective treatment to cure the trees after they are infected. It is extremely important to develop robust detection methods in order to remove infected trees promptly, thereby restricting the further spread of the disease. This project addresses these urgent needs. Antibody-based detection methods will be used together with the qPCR-based direct detection method to support field surveys.

IRCHLB-P4-41

Biochemical characterization of *Liberibacter crescens* lipopolysaccharide (LPS)

Mukesh JAIN[1], Ian BLACK[2], Eva HAWARA[3], Parstoo AZADI[2], Russ CARLSON[2], Wenbo MA[3], Kathryn M. JONES[4], Dean W. GABRIEL[1]

[1]Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
[2]Complex Carbohydrate Research Center, University of Georgia, Athens, GA 30602, USA
[3]Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521, USA
[4]Department of Biological Science, Florida State University, Tallahassee, FL 32306, USA

Abstract: Lipopolysaccharide (LPS) is the major surface molecule of Gram-negative bacteria and consists of three distinct structural domains: the outermost O-antigenic polysaccharides, the core and the hydrophobic lipid A anchored in the outer leaflet of the bacterial outer membrane. The lipid A moiety is a glucosamine-based phospholipid endotoxin implicated in the Gram-negative septic shock. *Ca.* Liberibacter spp. are uncultured phloem-limited bacterial plant pathogens and insect

endosymbionts associated with devastating plant diseases such as citrus greening (Huanglongbing or HLB) and zebra chip of potato. Nonpathogenic *L. crescens* (Lcr) is the most basal Liberibacter lineage, and a cultured surrogate for functional genomic studies of pathogenic *Ca.* Liberibacters, none of which have been cultured. Compositional analysis of the LPS extracted from Lcr revealed that its LPS is comprised primarily of ribose, galactose and rhamnose (43.8, 22.9, and 11.9 Mol %, respectively) with a smaller amount of glucose (8.3 Mol %) representing the O chain of the LPS and the outer core oligosaccharide. 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) and a minor amount of heptose likely represent the inner core. The lipid A from *L. crescens* has a traditional penta-acylated chitobiose backbone, containing two ester- inked C16:0 (3-OH) fatty acid residues, two amide-linked C14:0 (3-OH) residues and a very long chain fatty acid (VLCFA) C28:0 (26-OH). All the structural genes of the Raetz pathway for lipid A biosynthesis (except for *lpxM* encoding myristoyltransferase) have been annotated in the genomes of Lcr, *Ca.* L. asiaticus, *Ca.* L. africanus and *Ca.* L. solanacearum. *Sinorhizobium meliloti* genes *lpxXL* (SMc04268) and *acpXL* (SMc04278) encode a lipid A C28 acyltransferase and an acyl carrier protein involved in the biosynthesis of VLCFA-modified lipid A. Homologs for both these genes are present in Lcr (B488_RS04675 and B488_RS04700,
respectively) but absent in all pathogenic (and uncultured) Liberibacters. Multiple attempts to inactivate *lpxXL* in Lcr by marker interruption failed, leading us to speculate that uncultured Liberibacters may require VLCFA-modified lipid A for free living growth in culture.

Non-technical summary:
Comparative genomics and metabolic pathway analyses have revealed a trend for the reduction or complete absence of several biosynthetic pathways, metabolic enzymes, and secretion systems in the genomes (1.2 Mb) of uncultured pathogenic Liberibacters. The only Liberibacter sp. cultured to date, *L. crescens* with a genome size of 1.5 Mb, has provided valuable clues about the genes essential for free living growth in culture. Biochemical analyses revealed the presence of a very long chain fatty acid (VLCFA)- modified lipid A in *L. crescens* (Lcr) LPS. VLCFA-modified lipid A is a unique feature of several bacteria such as *Sinorhizobium meliloti* and *Brucella abortus* that form chronic intracellular infections within legumes and mammalian hosts. In *S. meliloti* symbiosis, absence of VLCFA-LPS results in increased sensitivity to salt, detergents and pH etc. Knockout mutations of various Lcr genes are readily obtained, but not mutations affecting formation of VLCFA-modified lipid A. We hypothesize that VLCFA-modified lipid A is essential for axenic growth of pathogenic Liberibacters.

Citations:

Ferguson GP, Datta A, Carlson RW and Walker GC. 2005. Mol. Microbiol. 56:68-80
Raetz CR, Reynolds CM, Trent MS and Bishop RE. 2007. Annu. Rev. Biochem. 76:295-329

IRCHLB-P4-42

Closer to a culture: creation of a minimal media in *Liberibacter crescens* reveals essential growth requirements

Joseph R. PETRONE[1], Alam MUNOZ-BERISTAIN[1], and **Eric W. TRIPLETT[1]**

[1]University of Florida, IFAS, Microbiology and Cell Science Department, Gainesville, USA

Abstract: Huanglongbing disease is threatening to wipe out citrus production globally. The inability to culture the associated agent *in vitro* prevents traditional microbiological approaches towards disease treatments. *Liberibacter crescens*, as the closest cultured relative to the *Candidatus* Liberibacter pathogens, is a good model to study metabolism within the genus. Insights into the media requirements of

Liberibacter will help develop an understanding of optimal conditions towards culturing and treatment strategies. Creating a minimal growth medium in M15, the chemically defined media, revealed the essential amino acids for axenic culture. The three branched-chain amino acids (leucine, isoleucine, and valine) are all strictly required for growth and cells are unable to grow in the absence of one or more of the BCAA's. Current annotation of the BT-1 genome suggests *L. crescens* is unable to biosynthesize or degrade these BCAA's, yet experimentation reveals an inaccurate functional annotation of the degradation pathways. Cysteine was also found to be among the essential compounds, even though the current available genomes would suggest an ability to biosynthesize this amino acid. Cysteine may be required in culture as part of a reactive oxygen species scavenging system. Aromatic amino acids are among the non-essential AA's required for growth, suggesting *L. crescens* has the ability to synthesize these *in vitro* to some extent. The biosynthesis pathway for aromatics in the pathogenic Liberibacters are generally annotated as incomplete, but varies across genomes and other species of HLB-associated pathogens. More research into functional annotation and amino acid biosynthesis abilities of the *Candidatus* Liberibacter is needed, but manipulation of these pathways in planta could lead to effective treatment strategies.

Non-technical Summary: A minimal media was created for the closest cultured relative to *Candidatus* Liberibacter asiaticus, *Liberibacter crescens.* Amino acids such as the BCAA's and cysteine were found to be essential for growth. Cysteine requirement may suggest a need to detoxify ROS in culture.

IRCHLB-P4-43

Detection of huanglongbing (HLB)-associated bacterium, *Candidatus* Liberibacter asiaticus (CLas) in citrus seedlings

Cheol Woo CHOI[1], Jae Wook HYUN[1], Rok Yeon HWANG[1], and Charles A POWELL[2]

[1]Citrus Research Institute, National Institute of Horticultural and Herbal Science, R.D.A. Jeju, 63607, S. Korea

[2]University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, 2199 South Rock Road,
Fort Pierce, FL 34945, USA

Abstract: Huanglonbing (HLB, Citrus greening disease) is one of most destructive plant disease in citrus production countries. HLB-associated bacterium, *Candidatus* Liberibacter asiaticus (CLas), is phloem- inhabiting and causes phloem collapse and phloem plugging, resulting in impairment of phloem transport of citrus. The use of disease- free seedlings is one of the strategies to manage the HLB disease and, when the seedlings are produced, two or three years old plants grown from seed of swingle, lemon or trifoliate were used as a rootstock. In this study, we confirmed quantitative polymerase chain reaction (qPCR) targeting 16s rDNA of CLas to detect the pathogen in citrus seedling. On May 16 of 2018, fruits of lemon, mandarin and sweet orange were sampled, in open field of IRREC (Indian River Research and Education Center, Fort pierce, FL). The seeds collected from these fruits were washed with 2% sodium-hypochloride, dried and sowed on the clean soil in the pot. After sowing, from June to October, three plants per each varieties, were sampled every month and genomic DNA extracted from these leaf, stem and root, were used to conduct quantitative polymerase chain reaction (qPCR) detection for CLas pathogen in these plant. In result of qPCR, the pathogen were detected in root of these varieties of a month old plants and in root, leaf and stem of three old plants. This result suggests that seeds produced from healthy trees should be used as rootstock for seedling production.

Non-Technical summary: The use of disease-free nursery trees is one of the strategies to manage the HLB disease. When producing the nursery tree, budwoods were produced from free-disease mother tree made by heat-treat and micro shoot graft, and two or three years old seedlings grown from seed of swingle, lemon or trifoliate were used as a rootstock. We conduct qPCR and HLB pathogens were detected in one to three olds seedling. This result suggests that healthy trees should be used as rootstock for nursery production.

Citations:

Li, W., Levy, L., and Hartung, J.S. 2009. Quantitative distribution of '*Candidatus* Liberibacter asiaticus' in citrus plants with citrus Huanglongbing. *Phytopathology*, 99: 139–144. doi:10.1094/PHYTO-99-2-0139

Li, W., Hartung, J. S. and Levy, L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. *J. Microbiol. Methods* 66:104–115. DOI:10.1016/j.mimet.2005.10.018

Morgan, J, K., Zhou, L., Li, W., Shatters, R. G., Keremane, M. and Duan, Y. P. 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.* **26(2):**90–98, doi: 10.1016/j.mcp.2011.12.001

IRCHLB-P4-44

Developing strategies and protocols for efficient testing of field trees for HLB

MaryLou POLEK
and Manjunath KEREMANE

USDA ARS National Clonal Germplasm Repository for Citrus and Dates, Riverside, CA USA

Abstract: *Candidatus* Liberibacter asiaticus (CLas) was detected in both psyllids and plants within three miles from the USDA ARS citrus germplasm repository and the University of California, Riverside campus in July 2017. The need to increase surveillance of the field planted germplasm collection became evident. However, the standard collection, processing and DNA extraction were labor intensive and costly. Therefore, a pilot study was conducted to develop alternative protocols to rapidly test field trees for the presence of CLas at a reasonable cost. A block of about 800 trees with multiple varieties was selected for this study. About 17,000 extractions were tested by real time PCR with no positive finds. To reduce the costs involved with commercial DNA extraction kits and associated plasticware, several protocols were evaluated. We report a high throughput DNA extraction which is significantly less expensive (by about 65%) but provides the same quality of DNA as commercial kits. For evaluation of the new procedure, both known stubborn and HLB infected tissue were used as controls. HLB infected tissue was received under permit from the University of California, Davis Contained Research Facility. In this new protocol, one person can process about eight 96-well plates in a day. This method does not fulfill regulatory requirements, and any suspect results must be reported to and confirmed by regulatory agencies.

Non-Technical summary: We conducted a pilot study to develop a less expensive method to rapidly survey a large number of field trees for the presence of HLB associated bacteria. A new sensitive high throughput protocol for DNA extraction was developed which resulted in saving about 65% in costs when compared to standard protocols using commercial kits.

IRCHLB-P4-45

Development of a tandem repeat-based polymerase chain displacement reaction method for sensitive detection of '*Candidatus* Liberibacter asiaticus'

Binghai LOU[1], Yaqin SONG[1], Moytri ROYCHOWDHURY[2], Chongling DENG[1], Ying NIU[1], Qijun FAN[1], Yan TANG[1], and Changyong ZHOU[3]

[1]Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops, Guilin, Guangxi 541004, P. R. China

[2]Department of Biological Sciences, Idaho State University, 650 Memorial Dr., Pocatello, Idaho 83201, USA

[3]Citrus Research Institute, Southwest University, Chongqing 400715, P. R. China

Abstract: Huanglongbing (HLB) is one of the most destructive diseases in citrus industry worldwide. Early detection of HLB pathogens can facilitate timely removal of infected citrus trees on field. However, low titer and uneven distribution of HLB pathogens in host plants make reliable detection challenging. Therefore, the development of effective detection methods with high sensitivity for the pathogens of HLB is important. Here we report the development of a novel method, tandem repeat-based polymerase chain displacement reaction (TR-PCDR), for the detection of '*Candidatus* Liberibacter asiaticus' (CLas), a widely distributed HLB- associated bacterium. A specially designed primer set TR2-PCDR-F/TR2-PCDR-1R and a thermostable *Taq* DNA polymerase mutant with 5' to 3' strand displacement activity were used for TR-PCDR amplification. Performed in a regular thermal cycler, TR-PCDR could produce more than two amplicons after each amplification cycle. Sensitivity of the developed TR-PCDR was 10 copies of target DNA fragment. The sensitive level was proven to be 100 times higher than conventional PCR and similar to real-time PCR. Data from the detection of Las with filed samples using the above three methods also showed similar results. No false-positive TR-PCDR amplification was observed from healthy citrus samples and water controls. These results thereby illustrated that the developed TR-PCDR method can be applied to the reliable, highly sensitive and cost- effective detection of Las.

Non-Technical summary: A new method with high sensitivity, named as tandem repeat- based polymerase chain displacement reaction (TR-PCDR), was developed for the diagnosis of Citrus Huanglongbing. It can enhance the accuracy of molecular diagnosis of Huanglongbing.

IRCHLB-P4-46

Development specific markers for Psy62, Gxpsy and Ishi-1 strains huanglongbing (HLB)-associated bacterium *Candidatus* Liberibacter asiaticus.

Cheol Woo CHOI[1], Jae Wook HYUN[1], Rok Yeon HWANG[1], and Charles A POWELL[2]

[1]Citrus Research Institute, National Institute of Horticultural and Herbal Science, R.D.A. Jeju, 63607, S. Korea

[2]University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, 2199 South Rock Road, Fort Pierce, FL 34945, USA

Abstract:

Huanglonbing (HLB, Citrus greening disease) is one of most destructive plant disease in citrus production countries. HLB-associated bacterium, *Candidatus* Liberibacter asiaticus (*C*Las), is phloem-inhabiting and causes impairment of phloem transport of citrus leading to tree decline and dead. Leaf symptoms of HLB were similar to micro- element deficiencies, such as zinc, iron and boron, so it is difficult to diagnose with the naked eye. Whole genome sequences of *C*Las strains, Psy62 from Florida, Gxpsy from china and Ishi-1 from japan, have been completed and genome structural variations of these strains were found by mapping and linear alignment of these strains genomes as

previously study. In this study, we develop three sets of PCR primers as specific markers for Psy62, Gxpsy and Ishi-1 based on genome variations and RT-PCR were conducted with genomic DNA of HLB-diseased leaf of grapefruit, sweet orange and clementine, as template. In result, Psy62 F/R primer set amplified an expected 206-bp band with genomic DNA of grapefruit and sweet orange, Gxpsy F/R primer produced a 218-bp band with that of grapefruit and clementine, and no products were amplified when using Ishi-1 F/R primer with these genomic DNA, as templates. With these markers, we'll try to test the relationship between HLB symptoms and pathogens.

Non-technical summary: There are insertion/deletion variations of three strains of huanglongbing (HLB)-associated bacterium *Candidatus* Liberibacter asiaticus, Psy62, Gxpsy and Ishi-1, and we assumed that there would be a difference of pathogenic features due to this genomic variation. We developed specific markers for these strains. Using these markers, it is expected that we will understand relationship between these strains and HLB symptoms of citrus host plants.

Citations:

Duan, Y. P., Zhou, L. J., Hall, D. G., Li, W., Doddapaneni, H., Lin, H., Liu, L., Vahling, C. M., Gabriel, D. W., Williams, K. P., Dickerman, A., Sun, Y. and Gottwald, T. 2009. Complete genome sequence of citrus Huanglongbing bacterium, 'Candidatus Liberibacter asiaticus' obtained through metagenomics. Mol. Plant-Microbe Interact. 22: 1011–1020. doi: 10.1094/MPMI-22-8-1011

Katoh, H., Miyata, S., Inoue, H. and Iwanami, 2014. Unique Features of a Japanese '*Candidatus* Liberibacter asiaticus' Strain Revealed by Whole Genome Sequencing. *PLoS ONE* 9(9): e106109.DOI: 10.1371/journal.pone.0106109

IRCHLB-P4-47

Duplex droplet digital PCR for detection of *Candidatus* Liberibacter asiaticus using 16S rRNA and ribonucleotide reductase genes

Yogita MAHESHWARI[1], Vijayanandraj SELVARAJ[1], Subhas HAJERI[2], **Raymond YOKOMI[1]**

[1]USDA-ARS, SJVASC, Parlier, CA, USA;
[2]CPDP, CCTEA, Tulare, CA, USA

Abstract: Huanglongbing (HLB) has now been detected in over 850 citrus trees across three urban counties of southern California. HLB is associated with "*Candidatus* Liberibacter asiaticus" (CLas), an uncultivable bacterium, and is vectored by the Asian citrus psyllid (ACP). Quarantines delimit initial spread and management of CLas and improved methods for early detection and rapid removal of CLas-infected trees are needed. CLas detection is challenging due to its uneven distribution, variable titer and long latent period in infected trees. Since the chromosome of CLas has three copies of the 16S rRNA gene and five copies of nrdB, encoding the β-subunit of ribonucleotide reductase (RNR), this study developed a duplex droplet digital PCR (ddPCR) procedure to detect CLas at low titer using primers for the 16S and RNR genes. Standard curve analyses on tenfold dilution series of CLas plasmid, plant leaf and insect DNA showed that the ddPCR assay exhibited excellent linearity and efficiency for both targets. The sensitivity of the duplex assay of the 16S and RNR plasmids were both 2 copies/20µl reaction. Using actual CLas low titer samples in leaf tissue and ACP, the limit and percent detection of CLas was greater with the RNR than the 16S gene target. Therefore, the duplex ddPCR described here can be used to confirm qPCR results have high or marginal Ct values.

Non-Technical summary: Droplet digital PCR (ddPCR) partitions a test sample into 20,000 uniform nanoliter-sized droplets, each droplet acts as an individual PCR reaction. This increases the ratio of target DNA to background DNA and dilutes inhibitory agents resulting in absolute quantification of PCR amplification. ddPCR was coupled with the 16S- and RNR-specific primer sets and resulted in absolute quantitation and unambiguous detection of low titer CLas infection from citrus leaves and ACP.

Citations:

Selvaraj, V., Maheshwari, Y., Hajeri, S., Chen, J., McCollum, T.G., and Yokomi, R. 2018. Development of a duplex droplet digital PCR assay for absolute quantitative detection of "Candidatus Liberibacterasiaticus". PLoS ONE 13(5): e0197184. https://doi.org/10.1371/journal.pone.0197184.

IRCHLB-P4-48

Enhancing PCR capacity in early detection of "*Candidatus* Liberibacter asiaticus" utilizing whole genome sequence information

Minli BAO[1,2], Zheng ZHENG[1], Xiaoan SUN[3], Jianchi CHEN[2], Xiaoling DENG[1] [1]South China Agricultural University, Guangzhou, China, [2]U.S. Department of Agriculture, Parlier, CA, USA, [3]Florida Department of Agriculture and Consumer Services, Gainesville, FL, USA

Abstract: "*Candidatus* Liberibacter asiaticus" (CLas), an unculturable α- proteobacterium, is associated with citrus Huanglongbing (HLB, yellow shoot disease). PCR procedures that effectively confirm or exclude CLas infection in asymptomatic or high Ct (>35) samples are important for HLB management. CLas was defined mainly by a 23-bp signature oligonucleotide sequence (OI1) in the 16S rRNA gene (*rrs*) acquired through Sanger sequencing in 1994. OI1 contained single nucleotide polymorphisms (SNPs) separating CLas from non-CLas species. The SNP region was used in primer HLBas, a key component of the current USDA TaqMan PCR (HLBas/HLBprobe/HLBr) system for CLas detection. By analyzing the 11 whole genome sequences of CLas currently available in GenBank database, a missing nucleotide G in OI1/HLBas was found. This study evaluated the effect of the missed-G in CLas detection. A corrected primer CLas-4G was developed to replace HLBas in the TaqMan PCR system. The CLas-4G PCR system reduced Ct by 0.76 (n=175) compared to that of the HLBas PCR system. The second study developed a gene-copy-ratio PCR (gcrPCR) system (used in both TaqMan probe and CYBR green formats). Primer set RNR1f/RNR1r, derived from the 5-copy *nrdB* gene encoding ribonucleotide reductase (RNR), was referenced to primer set CLas- 4G/HLBr, derived from the 3-copy *rrs* gene. The Ct ratio (Rrnd:rrs) of the gcrPCR effectively differentiated the result of CLas positive (Rrnd:rrs <1) from that of CLas negative (Rrnd:rrs >1) at high Ct situation. And, thirdly, a new primer set, 4CPf/4CPr from a 4-copy genomic locus of CLas was developed. PCR with 4CPf/4CPr had a Ct reduction of at least 0.6 (n=100) from those of the *rrs*-based primer sets. However, the genetic nature of the 4CPf/4CPr locus remained unknown.

Non-Technical summary: This research explored the use of available whole genome sequence information of the citrus HLB pathogen to improve HLB diagnostics. First, a sequence error in the current standard PCR system was detected and corrected; second, a gene-copy-ratio PCR (gcrPCR) system was developed. The gcrPCR effectively resolved the confusion between HLB positive and HLB negative interpretations during early detection practice; and third, a new primer set based on a 4-copy gene of HLB pathogen was developed, which demonstrated a high level of detection sensitivity. All these improvements will benefit current world-wide efforts in HLB management.

IRCHLB-P4-49

Evidence for natural transformation in *Liberibacter crescens* strain BT-1

Lulu CAI, Mukesh JAIN, Alejandra MUNOZ-BODNAR, **Dean W. GABRIEL**

Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA

Abstract: Natural DNA transformation is a horizontal gene transfer mechanism of bacterial uptake of naked DNA through Type IV pilus (T4P) and its stable integration in their genomes[1,2]. Natural transformation drives bacterial genome plasticity and adaptability, and has been demonstrated in several bacterial species such as *Streptococcus pneumoniae*, *Helicobacter pylori*, *Xylella fastidiosa*, *Vibrio cholera*, *Escherichia coli*, *Agrobacterium tumefaciens* and *Pseudomonas fluorescens* etc. Fastidious α- proteobacteria *Candidatus* Liberibacter asiaticus (CLas), *Ca.* L. americanus and *Ca.* L. africanus (associated agents for Huanglongbing or citrus greening), and *Ca.* L. solanacearum (causal agent for potato zebra chip) remain uncultured to date. Nonpathogenic *Liberibacter crescens* strain BT-1 (Lcr) is a phylogenetically related and the only culturable species of the Liberibacter genus[3]. All the structural genes required for assembly of T4P are annotated in the Lcr genome, and microscopic evidence for T4P will be presented. We provide first empirical evidence that Lcr is naturally competent for direct uptake and chromosomal integration of both linear and circular plasmid DNA in the presence of xanthan gum or chitin. Chromosomal integration of naturally acquired DNA (both linear and plasmid, carrying gentamycin resistance gene) in Lcr was achieved by homologous recombination within a non-essential target locus (B488_RS03405). Addition of chitin (1 mM) or xanthan gum (0.05%) in the Lcr cells/DNA transformation mix reliably yielded 3-4 transformation events per µg DNA. Likewise, ~10-12 transformation events were obtained using closed circular (non-replicative) plasmid DNA. Similar transformation efficiencies are routinely obtained with electroporated circular suicide plasmids for insertional mutagenesis of target loci. Natural uptake of broad host range shuttle plasmid pUFR071 (RepW) yielded ~900 transformation events. In comparison, 6×10^4 transformed Lcr cells were obtained via electroporation of pUFR071.

Non-technical summary:
Liberibacter crescens (Lcr) is a useful genetically tractable surrogate host for functional genomics of uncultured pathogenic Liberibacters. Presence of T4P and natural competence for DNA uptake and integration in Lcr genome has been demonstrated for the first time for any Liberibacter sp. These data are significant in the context of previously reported lack of enzyme systems for metabolism of purines and pyrimidines in the genome of *Ca.* L. asiaticus (Las)[4]. We hypothesize that natural uptake of DNA likely fulfills the nucleotide requirements for Las, highlighting the need for adding nucleotides to overcome nutritional bottlenecks for successful culturing of pathogenic Liberibacters in axenic media.

IRCHLB-P4-50

How early is “early detection”? Greg MCCOLLUM

USDA, ARS, US Horticultural Research Laboratory, Fort Pierce, FL 34945

Abstract: To be considered “early detection,” *Candidatus* Liberibacter asiaticus (CLas) infections must be identified prior to the appearance of HLB symptoms. HLB symptoms can be ambiguous, however, the greatest number of confirmed infections from field surveys are from symptomatic leaves. Experiments described herein were conducted to determine the effectiveness of qPCR to predict HLB development in eleven commercial citrus scion types. CLas inoculations were conducted by confining populations of experimental trees with CLas- infected ACP (ca. 3 wk exposure). Following ACP exposure the trees were disinfested of ACP and then held free of ACP during subsequent HLB symptom evaluations. Petiole samples were collected from young leaves that had been fed on by the inoculative ACP, and assayed for CLas infection by qPCR, typically one month following ACP exposure, but in some experiments subsequent assays were conducted multiple times post exposure. In addition to testing for CLas, trees were evaluated for the development of HLB symptoms. Based on results of the first CLas assay conducted following ACP exposure, 79% of trees that tested CLas negative at the first time following ACP exposure were never rated HLB symptomatic, and 75% of the trees that tested CLas positive were subsequently rated HLB symptomatic. In trees that were rated HLB symptomatic, the time of symptom appearance was typically within four to six months following ACP exposure, at which time CLas titers were greater than 10^3 copies per mg fresh wt of petiole. These results demonstrate that PCR can predict the development of HLB with 79% accuracy at one month following exposure to CLas infected ACP and that HLB symptoms become apparent within six months following ACP exposure. Although these experiments were conducted with small trees in the greenhouse, it is not unlikely that the results will be consistent for citrus in the field.

Non-Technical Summary: “Early detection” of CLas infections in citrus trees followed by removal of infected trees is one of the three tenets of HLB management. Although early detection actually refers to detection prior to visible HLB symptoms, the window of time between infection and symptom expression is a rather nebulous concept. This research was conducted to determine the efficacy of PCR for pre-symptomatic detection of CLas infections and to quantify the time between infection and appearance of HLB symptoms.

IRCHLB-P4-51

Isolation of CLas specific monoclonal antibodies (mAbs) for development of immunological tools for early detection of HLB pathogen

Vydehi KANNEGANTI[1], **Chandrika RAMADUGU[2]**, Sujata G PANDIT[3], and David P AUCOIN[1,3]

[1]DxDiscovery Inc, Reno, NV; [2]University of California, Riverside, CA; [3]University of Nevada, Reno, NV

Abstract: Early detection of *Candidatus* Liberibacter asiaticus (CLas), the associated pathogen of huanglongbing (HLB), is crucial to preventing further spread of this devastating citrus disease. There is a critical need to develop methodologies for reliable immunological detection of CLas in non-symptomatic citrus and in the psyllid, the insect vector. Our goal is to develop cost effective, sensitive, specific, antibody-based immunoassays that will facilitate HLB surveillance. The key to developing effective diagnostic immunoassays to detect CLas is the production of a library of monoclonal antibodies (mAbs) specific to CLas diagnostic targets. Isolation of CLas-specific mAbs is not straightforward because of its non-culturable status. In order to isolate mAbs against CLas, we utilized two different approaches. One is to utilize the targeted approach of immunizing mice with keyhole limpet hemocyanin (KLH) conjugated peptides specific to major outer membrane protein A (OmpA) of CLas. Polyclonal antibodies (pAbs) made against CLIBASIA OmpA have been shown to react with tissue prints made from HLB infected citrus plants (1, 2). Production of mAbs will generate a stable antibody source that can be easily maintained. Second and novel approach is to immunize mice with CLas infected psyllid

lysates. The antisera from the immunized mouse reacted well with CLas infected citrus leaf lysates, as well as OmpA peptide cocktail, indicating the production of CLas specific mAbs. Using this novel approach, we successfully isolated two CLas specific mAbs, that react specifically with one of the OmpA peptides tested. We anticipate to isolate a panel of CLas specific mAbs and generate valuable detection tools designed to monitor and reduce the spread of HLB disease.

Non-technical summary: There is a pressing need to develop diagnostic reagents for rapid in field and laboratory-based identification of HLB. Early detection of HLB disease leads to removal of compromised trees that would otherwise serve as infection source. At present, Fantibodies specific to CLas, that could be incorporated in a large-scale detection method are not available to the citrus industry, this is a major problem. Our work can result in the production of several antibodies that are critical to achieve our long-term goal to develop field-friendly dipstick assays for early detection of HLB pathogen (CLas) from both psyllids and citrus plants.

Citations:

Ding, F., Duan, Y., Paul, C., Brlansky, R. H. & Hartung, J. S. Localization and Distribution of ‘Candidatus Liberibacter asiaticus’ in citrus and periwinkle by direct tissue blot immuno assay with an anti-ompa polyclonal antibody. *PLoS One* (2015).

Bertolini, E. *et al.* Tissue-print and squash real-time PCR for direct detection of ‘Candidatus Liberibacter’ species in citrus plants and psyllid vectors. *Plant Pathol.* (2014).

IRCHLB-P4-52

Liberibacter crescens exhibits both flagellar swimming and Type IV pili-mediated twitching motility in culture

Lulu CAI[1], Mukesh JAIN[1], Alejandra MUNOZ-BODNAR[1], Laura A. FLEITES[2], Marta SENA-VELEZ[3], Michelle HECK[2], Kathryn M. JONES[3], **Dean W. GABRIEL[1]**

[1]Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA
[2]USDA Agricultural Research Service, Robert W. Holley Center for Agriculture and Health, Ithaca, NY 14853, USA
[3]Department of Biological Science, Florida State University, Tallahassee, FL 32306, USA

Abstract: Bacterial swimming and swarming in aqueous environments are mediated by rotary propulsion of flagella[1]. Under conditions of low water content such as within biofilms, Type IV pili (T4P) are used for long distance surface translocation or twitching[2]. Active movement in diverse microenvironments is essential for colonization, nutrient resourcing, host recognition, and pathogenesis[1, 2]. Liberibacter *crescens* (Lcr), the only Liberibacter cultured to date, as well as the plant pathogens *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter americanus (CLam) and *Candidatus* Liberibacter africanus (CLaf) (associated agents of Huanglongbing) and *Candidatus* Liberibacter solanacearum (Lso, associated agent of potato zebra chip) all have a complete repertoire of genes annotated as encoding flagella and T4P. However, neither flagella nor pili have been observed in electron micrographs of Lcr or any of the citrus pathogens, despite numerous observations of these pathogens in citrus. Both flagella and pili have been observed in micrographs of Lso within infected potato psyllids[3], but functional translocation has not been established. Lcr has served as a genetically tractable surrogate model system for the uncultured pathogenic Liberibacters. We provide ultrastructural evidence for the presence of both flagella and T4P in Lcr and demonstrated their functionality. Lcr demonstrated swimming as uniform outward growth from the inoculation point along both the surface and within the medium when grown in BM7 medium in low (0.25%) agar concentration. Lcr twitching was evident in high

(0.75%) agar concentrations as surface restricted growth if stabbed through half the depth of medium and restricted along the medium/polystyrene interface if stabbed to the bottom of the plate. Replacing fetal bovine serum (FBS) in BM7 medium with methyl-β-cyclodextrin reduced both swimming and twitching by 75%. Insertional mutations of flagellar hook-associated protein gene *flgK* (B488_RS04530) and basal-body rod protein gene *flgF* (B488_RS00930) reduced swimming by 33% and 20%, respectively, in 0.25% agar plates.

Non-technical summary: CLas is limited to living phloem cells in citrus, where motility may not be needed. By contrast, in the psyllid insect that vectors this pathogen, CLas becomes systemic, circulative and present in multiple organs. Psyllid nymphs acquire CLas from citrus phloem while feeding, and the bacterium quickly invades midgut cells, becoming intracellular and then killing the invaded midgut cells before exiting the midgut into the insect hemolymph, where it then forms a biofilm and multiplies on the outside of the adult insect gut. Some CLas cells break free and migrate in the hemolymph to subsequently invade the salivary glands, eventually to exit into the lumen of the salivary gland for subsequent transmission to uninfected citrus. Active CLas motility may be required, but only in specific physiological situations so as to avoid triggering effective host defenses. All the genes required for flagella and Type IV pili are present in CLas, but neither active motility nor flagella or pili have been reported for CLas. The closely related *L. crescens* is demonstrably useful as a proxy for CLas pathogenicity factors, and in this work is shown to possess flagella, Type IV pili, and to both exhibit two forms of motility, as well as to form biofilms under specific conditions. Specific adaptions required to survive highly specific physiological situations are usually vulnerable to chemical interference, and can point the way to control the disease.

Citations:

Chaban B, Hughes HV and Beeby M. 2015. Semin. Cell Dev. Biol. 46:91.

Chang YW, Rettberg LA, Treuner-Lange A, Iwasa J, Sogaard-Andersen L and Jensen GJ. 2016. Science 351:aad2001.

Cicero JM, Fisher TW and Brown JK. 2016. Phytopathol. 106:142.

IRCHLB-P4-53

Loop-mediated isothermal amplification (LAMP) assay for detection of *Candidatus* Liberibacter asiaticus (*C*Las), the bacterium associated with citrus huanglongbing (HLB)

Cheol Woo CHOI[1], Jae Wook HYUN[1], Rok Yeon HWANG[1], and Charles A POWELL[2]

[1]Citrus Research Institute, National Institute of Horticultural and Herbal Science, R.D.A. Jeju, 63607, S. Korea

[2]University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, 2199 South Rock Road, Fort Pierce, FL 34945, USA

Abstract

The huanglongbing (HLB, Citrus greening disease) is one of the most devastating diseases that threaten citrus production worldwide. We developed newly loop-mediated isothermal amplification (LAMP) method targeting gene contained tandem repeat for more rapid and sensitive detection of *Candidatus* Liberibacter asiaticus (*C*Las), the bacterium associated with citrus huanglongbing. This new LAMP method was 10 fold more sensitive than conventional PCR in detection of *C*Las pathogen and similar to that of real-time PCR in visual detection assay by adding SYBR Green I to mixture and 1% agarose gel electrophoresis. Positive reactions were achieved in reaction temperature 57, 60 and 62°C but not 65°C. Although this LAMP method was not more sensitive than real-time PCR, it was not require a thermocycler for amplification or agarose gel electrophoresis for resolution. Thus, we expect that this LAMP method shows strong promise as a reliable, rapid, and cost-

effective method of detecting *C*Las pathogen in citrus and can be applied for rapid diagnosis is needed.

Non-technical summary: LAMP is one of methods for detecting HLB pathogen. LAMP has the advantage of being highly specific, rapid, efficient, and labor-saving. we expect that this LAMP method shows strong promise as a reliable, rapid, and cost-effective method of detecting the *C*Las pathogen in citrus and can be applied where rapid diagnosis is needed like such as in the field.

Citation:

Bove, J. M. 2006. Huanglongbing: a destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol*. 88(1):7-37.

Katoh, H., Miyata, S., Inoue, H. and Iwanami, T. 2014. Unique Features of a Japanese '*Candidatus* Liberibacter asiaticus' Strain Revealed by Whole Genome Sequencing. *PLoS ONE* 9(9): e106109. DOI: 10.1371/journal.pone.0106109

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., and Hase, T. 2000. Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res*. **28**(12):

IRCHLB-P4-54

Multiplex qPCR detection of three *Candidatus* liberibacter species

Fatima OSMAN[1], Deborah PAGLIACCIA[2], Sohrab BODAGHI[2], Tyler DANG[2], and Georgios VIDALAKIS[2]

[1]University of California, Davis, CA, USA; [2]University of California, Riverside, CA, USA.

Abstract: A real-time multiplex quantitative polymerase chain reaction (qPCR) assay for the simultaneous detection of three *Candidatus* Liberibacter species; *Candidatus* Liberibacter asiaticus (*C*Las), americanus (*C*Lam) and africanus (*C*Laf) was developed and validated. The capacity of the multiplex qPCR assay in detecting the targeted species of the pathogen was compared to singleplex qPCR designed specifically for each species. The multiplex qPCR was assessed using *C*Las, *C*Lam and *C*Laf isolates from diverse citrus species and citrus producing areas. No significant differences in detection limits were found and specificity was not affected by the inclusion of the three assays in the multiplex reaction. Comparison of the pathogen load for each bacterium using singleplex and multiplex qPCR assays, revealed no significant differences in the multiplex qPCR capacity for pathogen detection. Optimizing the DNA extraction technique for citrus tissues and testing the quality of the extracted DNA using qPCR targeting the cytochrome oxidase citrus gene (COX) as a specific internal control proved to generate better diagnostic assays. The designed qPCR assay was compared to the currently used assay for official HLB diagnosis designed by Li et al., 2006, 2007 and was shown to identify more positive samples as well as decrease the Cq values of the reactions. Results in this study showed that the developed multiplex qPCR can streamline pathogen testing of citrus nursery stock and field surveys by detecting all three *C.* liberibacter species, thus reducing time and labor while retaining the same sensitivity and specificity.

Non-Technical summary: A multiplex qPCR diagnostic test was developed to simultaneously detect all three *Candidatus* Liberibacter species associated with huanglongbing (HLB). Various HLB survey, citrus quarantine, germplasm, and nursery stock programs can benefit from the developed test as it increases the diagnostic capacity of qPCR without a significant increase in cost or time for testing.

IRCHLB-P4-55

Optimization of grapefruit fruit juice for culturing of '*Candidatus* Liberibacter asiaticus'

Marcus V. MERFA[1], Leonardo DE LA FUENTE[1]

[1]Department of Entomology and Plant Pathology, Auburn University, Auburn, USA

Abstract: Citrus huanglongbing (HLB) is currently the most devastating citrus disease worldwide. In the US, it is associated with the unculturable bacterium '*Candidatus* Liberibacter asiaticus' (CLas) (**1**). CLas has only been established in transient cultures (**2**), and has not been transferred to either insect or host plants, precluding fulfilment of Koch's postulates and effective screening of antimicrobials, which hampers the study of HLB. Recently, studies focused on understanding the conditions where CLas lives were performed, and this information could be used towards the goal of culturing this bacterium. The haemolymph of the insect vector (Asian Citrus Psyllid – ACP) and phloem sap content of host plants were characterized (**3)**, and the energetic needs of CLas was defined. Thus, in this study we aimed at using this generated knowledge to optimize grapefruit juice (GJ), the culture medium previously used by our group to establish transient cultures of CLas (**2**). To test conditions using GJ as basis medium, CLas inoculum was obtained from seeds of infected citrus plants or guts of infected ACPs, and was inoculated in 24-well plates containing GJ and GJ modified by addition of different compounds. Plates were incubated at 28ºC for 21 days, and the amount of CLas was determined by RT-qPCR. Sub-cultures of each condition were also performed to obtain replicates. Results showed that adjusting the pH of GJ from 3.5 to 5.85 increased growth of CLas. In addition, a highly repeatable growth pattern was observed in which CLas grows better starting from a low number of cells, but grows only to a certain limit before the culture starts to die. These results provide information towards efforts of culturing CLas in vitro.

Non-technical summary: The lack of culturing of CLas, the associated agent of HLB, prevents studies of the disease and development of effective management strategies. Since recent studies have elucidated the chemical conditions of the environments where this bacterium grows in nature, our objective was to use this knowledge to increase the viability of CLas in laboratory conditions. We aim to contribute to the final goal of culturing this bacterium that will ultimately lead to a better understanding of CLas, so appropriate management strategies can be developed.

Citations:

Gottwald, TR. 2010. Current epidemiological understanding of citrus Huanglongbing. Annu. Rev. Phytopathol. 48: 119-139.

Parker, JK, Wisotsky, SR, Johnson, EG, Hijaz, FM, Killiny, N, Hilf, ME, and De La Fuente, L. 2014. Viability of '*Candidatus* Liberibacter asiaticus' prolonged by addition of citrus juice to culture medium. Phytopathology 104: 15-26.

Killiny, N. 2017. Metabolite signature of the phloem sap of fourteen citrus varieties with different degrees of tolerance to Candidatus Liberibacter asiaticus. Physiol. Mol. Plant Pathol. 97:20–29.

IRCHLB-P4-56

Optimization of *Liberibacter crescens* growth suggests ammonium and phosphate as important factors in the plant-host interface.

Joseph R. PETRONE[1], Maritsa CRUZ-MUNOZ[1], Alam MUNOZ-BERISTAIN[1], Eric W. TRIPLETT[1]

[1]University of Florida, IFAS, Microbiology and Cell Science Department, Gainesville, USA

Abstract: *Liberibacter crescens* is the closest cultured relative to the *Candidatus* Liberibacter pathogens. Sharing over 75% of nucleotide level similarity with *Candidatus* Liberibacter asiaticus suggests that *L. crescens* is a good model to study growth conditions. As not much is known about the handling and optimal growth conditions, more insight is found about the Liberibacter genus for culturing and treatment efforts. Here we describe the growth optimization in a chemically defined medium and the significance of our findings. The pH, aeration, temperature, and buffering capacity were all empirically evaluated. Our results revealed *L. crescens* as a sensitive neutrophile, favoring a 10mM sodium phosphate buffer with shaking incubation at 28ºC and 250rpm. Phosphate was a preferred buffering source amongst potential systems, suggesting a phosphate requirement above buffering capacity. Optimal growth was found as phosphate levels increased from 5mM to 10mM, approaching the biological ranges found in phloem. These results suggest a phosphate requirement of *Liberibacter crescens* growth, which may help explain the observed phosphorous deficiency in HLB-infected plants. An observed rise of the pH in the external medium is positively correlated with growth, which can be attributed to a measured accumulation of ammonia in the media. While more is to be discovered, we hypothesize that deamination within the cell is leading to the excretion and buildup of ammonia in the external environment. The accumulation of ammonium in citrus leads to ionic imbalances, oxidative stress, cell membrane pH dysregulation, and stunted growth and chlorosis; all of which are HLB symptoms. This finding suggests that disease in planta may be worsened by the effects of ammonium toxicity. Fertilization strategies to relieve ammonium buildup may alleviate some HLB symptoms.

Non-technical summary: Optimal growth conditions were studied in *Liberibacter crescens*, the closest relative to *Candidatus* Liberibacter asiaticus. Ammonia accumulation and a rise in pH were noticed with growth. This may lead to ammonium toxicity in the phloem and contributing to disease symptoms. Phosphate was the preferred buffer at physiological ranges in the plant which suggests that phosphate deficiency in planta may be caused by the pathogen. Fertilization strategies can relive these effects.

IRCHLB-P4-57

Progress on the development of a field-use optical sensor for screening of citrus pathogens in California

Perry EDWARDS[1], Brad OCHOCKI[1], Zhiwen LIU[1,2], Subhas HAJERI[3], and Ray YOKOMI[4]

[1]Croptix, State College, PA
[2]Department of Electrical Engineering, the Pennsylvania State University, University Park, PA
[3]Citrus Pest Detection Program, Central California Tristeza Eradication Agency, Tulare CA
[4]USDA-ARS SJVASC, Parlier, CA

Abstract: A compact, field-use optical sensor to detect plant disease status was evaluated. The sensor clips to leaves and uses broadband visible and near-infrared diffuse reflectance spectroscopy to examine leaves internally to assess physiological changes associated with specific plant health conditions. The spectral data are analyzed using machine learning models that are trained on spectral profiles collected from leaf samples of citrus trees associated with specific disease or healthy states. The sensor platform can be used as an early screening tool for citrus diseases including '*Candidatus* Liberibacter asiaticus' (CLas) associated with Huanglongbing (HLB). The sensor is currently being trained on field trees infected with *Citrus tristeza virus* (CTV) and *Spiroplasma citri*, causal agent of citrus stubborn disease, in commercial groves in California. These pathogens are phloem-limited like CLas and produce symptoms in citrus that can be confused for HLB. These data will facilitate sensor calibration to distinguish internal spectral profiles of asymptomatic CLas-infected citrus leaves as different from spectral profiles of CTV- and *S. citri*-infected leaves. Progress on data collection and training efforts with the sensor as a California citrus disease screening tool will be presented.

Non-Technical summary: A compact, field-use optical sensor to detect plant disease status is being developed to enable large-scale screening at a minimal cost due to its ability to take measurements directly in the field with no sample preparation. This provides growers with a large-throughput screening tool, which can identify suspect trees for follow-up confirmation by qPCR or other more costly diagnostics. The sensor is currently being trained and tested in commercial citrus groves in California on *Citrus tristeza virus* (CTV) and Stubborn diseases to provide better capability to distinguish between those diseases and the arrival of Huanglongbing (HLB) disease.

IRCHLB-P4-58

Targeted early detection of citrus Huanglongbing causal agent '*Candidatus* Liberibacter asiaticus' before the symptoms appear

Sheo Shankar PANDEY and Nian WANG

University of Florida, Lake Alfred, FL, USA

Abstract: Citrus Huanglongbing (HLB) is the most severe disease of citrus plants associated with *Candidatus* Liberibacter asiaticus (CLas) and transmitted by the insect vector Asian citrus psyllid (ACP). To date, no effective curative measure is available against HLB. For citrus production areas without HLB or with low HLB disease incidence, removal of CLas inoculum is critical to prevent HLB spread. Such strategy requires robust early diagnosis of HLB for inoculum removal to prevent ACP acquisition and transmission of CLas. However, early diagnosis of HLB is challenging because the citrus trees remain asymptomatic for several months to years after CLas transmission by ACP. In this study, we report a new method for a targeted early detection of CLas in Valencia sweet orange before HLB symptom expression. We take advantage of the fact that CLas remains around the ACP feeding site immediately following transmission into the young flush and before flush maturation. The ACP secretes salivary sheaths at their feeding sites, which can be visualized using Coomassie brilliant blue staining due to the presence of salivary sheaths secreted by ACP. Epifluorescence and confocal microscopy indicate the presence of salivary sheaths beneath the blue spots on ACP-fed-leaves. Quantitative real-time PCR (qPCR) and conventional PCR assays are able to detect CLas in the ACP feeding surrounding areas as early as 2-20 days after ACP feeding. This finding lays a foundation to develop much-needed tools for early diagnosis of HLB before symptom expression, thus assisting CLas inoculum removal and preventing HLB from spreading.

Non-Technical summary: We have developed a targeted early detection of the HLB associated agent '*Candidatus* Liberibacter asiaticus' before the symptom appearance. Our study has potential to assist Las inoculum removal and preventing HLB spreading.

IRCHLB-P4-59

Universal detection system for Liberibacters

Manjunath KERAMANE[1], Chandrika RAMADUGU[1], Richard STOUTHAMER[1], Mikeal ROOSE[1] , **Richard LEE**[2]

[1]University of California, Riverside, CA, USA
[2]USDA-ARS retired

Abstract: Asian citrus psyllids (ACP) and citrus are associated with at least five different species of Liberibacters; *Candidatus* Liberibacter asiaticus (CLas), *Ca*. L. africanus (CLaf), *Ca*. L. americanus (CLam), *Ca*. L. caribbeanus (CLca) and *L. crescens* (Lcr). Efficient HLB prevention and quarantine measures would require reliable technology for detection of all known Liberibacter species associated with citrus and ACP. We have identified genomic regions conserved in all relevant Liberibacters like CLas, CLam, CLaf, CLso, and Lcr and developed primers for specific amplification of DNA from target species. Several primer pairs were

designed to amplify all the above mentioned five Liberibacter spp. and *Ca*. L. europaeus (using Gblocks with synthetic DNA fragments). Evaluations were done by qPCR analysis for selection of highly specific primer sets that do not amplify closely related members of Rhizobiales normally associated with citrus roots. We have developed a Taqman probe-based real time PCR assay capable of detecting all Liberibacters, but not species of *Rhizobium*, *Agrobacterium*, or *Sinorhizobium*. Similarly, no reaction was observed with DNA extractions from healthy citrus or Liberibacter-free ACP and tomato psyllids. This assay for detection of all citrus-associated Liberibacters would be very useful in routine testing.

Non-technical summary: New species of Liberibacters not detectable by available testing methods have led to HLB epidemics in the past. Effective prevention and exclusion measures for citrus HLB are likely to be more effective with testing methods capable of detecting a wider range of pathogen forms. A testing system capable of detecting all known forms of Liberibacters was developed in this study.

IRCHLB-05:
Epidemiology Oral Presentations

IRCHLB-O3a-01

A decade of '*Candidatus* Liberibacter asiaticus' and its Asian citrus psyllid vector in Texas: an epidemiological insight

Olufemi J. ALABI[1], Mamoudou SÉTAMOU[2], Madhurababu KUNTA[2], Jon DALE[3], John V. da GRAÇA[2]

[1]Department of Plant Pathology & Microbiology, Texas A&M AgriLife Research and Extension Center, Weslaco, USA; [2]Texas A&M University-Kingsville Citrus Center, Weslaco, USA; [3]Texas Citrus Pest & Disease Management Corporation, Mission, USA

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is consistently associated with Huanglongbing (HLB) in US citrus-producing states, where the Asian citrus psyllid (ACP) vector of CLas is also widely prevalent. Surveys were conducted in Texas over an 11- year period (2007-2017) to assess the prevalence and titer of CLas in ACP and citrus trees. ACP cohorts and leaf tissues from suspect trees were sampled in residential areas and commercial groves and tested for CLas by qPCR using standard assays. CLas detection in ACP (2011) preceded that of plant tissue (2012) by 10 months. Annual percentages of CLas-positive ACP and leaf tissue over the detection period followed an exponential growth pattern, varying from 0.03% in 2011 to 28.7% in 2017 and from 1.2% in 2012 to 36.5% in 2017, respectively. The proportion of HLB detection sites increased with time reaching 30% of all commercial blocks and 40% of all residential trees surveyed by 2017. Seasonal variations were observed in the percentages of CLas-positive ACP within a given year such that significantly more CLas- positive ACP were recorded during fall and winter relative to the very hot south Texas summer months of June to September. The mean Ct values of CLas-positive ACP and leaf tissue significantly decreased with time during the study period (2011-2017) indicating higher bacterial titers in both tissue types. The results showed a slow but steady spread of CLas in Texas during the first four years of the epidemics, followed by an exponential phase of disease progression.

Non-Technical summary: We observed seasonal variations in the CLas bacterium concentrations in citrus trees as well as in the ACP vector. This may account for the relatively slow but steady spread of HLB under TX growing conditions. The results are being used to tweak our ongoing area-wide ACP management to ensure targeted control of the vector in a sustainable manner.

IRCHLB-O3a-02

Modelling of HLB invasion: prediction of southern California and Central Valley spread using epidemiological parameters estimated from Texas survey data

Viet-AnhNGUYEN[1], David W. BARTELS[2], Christopher A. GILLIGAN[1]

[1]Epidemiology and Modelling Group, Department of Plant Sciences, University of Cambridge, UK

[2]Animal and Plant Health Inspection Service,
T. S. Department of Agriculture, USA

Abstract: We have developed a stochastic, spatially-explicit model for HLB disease spread at landscape scale. The model can be used to predict future invasion of the pathogen and to evaluate the impact of area-wide control strategies on epidemic outcomes. We model the transmission of the pathogen from a 1km-by-1km site with infected trees to a healthy site by accounting for (1) the underlying vector dynamics from the donor to the receptor sites, which are driven by citrus densities and the effectiveness of control practices at each site, and (2) the risk of introducing infections from external sources. We fitted the model to HLB survey data in the Lower Rio Grande Valley, Texas, using data- augmented, Markov Chain Monte Carlo methods under a Bayesian framework. Posterior estimates of epidemiological parameters are then used to generate predictions for future spread of HLB disease in southern California and the Central Valley. In adapting the model from Texas to California we show how to overcome challenges associated with different conditions in the two states. These include differences in the distribution of underlying insect vectors, weather suitability, and the level of human intervention. We also use the model to reproduce the historic spread of the disease in Florida.

Non-Technical summary: We have developed a model for large-scale HLB spread in the Lower Rio Grande Valley, Texas, and calibrated the model using Texas HLB survey data. We have shown that the model also accounts for historic spread in Florida. The model is being used to predict how far and how fast HLB is likely to spread in California. The model also enables the impact of different management scenarios to be screened and evaluated.

IRCHLB-O3a-03

CLas titer in ACP, not infected citrus, is the driving force for the spread of HLB

Greg MCCOLLUM, David HALL, Weiqi LUO

USDA, ARS, US Horticultural Research Laboratory, Fort Pierce, FL 34945

Abstract: Transmission of *Candidatus* Liberibacter Asiaticus (CLas) from ACP progenitor to progeny (generational transmission) requires that CLas pass through citrus. Transovarial transmission of CLas is apparently insignificant. Acquisition of CLas by ACP from citrus occurs more efficiently by nymphs than by adults, presumably as a result of nymphs feeding for longer durations in close proximity to sites where eggs were laid, and where CLas was originally transmitted to citrus by infected progenitors. Adult ACP not only acquire CLas less efficiently than nymphs, but ACP that acquire CLas as adults are less efficient at transmission than nymphs. All of these observations suggest that citrus is merely a conduit for generational transmission of CLas and that CLas titer in citrus trees may not play an important role on CLas dispersal. We conducted experiments to determine how a generational transmission of CLas is impacted by titer in progenitor ACP adults, and in citrus. Our results indicate that CLas titer in ACP is the major driver in generational transmission. The probability of successful transmission starts to increase when CLas titers in progenitor adults exceeds ca. 10^3 copies per insect, the probability of infection increases dramatically to

over 80% when CLas titer exceed 10^4 per insect. In both ACP nymphs and adults CLas titer increased with time after initial acquisition; titers of 10^6 copies per ACP adult were not uncommon. This increase in CLas titer during development occurred regardless of infection status of the tree prior to exposure to CLas infected progenitors. CLas titer in ACP in adults also impacted the titer of CLas detected both in leaves on whole trees or on detached leaves. In a comparison of transmission when CLas-infected progenitors were placed onto citrus either free of or infected with CLas, there was no significant difference in transmission efficiency in the experiment. Our data support the hypothesis that infection status of trees has little significance in generational transmission.

Non-Technical Summary: Transmission of CLas, presumptive causal agent of HLB, from one generation of ACP adults to the next requires the insects transmit the pathogen through citrus where it can subsequently be acquired by progeny ACP. Our results indicate that it is not the CLas infection status of citrus trees that drives generational transmission of CLas, but rather, CLas titer in progenitor insects is the driver. These results support the importance of proactive ACP control to prevent development of HLB epidemics.

IRCHLB-O3a-04

Predict likelihood of ACP/HLB dispersal into CA commercial citrus under different control protocols

W. LUO[1,2], D. POSNY[1,2], S. ZHANG[1,2], T. GOTTWALD[1] and F. LOUWS[2]

[1] U.S. Department of Agriculture, Agricultural Research Service, Fort Pierce, FL, USA

[2] Center for Integrated Pest Management, North Carolina State University, Raleigh, NC, USA

Abstract: Since the 2012 discovery of a HLB tree in the Hacienda Heights residential area, subsequent findings of HLB and ACP have increased in California indicating epidemic development, amplifying the risk of ACP endemicity as well as HLB introduction into commercial citrus. This poses a major threat to the viability of the citrus industry, emphasizing the urgent need for proactive multiscale intervention. Understanding the spread of ACP and HLB across California’s residential, commercial, and mixed citrus landscapes is crucial in successfully designing /prioritizing mitigation efforts/resources while minimizing economic losses. Therefore, we developed a spatially explicit agent-based model to investigate how ACP and HLB spread in actual CA landscapes, and analyze the efficiency of management protocols through scenario- based simulations and cost-benefit analyses. Given ACP/HLB incidence or detection data for a particular region and a CA-recommended/proposed management program (grove-level, residential/dooryard, multiscale, area-wide), we can estimate the likelihood of ACP/HLB establishment and predicted spread to evaluate the effectiveness and expected cost of control. Control or simulation scenarios can include different combinations of survey, early detection, biological control, tree removal and replantation, grower/residential compliance level and disease management cost. For example, resource allocation (e.g. required sampling effort or as part of a delimiting response after infestation/ infection confirmation) and minimum coordination/participation rates (area-wide, PMA, residential) are evaluated for different Southern California regions. Informative analyses/outputs will assist in guiding spatiotemporal ACP and/or HLB targeted management strategies across CA, and can provide unique insight into the effectiveness of HLB mitigation.

Non-Technical summary: The recent outbreaks of HLB in the Southern CA increases the awareness for potential HLB introduction and establishment in commercial citrus. A mathematical model incorporating ACP and

HLB biology was developed to simulate how disease spreads in mixed residential and commercial citrus landscapes. Commercial citrus within close proximity to existing residential HLB finds is used as a case study. Scenario-based simulation analyses improve our understanding of the ACP/HLB dynamic under realistic weather conditions in actual CA landscape. In addition, it can evaluate the behavior and distribution of ACP/HLB subject to different management strategies, and assist in the development of enhanced surveillance strategies to optimize the development of sampling resources.

IRCHLB-O3a-05

Update on Canine assisted early detection of HLB

[1]**T. GOTTWALD,** [1]G. POOLE, [1]T. MCCOLLUM, [2]W. LUO, [2]D. POSNY, [1]J.H. BAI and [1]E. BALDWIN

[1]USDA-ARS, Fort Pierce, FL, USA.; [2]North Carolina State University, Raleigh, NC, USA

Abstract: For all exotic diseases, early detection followed by early response is the underpinning for control and HLB is no exception. By the time trees are symptomatic for HLB, multiple ACP mediated dispersal events have likely occurred. Thus subclinical detection immediately post infection is required to minimize pathogen spread. We have previously reported that 20 dogs were trained for early detection via a USDA, APHIS HLB Mac grant. A 10,000 tree study using 10 canines was assessed via latent class analysis (LCA) and ascertained overall 87.6% sensitivity, 99.6% specificity, and 99.17% detection accuracy. A longitudinal study of plants infected via ACP-mediated transmission was conducted monthly for 24 months to determine the earliness of canine versus PCR detection. Using 10 canines, 65% detection was immediate upon first exposure to the presumptive infected plants within 1 month rapidly increasing to 98% accuracy 7 months post inoculation. However some canines detected all plants correctly immediately. Whereas, qPCR only detected 10% or less of the inoculated plants for the first three months with an average of ~25% detection accuracy thereafter. Subsequent tests or newly inoculated trees indicate that dogs can detect early CLas infections within 2 weeks post inoculation via ACP. In blind, randomized tests, canines correctly detected a broad range of CLas-infected plants, i.e., multiple citrus species and cultivars, periwinkle, tobacco, and dodder as well as CLas+ ACP. Therefore we believe that the canines are actually detecting CLas bacteria rather than pathogen-induced host response volatiles. Canines are scheduled for deployment to southern California for CLas detection validation tests in late 2018-early 2019.

Non-Technical Summary: Dogs have been trained to detect HLB-infected trees with an accuracy of >99% and can be used as an early detection method for commercial and residential survey. Canine detection is consistently months earlier than PCR. When linked with a rapid response, canine detection will be a powerful tool which will help minimize disease spread and optimize planting longevity.

IRCHLB-O3a-06

Comparative economic analyses of EDTs for sustainable HLB survey design via modeling and simulation

D. POSNY[1,2], W. LUO[1,2], E. TAYLOR[1], T. GOTTWALD[1] and F. LOUWS[2]

[1] U.S. Department of Agriculture, Agricultural Research Service, Fort Pierce, FL, USA

[2] Center for Integrated Pest Management, North Carolina State University, Raleigh, NC, USA

Abstract: As EDTs progress and develop, economic sustainability of the detection

technology implementation and the detection efficiency are both of critical importance. Mathematical modeling and geostatistical technologies are able to grasp the spatiotemporal complexity of pest and disease invasion dynamics as well as the implementation and evaluation of viable management strategies. Using survey and removal as the sole controls implemented in the simulation study and ACP modeled as a relative density at the grove-level to incorporate general population dynamics (e.g, endemic or seasonal/cyclic populations) affecting transmission risk, we investigated the cost-benefit of HLB detection techniques (e.g., visual detection, PCR, Canines). We compared different survey deployment options including survey design (e.g., full, perimeter/ edge, stratified), timing/frequency, and detection technique. After disease confirmation, removal of infected trees is the primary combatant in reducing the local inoculum pressure. We considered removal of individually confirmed HLB+ trees as well as culling at a specified radius (e.g. 50m). It is important to note that both infection confirmation (i.e., lab capacity for PCR) and removal often occur after variable time delays; to account for such variability, confirmation and removal times can be sampled from normal distributions with a specified mean and standard deviation. Under different combinations of these control measures and EDTs, we modeled the spread of HLB, estimated the yield/production, and conducted full cost-benefit analyses. Regulatory agencies, growers and decision makers can benefit from such simulation studies to assist in making cost-effective decisions for proposed adjustments in ongoing management programs and as detection technologies change and improve. An interactive web application was developed to allow users to investigate the spatiotemporal disease dynamics, detection technique efficiency, and sustainability from the associated costs.

Non-Technical summary: Simulation study comparing detection methodologies for early detection of HLB in a commercial citrus grove. Cost- benefit analyses were conducted to evaluate mitigation efficiency and economic sustainability of HLB survey and removal program.

IRCHLB-05: Epidemiology Poster Presentations

IRCHLB-P5-60

Analyzing the impact of sampling on PCR efficiency from field studies under HLB epidemic development in FL

D. POSNY[1,2], W. LUO[1,2], E. TAYLOR[1], T. GOTTWALD[1] and F. LOUWS[2]

[1] U.S. Department of Agriculture, Agricultural Research Service, Fort Pierce, FL, USA

[2] Center for Integrated Pest Management, North Carolina State University, Raleigh, NC, USA

Abstract: Huanglongbing (HLB) detection via PCR is greatly hindered by the sampling selection procedure (typically, 4-8 leaves per citrus tree), particularly in mature citrus trees which can have tens to hundreds of thousands of leaves. The probability of selecting *Candidatus* Liberibacter asiaticus (CLas)- infected tissue in an early infection (i.e. in the cryptic asymptomatic stage) is extremely low, and consequently, delays disease control measures. Even as the infection progresses within the tree, the distribution most likely remains uneven, still limiting the probability of detection. Only when the citrus tree is in a late state of infection is detection via PCR more likely. Therefore, given the unavoidable sampling issue and other potential inaccuracies associated with PCR, citrus confirmed to be HLB-positive have likely been infected for a while, contributing to unmitigated HLB propagation. We investigated PCR efficiency through several Florida field studies, where commercial citrus blocks were regularly surveyed and tested by PCR. On average, initial Ct-values were above 36

(assumed threshold for HLB confirmation), and thus, deemed HLB-negative. Once a Ct- value dropped below 36, the citrus host was confirmed to be HLB+, however, subsequent tests produced inconsistent, inconclusive (36-38) or negative results for the same tree in some cases. Initially, inconclusive trees in a Florida commercial block took 9+ months to confirm, with Ct value dramatically dropping into the 20's soon thereafter. Through simulation analyses, the HLB epidemic development was estimated to evaluate the efficiency of PCR detection via best-case and worst-case scenarios.

Non-Technical summary: Data from field and laboratory studies in Florida have been compiled to reconstruct HLB epidemic timelines (i.e., estimates of the actual infection curves). Using a Ct-value threshold of 36 for HLB confirmation, we analyze the efficiency of PCR detection in a real-world setting.

IRCHLB-P5-61

High-risk based field survey and high-throughput qPCR detection system for Huanglongbing-associated bacteria in commercial groves of California

Subhas HAJERI[1], Wei LIAO[12], Karen WESTERMAN[1], Jill BARNIER[1], Weiqi LUO[2], Tim GOTTWALD[2], and Ray YOKOMI[3].

[1]Citrus Pest Detection Program, Central California Tristeza Eradication Agency (CCTEA), Tulare CA 93274.

[2]USDA-ARS U.S. Horticultural Research Laboratory, Fort Pierce, FL 34945.

[3]USDA-ARS Crop Diseases, Pests and Genetics Research, Parlier, CA 93648.

Abstract: Citrus is a major economic crop of California with an impact of over $7 billion. In California, '*Candidatus* Liberibacter asiaticus' (*C*Las) is associated with huanglongbing (HLB), which is transmitted by Asian citrus psyllid (ACP), and is a serious threat to the citrus industry. More than 900 *C*Las-positive trees in southern California have been identified and removed by California Department of Food and Agriculture (CDFA) since first detection in 2012. All finds have been in dooryard trees and none in commercial citrus production to date. CCTEA has been serving the San Joaquin Valley growers over 50 years by surveying commercial citrus for *Citrus tristeza virus* (CTV). With imminent danger of HLB, CCTEA is operating as Citrus Pest Detection Program (CPDP) and has switched its emphasis from CTV to HLB. Working in collaboration with ARS, CCTEA has evolved in both field and lab operations with changing needs and available technology. Field operations have shifted to high-risk based perimeter survey for HLB, emphasizing visual inspection for HLB symptoms and ACP presence, and spot-sample collection. Lab operations have evolved from bio-indexing to ELISA to qPCR, making significant improvements by establishing a PCR laboratory and optimizing high-throughput nucleic acid extraction and multiplex qPCR systems. After obtaining State Plant Pest Permits from CDFA, CPDP tested over 4,000 ACP samples and over 30,000 plant samples for *C*Las in 2018. The CPDP can also conduct high-throughput qPCR for CTV genotypes and *Spiroplasma citri*, causal agent of citrus stubborn disease. CPDP is equipped to process and test around 100,000 samples a year with the current laboratory infrastructure and can play an important role in large-scale insect and plant sample screening for HLB in the coming years.

Non-Technical Summary: The grower funded Citrus Pest Detection Program (CPDP), operated by the Central California Tristeza Eradication Agency (CCTEA), has been serving the San Joaquin Valley citrus industry over 50 years by surveying commercial citrus for various citrus pests and diseases, evolving from focus on red scale, to *Citrus tristeza virus*, to Huanglongbing.

Field operations have shifted from systematic subsampling and follow-ups to high-risk based surveys, with an emphasis on visual inspection of grove perimeters for HLB symptoms and ACP presence, and spot sample collection. Lab operations have progressed from bio-indexing to ELISA to qPCR, which provides the ability to test for HLB-associated bacteria as well as citrus stubborn bacteria and CTV genotypes. The CPDP has been granted permits by CDFA to sample and test for HLB and is now is equipped to process and test around 100,000 samples a year with the current laboratory infrastructure. This will allow the CPDP to play an important role in large-scale insect and plant sample screening for HLB in coming years.

IRCHLB-P5-62

Rates of disease progress in peripheral and internal citrus blocks emphasize role of primary spread in *Huanglongbing* epidemiology

Kelly PAZOLINI, Armando Bergamin FILHO and José BELASQUE

“Luiz de Queiroz” College of Agriculture, University of São Paulo, 13418-900 Piracicaba, São Paulo, Brazil.

Abstract: *Huanglongbing* (HLB) is the main citrus disease and despite many efforts to its control disease incidence continues to increase in São Paulo. The objectives of this study were to better understand HLB epidemics under strict disease management and to demonstrate the importance of external sources of inoculum (primary spread) in HLB epidemics. Our study was carried out in a large commercial citrus property (~8.000 ha, 314 citrus blocks) located in the central area of São Paulo, Brazil. This property performed strict HLB management (frequent symptomatic trees eradication and vector control). Gompertz and logistic temporal models were fitted to annual disease progress of 177 citrus blocks and average *Diaphorina citri* (ACP) per trap were determined in 296 citrus blocks. Disease progress rate, HLB final incidence and average ACP per trap were compared between blocks located at the periphery (edge) or internally (surrounded by citrus) in the property. The findings, then, were related to external HLB inoculum sources. Both, Gompertz and logistic temporal models fitted well to HLB annual incidences, but Gompertz was chosen as the best model to describe HLB epidemics (higher R^2 and better distribution of residues). Values of Gompertz progress rates (ranging from 0.04 to 0.28 per year), average HLB incidence (0.11), and average ACP per trap (0.016) were low compared with data in the literature. The variables differed ($p \leq 0.05$) between peripheral and internal blocks, higher values being related mainly with blocks in property’s periphery. A great amount of noncommercial trees serving as HLB primary inoculum was found near citrus blocks presenting higher rates of disease progress, HLB incidences, and ACP per trap. The regional management of HLB should include, besides commercial orchards, noncommercial trees.

Non-Technical summary: The concept of area-wide management has become the main stay of HLB control programs. In Brazil, voluntary groups of citrus growers control ACP population with three to four coordinated area-wide insecticide sprays in defined short periods based on Fundecitrus alert system. Our results show that, in addition to this practice, attention must be directed also to noncommercial trees located outside the groves.

IRCHLB-P5-63

Risk based HLB survey extension: optimizing delimitation radius for cost- effective disease control

W. LUO[1,2], D. POSNY[1,2],T. GOTTWALD[1] and F. LOUWS[2]

[1] U.S. Department of Agriculture, Agricultural

Research Service, Fort Pierce, FL, USA

[2] Center for Integrated Pest Management, North Carolina State University, Raleigh, NC, USA

Abstract: With increasing HLB pressure in the LA basin and surrounding areas, the disease has manifested itself in urban neighborhoods, spreading further into Orange and Riverside counties. As of Nov 15, 2018, the number of confirmed HLB+ trees has exceeded 900 and more confirmations continue on a weekly basis. Concurrently, the number of LAS+ ACP finds has increased substantially, and given the strong spatial correlation with HLB+ tree finds, indicates the possibility of new discoveries of HLB clusters. Following HLB+ tree discoveries, it is necessary to design a high intensity/targeted survey to maximize detection of additional HLB+ trees in new outbreaks to prevent further spread, e.g. delimiting survey designed for new epicenters (e.g., San Gabriel, Pico Rivera, Whittier, Anaheim, Garden Grove). In association with the risk map, we investigate cost-effective approaches in delimiting survey efforts for improved resource allocation and early detection optimization through two simultaneous approaches. First, we perform spatiotemporal cluster analyses of outbreaks to optimize delimiting survey distance. Initial analyses indicate that the existing 800m delimiting survey distance can be significantly reduced while still maintaining >90% detection of infections emanating from the central focus of inoculum. Such reduction would return significant manpower and resource allocations to be used elsewhere in the program. New outbreaks stimulate the risk-based model to assign higher risk to surrounding STRs. Thus, any rare infections beyond the lesser delimiting distance would be acquired by the intensified risk-based survey in the area, compensating for the reduced delimitation survey. Secondly, we integrate a spatiotemporal HLB epidemiological model utilizing the survey result data (i.e. HLB confirmations, inconclusive and negative results) to anticipate potential subsequent spread. This approach will further assist in prioritizing survey deployment/sampling resources within delimitation survey protocols and provide another risk layer for the risk-based survey design (i.e. future risk outlook for shifting resources).

Non-Technical summary: It is hard to overstate the importance of delimiting survey in mitigation of HLB/ACP spread and development. Proper selection of suitable delimiting survey distances can provide a cost-effective balance between survey coverage and manpower allocation. Using both HLB+ ACP and tree samples with detection timeline within each cluster, and by expressing the cumulative distribution of detections as a function of distance from the initial detection, it was possible to show in simulated data-resampling experiments, that reducing the radius of the delimitation survey from 800m to 400m would recover >90% of the HLB detection by the larger radius.

IRCHLB-P5-64

Spatial distribution and temporal progress of *Huanglongbing* are strongly influenced by vector control and neighbouring non-commercial Rutaceae plants

José BELASQUE, Kelly PAZOLINI and Armando BERGAMIN FILHO

University of São Paulo, “Luiz de Queiroz” School of Agriculture, Piracicaba, São Paulo, Brazil

Abstract: Since the first detection of Huanglongbing (HLB) in São Paulo state, Brazil, in 2004, more than 42 million of commercial citrus plants were eradicated. The recommended disease management include the use of non-infected, insect-proof, nursery plants, frequent insecticide sprays for vector control, and roguing of HLB-symptomatic trees. Although those practices were recommended since 2004, roguing of symptomatic trees are only partially adopted by the citrus growers. Near 230 citrus plots, belonging to 4 farms in São Paulo, are

under evaluations to determine the progress of HLB- trees and its vector incidence and distribution. All citrus plots are maintained by at least 12 insecticide sprays/year and HLB-tree eradication (4 times/year). Until 2017, the average HLB-tree incidence/plot was 25.7% (from 5% to 80%). The number of adult vector/yellow stick card/assessment ranged from 0.00 to 0.18 (average 0.03). Surveys conducted to identify eggs, nymphs, and adults on trees resulted in less than 1% of infested branches/assessment. The monomolecular model were tested and fitted well for the cumulative proportion of removed trees (rate of progress ranged from 0.74 to 26.04, average 6.71). The highest proportions of removed trees and vector detections occurred only at plots located at the perimeter of all farms. The location of neighboring commercial citrus farms, where vector control is adopted, did not explained the most affected citrus plots in the studied farms. However, thousands of non-commercial citrus and Murraya spp. plants were identified until 5 kilometers from each farm. We hypothesize that the "primary infection", promoted by the dispersion of bacteriliferous adults from those non-commercial Rutaceae plants, are the main process of HLB infection in commercial citrus farms maintained with strong HLB management.

Non-technical summary: Non-commercial citrus and *Murraya* plants, neighboring citrus farms, are important sources of HLB/vector inoculum and actions to reduce or manage them are essential to prevent continuous infections of HLB on citrus plants under disease and vector management.

IRCHLB-06: Host Resistance and Tolerance Oral Presentations

IRCHLB-O3b-01

Identification of natural citrus genes and alleles associated with HLB tolerance in citrus breeding populations

Qibin YU[1], Mikeal L. ROOSE[2], Zhanao DENG[3] and Fred G. GMITTER Jr [1]

[1]University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, FL, USA
[2]University of California, Riverside, CA, USA
[2]University of Florida, IFAS, Gulf Coast Research and Education Center, Wimauma, FL, USA

Abstract: Field trials in Florida have revealed large variation in the severity of HLB symptoms in mandarin hybrid families within the UF-CREC breeding program. In this study, a genome-wide association study (GWAS) was conducted to identify single nucleotide polymorphisms (SNPs) associated with Huanglongbing (HLB) tolerance by genotyping and phenotyping hundreds of individuals within a triploid mandarin hybrid population. High-density SNP genotype data were generated with Axiom^{TM} Citrus 56AX (Affymetrix, Inc.). A mixed linear model (MLM) was used to make associations between HLB tolerance and SNPs. Population structure was corrected using a kinship matrix and principal components analysis (PCA) as covariance in the MLM model. Sub-network enrichment analysis was performed to narrow down the number of candidate genes with known functions in plant immunity and defense. Differential gene expression patterns of these candidate genes were investigated in a sub-set of the mandarin population. Results indicate that the expression level of several genes was significantly higher in randomly selected HLB-tolerant genotypes than in the HLB-susceptible ones in our citrus breeding population, suggesting that these genes may play important roles in HLB resistance. Results from this study have improved understanding of the genetic factors underlying HLB tolerance and demonstrate the power of GWAS analysis and the potential of using marker-assisted selection in the citrus breeding program for improving HLB tolerance.

Non-Technical summary: Genome-wide association study (GWAS) can empower citrus breeders to identify key genetic components and molecular markers for HLB tolerance in citrus. Using a GWAS, this study identified several genes that differed significantly in expression level between HLB-tolerant and HLB-susceptible mandarin selections. Molecular markers can be used to make a prediction of HLB-tolerant plants quickly and much earlier than artificial inoculation and field evaluation, and to deliver HLB-tolerant cultivars from citrus breeding programs more effectively.

IRCHLB-O3b-02

Resistance to huanglongbing developed in hybrids of citrus crossed with Australian limes.

Chandrika RAMADUGU[1], Manjunath KEREMANE[2], David G. HALL[3], Thomas G. McCOLLUM[3], Richard LEE[2*] and Mikeal ROOSE[1]

[1] University of California, Riverside, CA, USA[2] National Clonal Germplasm Repository for Citrus and Dates, USDA, Riverside, CA, USA; [3] US Horticultural Research Laboratory, USDA Fort Pierce, FL, USA [*] retired

Abstract: Sustainable, long-term cultivation of citrus in regions threatened by huanglongbing (HLB) will require development of disease resistant cultivars. Resistance to HLB is not documented in commercial citrus; we have identified citrus relatives with high tolerance or resistance to HLB based on field trials. Some natural hybrids of citrus crossed with HLB resistant taxa have high tolerance/resistance indicating that the resistance trait is heritable. We have conducted wide crosses between citrus and HLB resistant/tolerant citrus relative genera belonging to the genera *Eremocitrus* and *Microcitrus*, the Australian limes. The success rate of these crosses is low since only certain cultivars of citrus have sexual compatibility with the Australian limes. We have generated hybrids from about 50 types of parental combinations. Propagations of hybrid plants were evaluated for HLB resistance by exposing them to feeding by infected psyllids in a greenhouse for four weeks. Propagated hybrids were also exposed to no-choice psyllid feeding for two weeks. The psyllids were later eliminated and the plants were maintained in a greenhouse for two years, observed for symptoms and tested by qPCR for CLas. A third method of pathogen exposure was by repeated graft challenge and testing the plants for CLas. A percentage of hybrids appear to be resistant since we could not detect the CLas pathogen for over two years after exposure to CLas in the greenhouse. Confirmation of resistance will be possible after the putative HLB resistant hybrids have been tested in the field (on- going), in different locations, and with different CLas isolates. Further breeding to generate hybrids with fruit quality closer to the citrus parent is in progress. The breeding population will be used in comparative genomic studies to identify resistance- associated genes that can then be utilized by biotechnological methods to develop HLB resistant commercial cultivars.

Non-technical summary: We used known HLB resistant citrus relative genera to generate hybrids of citrus by traditional breeding methods. The hybrids have been shown to inherit resistance trait from the resistant parent. The fruits from some hybrids with HLB resistance have flavors close to limes or lemons with traces of off-flavor inherited along with the resistance trait.

IRCHLB-O3b-03

Application of KASP markers to improve studies of HLB tolerance

Mikeal L. ROOSE[1], Claire T. FEDERICI[1], Ed

STOVER[2], and Qingchun SHI[2]

[1]University of California, Riverside, USA, [2]USDA-ARS, Ft. Pierce, FL, USA

Abstract: Experiments to evaluate Huanglongbing (HLB) tolerance or resistance of specific varieties or genetic populations often use seedlings as replicates due to quarantine regulations that block transfer of large amounts of vegetative materials or because production of seedlings requires less labor than cuttings or grafted trees. However, seedling populations are not always genetically uniform because the percentage of nucellar seedlings varies among citrus genotypes. Analysis of seedlings with a moderately large set of DNA markers, including some markers homozygous and heterozygous in the female parent tree, can distinguish nucellar and zygotic seedlings, and those from outcrossing, with a high probability. To accomplish this efficiently, we developed sets of SNP markers that can be analyzed with the KASP technology, for which researchers need only collect tissue samples from each seedling. DNA extraction and marker analysis are outsourced to LGC Genomics. Efficient SNP sets include markers located at opposite ends of each chromosome so they are independent, and our SNP database of citrus varieties developed with Affymetrix Axiom arrays allows us to choose SNPs heterozygous in most individuals in populations of interest. An example of applying this approach to a population of Fortune x Fairchild hybrid seedlings being phenotyped for HLB tolerance at Ft. Pierce Florida will be described. Data from nucellar seedlings can be weighted more highly than that of zygotic seedlings in subsequent genetic analyses.

Non-Technical Summary: To improve genetic analyses of HLB tolerance and resistance that use seedling populations we developed inexpensive SNP assays to distinguish nucellar and zygotic seedlings. Application of these assays should improve interpretation of these experiments and more clearly define the basis of resistance and tolerance.

IRCHLB-O3b-04

Screening diverse citrus genotypes for plant immune perception

Jessica FRANCO[1],
Chandrika RAMADUGU[2]
and Gitta COAKER[1]

[1]Department of Plant Pathology, University of California, Davis; [2]Department of Botany and Plant Sciences, University of California, Riverside

Abstract: Plants possess receptors anchored at the cell surface known as pathogen recognition receptors (PRRs). PRRs can recognize conserved pathogen components termed pathogen-associated molecular patterns (PAMPs). PAMPs can represent variety of materials such as conserved proteins, carbohydrates, lipids or other features essential for pathogen growth and survival[1]. PAMPs have been identified from all pathogenic organisms, including bacteria and insects[1]. A hallmark of PAMP perception is the rapid production of reactive oxygen species (ROS)[1]. Robust ROS production is required for an effective immune response[1]. Despite the importance of various citrus pathogens, the diversity of citrus PAMP perception remains unknown. Piercing- sucking insects, such as the Asian citrus psyllid (ACP) can introduce insect and bacterial PAMPs during initial feeding, when they salivate in the apoplast before their stylet reaches the phloem[2]. All citrus varieties are susceptible to HLB, but previous work has shown that Australian wild citrus relatives, such as Australian desert lime and Australian finger lime, which are sexually compatible with cultivated citrus, are more disease tolerant to Huanglongbing (HLB)[3]. We

developed a microplate assay to quantify ROS production as a marker for PAMP perception in diverse citrus genotypes using PAMPs present in *Candidatus* Liberibacter asiaticus and ACP. We screened 78 diverse citrus genotype for their ability to perceive select PAMPs, including susceptible commercial citrus varieties and HLB resistant wild citrus relatives[3]. The susceptible sweet orange varieties, Washington Navel and Valencia orange, were least responsive to tested PAMPs. In contrast, citrus relatives exhibited segregation in both perception and magnitude of responses to tested PAMPs. Progress on the identification and characterization of corresponding citrus PRR receptors will be reported. A heightened understanding of PAMP responsiveness in citrus and identification of the corresponding PRRs will lead to targeted disease management strategies.

Non-technical summary: Plants can actively recognize conserved pathogen features as non-self and trigger defense responses resulting in tolerance and resistance. After pathogen perception, plants produce reactive oxygen species (ROS). We used ROS production as a proxy to identify which features from the HLB associated bacteria and insect vector are recognized in diverse citrus genotypes. Recognition of these pathogen features is not uniform nor is the response magnitude in HLB susceptible and resistant citrus varieties. We are currently identifying the corresponding citrus immune receptors. This study will provide candidates for the development of HLB resistant citrus varieties and accelerate breeding efforts.

Citations:

Boutrot, F. & Zipfel, C. Function, Discovery, and Exploitation of Plant Pattern Recognition Receptors for Broad- Spectrum Disease Resistance. *Annu. Rev. Phytopathol.* (2017). doi:10.1146/annurev-phyto-080614-120106

Chaudhary, R., Atamian, H. S., Shen, Z., Briggs, S. P. & Kaloshian, I. GroEL from the endosymbiont Buchnera aphidicola betrays the aphid by triggering plant defense. *Proc. Natl. Acad. Sci.* (2014). doi:10.1073/pnas.1407687111

Ramadugu, C. *et al.* Long-term field evaluation reveals Huanglongbing resistance in Citrus relatives. *Plant Dis.* (2016). doi:10.1094/PDIS-03-16-0271- RE

IRCHLB-O3b-05

Potential Mechanisms of NPR1 mediated resistance against Huanglongbing (HLB)

Wenming QIU[1, 2], Juliana Moreira SOARES[1], Jude W. GROSSER[1] and **Manjul DUTT[1]**

[1]Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850
[2]Institute of Fruit and Tea, Hubei Academy of Agricultural Sciences, Wuhan, China 430064

Abstract: Huanglongbing (HLB), a bacterial disease associated with *Candidatus* Liberibacter asiaticus (CLas), is a major threat to the worldwide citrus industry. An efficient approach to cope with HLB is the use of plants engineered with genes to improve resistance. In a previous study, several citrus transgenic trees expressing the *Arabidopsis thaliana NPR1* (AtNPR1) remained HLB free when grown in a field site under high HLB disease pressure (Dutt et al. 2015). To uncover the molecular basis by which AtNPR1 mediates tolerance to HLB, a transcriptome analyses were performed from AtNPR1, CsNPR1 (the AtNPR1 homolog present in sweet orange) overexpressing transgenic trees and non- transgenic controls. We identified 429 differentially expressed genes (DEGs) when comparing AtNPR1 and CsNPR1 transgenic lines. Data mining revealed enhanced transcription of genes encoding for Pathogen-associated molecular patterns (PAMP) transcription factors, leucine-rich repeat receptor kinases (LRR-RKs) and putative Ankyrin repeat-

containing proteins. These proteins were highly upregulated in the AtNPR1 transgenic line compared to CsNPR1 transgenic line. AtNPR1 overexpressing lines were also more sensitive to exogenous salicylic acid application than CsNPR1. Furthermore, analysis of protein-protein interaction indicated that AtNPR1 could interact with CsNPR3/4 and CsTGA2/5 in the nucleus. However, the interaction of CsNPR1 with CsNPR3/4 and CsTGA2/5 lacked nuclear localization and was observed in the cytoplasm. Our results suggested that AtNPR1 and CsNPR1 might present different regulatory mechanisms in citrus, which prevents the CsNPR1 from effectively protecting the plant against HLB.

Non-Technical Summary: Genetically improved citrus that can withstand HLB and remain productive even under high disease pressure can be a viable long-term strategy to keep the industry productive. Understanding how an engineered citrus can provide protection against HLB is critical in developing long term management strategies against HLB. The Arabidopsis NPR1 when inserted in a citrus tree can provide long term resistance by enhancing the activity of proteins that play a role in the plant's defense. Arabidopsis NPR1 also interacts with proteins in the plant that can negatively regulate the innate defense mechanisms, thereby boosting the resistance process.

Citations:

Dutt, M., Barthe, G., Irey, M., Grosser, J. Transgenic citrus expressing an Arabidopsis NPR1 gene exhibit enhanced resistance against Huanglongbing (HLB; Citrus Greening). PloS one. 2015.

IRCHLB-O3b-06

Molecular mechanism underlying a thornless bud sport derived from a HLB-tolerant seedling via transcriptome analysis

Bo WU[1], Feng LUO[1], Na LI[2] and YongPing DUAN[2]

[1]School of Computing, Clemson University, Clemson, USA

[2]USDA Agricultural Research Service, U.S. Horticultural Research Laboratory, Fort Pierce, USA

Abstract: Selection of bud sports with superior traits has contributed to the identification of many important citrus cultivars, though their molecular mechanisms were rarely understood. In our current breeding project, one spontaneous thornless bud mutant (TL) was selected from a thorny pumelo seedling with Huanglongbing (HLB) tolerance (wild type, W). When TL was propagated via bud grafting in numbers, reversion of the mutant phenotype was observed in a number of propagates that are referred to as thorny mutants (T). The tolerance to HLB was enhanced in both T and TL compared with W when evaluated with two different *Candidatus* Liberibacter asiaticus (CLas) isolates. To reveal the underlying mechanisms of the thornless mutant, the reversion of the phenotype and their enhanced HLB tolerance, we compared the transcriptome of leaves of wild-type (W), Thorny (T) and (Thornless) TL. We obtained a total of 4,165 putative gene loci and 38,253 transcripts. Though T and W were both thorny, significantly more genes were differentially expressed between T and W (3,541 genes) than between TL and W (1,660 genes). And 166 genes were up regulated and 308 genes were down regulated in both T and TL compared with W. Moreover, 96 and 72 genes were significantly up regulated and down regulated in TL compared with both T and W. These results suggested that the enhanced tolerance of T and TL could have derived either from the pathways altered in both mutants, or from the up regulation of different defense response genes in T and TL. Whole genome PacBio resequencing and transgenic experiments are being carried out to further identify candidate functional mutations and verify the function of the selected candidate genes.

Non-Technical summary: In the past, new cultivars were successfully developed from the observation and propagation of naturally occurring mutations with some perceived benefit. Understanding the genetic mechanism of selected mutants is important for the selection of naturally occurring HLB-tolerant/resistant cultivars.

IRCHLB-06: Host Resistance and Tolerance Poster Presentations

IRCHLB-P6-65

CRISPR/Cas9 editing of candidate susceptibility genes to improve citrus resistance to Huanglongbing

Saroj PARAJULI[1], Alfred HUO[2], Yi LI[3], Fred G. GMITTER, Jr.[4], and Zhanao DENG[1]

[1]Gulf Coast Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Wimauma, FL, USA
[2]Mid-Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Wimauma, FL, USA
3Department of Plant Science, University of Connecticut, Storrs, CT, USA
[4]Citrus Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Lake Alfred, FL, USA

Abstract: The citrus industry in the U.S. is being threatened by Huanglongbing (HLB) disease. There is an urgent need to develop a long-term effective genetic solution to protect the citrus industry from the devastating effects of HLB. Various gene expression and gene editing approaches are being explored to improve citrus resistance to HLB. Several disease susceptibility genes have been identified in model and crop plants. Among them are the *sugars will eventually be exported transporters* (*SWEET*) genes. In rice, *SWEET11*, *SWEET13* and *SWEET14* genes are involved in rice susceptibility to *Xanthomonas oryzae oryzae* (*Xoo*). Knocking-out of these genes increased rice resistance to *Xoo*. Gene expression studies in citrus have suggested that *SWEET* genes may be involved in citrus susceptibility to HLB. To understand the roles of citrus *SWEET* genes in HLB susceptibility, we targeted several *SWEET* genes in two citrus genotypes ('Duncan' grapefruit and Carrizo' citrange) and edited them using the CRISPR/Cas9 system. Six different types of insertion and deletion of nucleotides were observed in different transgenic lines, ranging from addition of a single nucleotide to the deletion of up to 23 nucleotides. Most of the mutations were either addition or deletion of a single nucleotide at the targeted sites. Deep sequencing of the targeted regions revealed that the mutation frequency ranged from 2% to 100%. Preliminary data seem to show that some of these mutant plants have increased resistance to citrus canker. The gene-edited plants will be useful for understanding the roles of *SWEET* genes in citrus susceptibility or resistance to HLB.

Non-technical summary:
The resistance/susceptible genes for increasing HLB resistance in citrus genotypes has not been identified yet. There are several potential candidate susceptibility genes identified from transcriptomics study and they need to be tested. SWEET genes are one of the susceptibility genes providing resistance to phloem limited bacteria in other crops. Therefore, we targeted and knocked out two different SWEET genes in Citrus. Our preliminary data shows those SWEET edited plants have increased resistance to citrus canker. The gene-edited plants will be useful for understanding the roles of *SWEET* genes in citrus susceptibility or resistance to HLB.

IRCHLB-P6-66

Development of HLB Resistant Citrus Varieties for California Using CRISPR- Cas9

Karl HARO VON MOGEL[1], Jason JUNIO[1], Martha L. OROZCO-CARDENAS[1], Nian WANG[2], Mikeal L. ROOSE[1]

[1]Botany and Plant Sciences, University of California, Riverside; [2]Citrus Research and Education Center, Department of Microbiology and Cell Science, University of Florida, Lake Alfred

Abstract:

Huanglongbing (HLB), also known as citrus greening, is emerging as a threat to the citrus industry in California. The disease is associated with *Candidatus* Liberibacter asiaticus (CLas), a phloem-limited gram- negative bacterium that has not yet been cultured in artificial media. One of the few potential solutions to this disease is incorporating resistance into citrus germplasm, however, breeding cycles for citrus are long and can change important varietal traits. Genome editing has the potential to precisely edit specific DNA sequences to generate resistant varieties without changing their horticultural traits, farm management, or fruit quality. This editing approach can also be used to generate varieties that do not contain DNA from other species and so will not be considered "transgenic" in the US, which may facilitate the regulatory process. We are targeting previously-identified candidate HLB-susceptibility genes for genome editing via protoplast isolation from embryogenic cells of Washington Navel Orange, Lisbon Lemon (Limoneira 8A), and Tango Mandarin. Plants will be recovered, cultivated, and top-grafted onto mature scions in a greenhouse. The candidate plants will be challenged with CLas to evaluate the level of resistance to HLB. Our goal is to develop and release HLB-resistant varieties important for California that can be effective and sustainable solutions to HLB. Research supported by NIFA award 2018-70016-27412.

Non-Technical Summary:

Huanglongbing (HLB) threatens to severely impact California citrus production in the coming years, and there are no ready solutions available. We are using genome editing to develop resistance to HLB in important local varieties by "silencing" genes that make the trees susceptible to the disease. We will present on our approach and current progress on this project.

Citations:

Wang, Y., Zhou, L., Yu, X., Stover, E., Luo, F., and Duan, Y. 2016. Transcriptome Profiling of Huanglongbing (HLB) Tolerant and Susceptible Citrus Plants Reveals the Role of Basal Resistance in HLB Tolerance. Front Plant Sci 7:933.

Cong, L., Ran, F.A., Cox, D., Lin, S., Barretto,

R., Habib, N., Hsu, P.D., Wu, X., Jiang, W., Marraffini, L.A., and Zhang, F. 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339:819- 823.

Jia, H., and Wang, N. 2014. Targeted Genome Editing of Sweet Orange Using Cas9/sgRNA. PLoS One 9:e93806.

IRCHLB-P6-67

Exploring Transient Expression of Cas9 Proteins and Heat Treatments for Increasing Gene Editing Efficiency in Citrus

Saroj PARAJULI[1], Alfred HUO[2], and Zhanao DENG[1]

1 University of Florida, IFAS, Gulf Coast Research and Education Center, Wimauma, FL,
2 University of Florida, IFAS, Mid-Florida Research and Education Center, Apopka, FL

Abstract: The CRISPR/Cas9 system has been used to edit genes in several citrus genotypes. Early demonstrations of successful genome editing in citrus were based on targeting the phytoene desaturase (PDS) gene and the citrus canker susceptibility gene *CsLOB1*. Despite of

these early successes, the gene editing efficiency of this system in citrus needs to be increased substantially for routine use. In this study, we demonstrated enhanced gene editing efficiencies in two citrus genotypes (‘Carrizo’ citrange and ‘Duncan’ grapefruit) by transient expression of two Cas9 proteins [SpCas9 and high-fidelity spCas9 (HFspCas9)] and heat treatments. Mutations were detected in citrus leaf and callus tissues 96 and 144 hours after *Agrobacterium* inoculation. Heat treatment at 37 ˚C for 48 hours increased the gene mutation efficiency by several folds. Transient expression of Cas9 proteins and gRNAs in citrus tissues can be a valuable tool for selecting gRNAs with high editing efficiencies and for producing stably edited citrus plants.

Non-technical summary: CRISPR/Cas9 system has been widely used to edit susceptibility genes and increase disease resistance. This system is new to citrus and efficiency for editing is lower as compared with other crops. We are exploring the technique to test gRNA efficiencies by transiently expressing different gRNAs in citrus leaves and selecting the best gRNA for stable transformation. Heat treatment of the ex-plants at 37 C increased the editing efficiencies in both callus and leaves tissues.

Citations:

Li et. al. (2013) Multiplex and homologous recombination-mediated genome editing in *Arabidopsis* and *Nicotiana benthamiana* using guide RNA and Cas9. Nature Biotechnology 31, 688-691.

IRCHLB-P6-68

Overexpression of the *Arabidopsis* NPR1 protein in citrus confers robust tolerance to Huanglongbing

Cecile J. ROBERTSON[1], Xudong ZHANG[2], Siddarame GOWDA[1], Vladimir ORBOVIĆ[1], William O. DAWSON[1], **Zhonglin MOU**[2]

[1]University of Florida, Lake Alfred, USA, [2]University of Florida, Gainesville, USA

Abstract: Huanglongbing (HLB), also known as citrus greening, is one of the most destructive diseases of citrus worldwide. It is associated with unculturable phloem-limited bacteria that belong to the *Candidatus* Liberibacter genus including *Candidatus* Liberibacter asiaticus (CLas), *Candidatus* Liberibacter africanus (CLaf), and *Candidatus* Liberibacter americanus (CLam). Currently, there is no effective control strategy for HLB and no known cure for the disease. In this project, we tested whether the *Arabidopsis thaliana NPR1* (*AtNPR1*) gene could be useful for managing the HLB disease. *AtNPR1* encodes a master transcription regulator of systemic acquired resistance, which is a plant defense mechanism providing long-lasting resistance to a broad spectrum of pathogens. The *AtNPR1* gene has been widely used in improving disease resistance in various crop plant species. We have generated transgenic ‘Duncan’ grapefruit and ‘Hamlin’ sweet orange expressing *AtNPR1*. Previous characterization of the transgenic lines indicated that overexpression of *AtNPR1* confers resistance to citrus canker, another serious bacterial disease in citrus. After long- term intensive testing of these transgenic lines for resistance or tolerance to HLB under greenhouse conditions, three independent transgenic lines (one line in the ‘Duncan’ grapefruit background and two lines in the ‘Hamlin’ sweet orange background) only occasionally displayed mild HLB symptoms in the presence of CLas and have kept growing normally over a period of nine years. Significantly, all vegetatively propagated progeny plants of these lines have retained the same levels of HLB tolerance. Furthermore, immunoblot analysis revealed that the three transgenic lines accumulate high levels of AtNPR1 protein. These results, together with the previous finding that the same three transgenic lines are resistant to citrus canker, demonstrate that overexpression of AtNPR1 in citrus is able to provide robust tolerance to HLB, and suggest that the citrus NPR1 signaling pathway may be

manipulated to create tolerance to HLB.

Non-technical summary: The *Arabidopsis thaliana NPR1* (*AtNPR1*) gene encodes a key positive regulator of plant systemic acquired resistance that provides resistance to a broad spectrum of pathogens. It was found that transgenic overexpression of *AtNPR1* in citrus not only increases resistance to citrus canker, but also confers robust tolerance to HLB. The citrus NPR1 signaling pathway is thus an ideal target for creating HLB tolerance using various biotechnological approaches.

Citations:

Robertson, C.J., Zhang, X., Gowda, S., Orbović, V., Dawson, W.O., and Mou, Z. (2018). Overexpression of the Arabidopsis NPR1 protein in citrus confers tolerance to Huanglongbing. J. Cit. Pathol. iocv_journalcitruspathology_38911.

Zhang, X., Francis, M.I., Dawson, W.O., Grahma, J.H., Orbović, V., Triplett, E.W., and Mou, Z. (2010). Over-expression of the Arabidopsis *NPR1* gene in citrus increases resistance to citrus canker. Eur.J. Plant Pathol. 128: 91-100.

Silva, K.J.P., Mahna, N., Mou, Z., and Folta, K.M. (2018). NPR1 as a transgenic crop protection strategy in horticultural species. Hort. Res. 5: 15.

IRCHLB-P6-69

Particle Bombardment for Transient Gene Expression in Citrus Plant Cells Using the Helios® Gene Gun.

Amit LEVY[1,2] ,Yosvanis ACANDA[1,2] and Chunxia WANG[1]

[1]Univeristy of Florida, Citrus Research and Education Center. Lake Alfred, USA, [2]Department of Plant Pathology, University of Florida, Gainesville, USA

Abstract: Transient gene expression can provide valuable data about various aspects of protein characteristics, such as its subcellular localization and intra/inter cellular trafficking, its expression levels, stability and degradation, its interactions with other proteins, and its activity. In citrus, transient gene expression is typically carried out using Agrobacterium inoculation, but the efficiency of this method varies considerably depending on the citrus variety and the experimental conditions. Particle bombardment is a very convenient method to deliver nucleic acids into the cells directly. Because it is a physical method, it does not depend on biochemical features of the structural components that are typically present on cell surfaces and can overcome physical barriers such as the cell wall. The Helios® Gene Gun is a hand-held device that uses a low-pressure helium pulse to accelerate DNA/RNA-coated sub-cellular sized particles into a wide variety of target cells either for *in vitro* or *in vivo* applications. Using this system, we optimized parameters for robust particle bombardment into citrus plant cells and transient gene expression. In addition, we also achieved stable protein expression in cultured tissues, which could lead to the production of transgenic plants.

Non-technical summary: Stable protein transformation is a long and difficult process in citrus. Transient gene expression is a much faster way to express proteins in the plant. In order to understand protein characteristics in their natural citrus environment, we have developed a method for robust gene expression in citrus. We can also use this method to generate transgenic plants.

IRCHLB-P6-70

Progress in the analysis of HLB tolerance in a segregating population

Maria B. BESILLA[1], Lorenzo ROSSI[2], Vicente FEBRES[1], **Gloria MOORE**[1], José X. CHAPARRO[1]

[1]University of Florida, Gainesville, Fl, USA, [2]Indian River Research and Education Center, FL, USA

Abstract: Huanglongbing (HLB) is a disease imparting enormous economic losses to the citrus industry around the world. No methods to cure HLB have been developed thus far. A possible solution to the disease is breeding efforts to produce resistant/tolerant trees. However, insufficient information about the inheritance of HLB tolerance makes it difficult for breeders to develop genetic sources for the trait. A segregating family, resulting from a cross between a breeding selection and commercial germplasm, has been evaluated in the field at IRREC in Fort Pierce and their clones have been maintained in a greenhouse in Gainesville, Florida. The field trees and their clonal counterparts were visually rated for HLB symptoms. The two year old clonal trees in the quarantine greenhouse represent a snapshot of HLB and canker infection under field conditions at IRREC. The proportion of escape trees is expected to decrease over time. Some trees from this family consistently expressed HLB and canker tolerant phenotypes in in the field in Fort Pierce and in the greenhouse in Gainesville. A HLB tolerance phenotype was detected in seven trees and their clones. This family thus presents germplasm for future crosses to develop varieties with HLB and canker tolerance.

Non-Technical Summary: Infected budwood taken from a HLB tolerant segregating family planted in Fort Pierce was grafted in a greenhouse in Gainesville, FL. The trees in Fort Pierce were in an HLB and canker high-pressure disease environment, while their clones in Gainesville were not exposed to further infection agents since they were grafted. We can identify HLB and canker tolerance in this germplasm by analyzing the phenotype and level of infection in this family.

IRCHLB-P6-71

Transgenic success-guided reproduction of Huanglongbing resistance/tolerance in citrus by gene editing

Ozgur BATUMAN[1], William DAWSON[2], Manjul DUTT[2], Choaa EL-MOHTAR[2], Jude GROSSER[2], Michael IREY[3], Amit LEVY[2], **Zhonglin MOU[4]**, Vladimir ORBOVIĆ[2]

[1]University of Florida, Immokalee, USA, [1]University of Florida, Lake Alfred, USA, [3]Southern Gardens Citrus, Clewiston, USA, [4]University of Florida, Gainesville, USA

Abstract: Huanglongbing (HLB), also called citrus greening, is a devastating citrus disease present in most of the citrus growing regions of the world. In Florida, HLB has become endemic while in Texas the disease is spreading into many commercial groves. In California, HLB has been detected in several residential trees and the vector continues to spread into commercial areas. Without new solutions, it is possible that the California and Texas citrus industries may follow a pattern similar to that in Florida. We have generated transgenic citrus trees overexpressing the *Arabidopsis thaliana* NPR1 (AtNPR1) protein, which exhibit robust tolerance to HLB and resistance to citrus canker. However, these trees are genetically modified organisms (GMOs) that will have to go through the time-consuming approval process, and if approved, the industry would have to sell a GMO product. On the other hand, recent development of the transgene-free CRISPR/Cas9 technology makes it possible to generate non-GMO citrus varieties that are resistant or tolerant to HLB. A major limitation of the CRISPR/Cas9 technology is finding the right gene to mutate. Our transgenic results have identified the NPR1 signaling pathway as an ideal target of gene editing to create HLB resistance or tolerance in citrus. Two negative regulators of the citrus NPR1 signaling pathway are being targeted by CRISPR/Cas9-

mediated mutagenesis. Moreover, a group of negative regulators of the citrus immune system are being tested using the *Citrus tristeza virus* (CTV)- mediated RNAi to pinpoint further targets for gene editing. In addition, outreach activities are being carried out, which include keeping citrus growers and state regulators aware of the progress of the project, educating the citrus communities about the transgene-free CRISPR/Cas9 technology and its benefits to the citrus industry, and educating the public for acceptance of fruit and juice produced with CRISPR/Cas9 products.

Non-technical summary: Transgenic citrus trees with robust tolerance to HLB have been generated, but these trees are genetically modified organisms (GMOs). The current project is using a more consumer-friendly gene editing technology to produce non-GMO citrus with HLB tolerance/resistance similar to that of the transgenic trees. The project also educates the citrus communities about the gene-editing technology and its benefits to the citrus industry and consumers, which will help establish market acceptance of non-GMO citrus products.

Citations:

Robertson, C.J., Zhang, X., Gowda, S., Orbović, V., Dawson, W.O., and Mou, Z. (2018). Overexpression of the Arabidopsis NPR1 protein in citrus confers tolerance to Huanglongbing. J. Cit. Pathol. iocv_journalcitruspathology_38911.

Dutt, M., Barthe, G., Irey, M. and J. Grosser (2015). Transgenic citrus expressing an *Arabidopsis NPR1* gene exhibit enhanced resistance against Huanglongbing (HLB; citrus greening). PLoS ONE 10: e0137134.doi: 10.1371/journal.pone.0137134.

Zhang, X., Francis, M.I., Dawson, W.O., Graham, J.H., Orbović, V., Triplett, E.W., and Mou, Z. (2010). Over-expression of the Arabidopsis *NPR1* gene in citrus increases resistance to citrus canker. Eur.J. Plant Pathol. 128: 91-100.

IRCHLB-P6-72

Use of high-density genetic maps to detect QTLs associated with Huanglongbing tolerance in citrus

Ming HUANG[1], Mikeal L. ROOSE[2], **Qibin YU[1]**, Dongliang DU[1], Yuan YU[1], Yi ZHANG[1], Zhanao DENG[3], Ed STOVER[4], Frederick G. GMITTER Jr[1]

[1]University of Florida, IFAS-CREC, Lake Alfred, FL, United States
[2]University of California, Riverside, CA, United States
[3]University of Florida, IFAS-GCREC, Wimauma, FL, United States
[4]USDA-ARS, Fort Pierce, FL, United States

Abstract: Sweet orange (*Citrus sinensis*) is highly susceptible to HLB, whereas trifoliate orange (*Poncirus trifoliata*, a close *Citrus* relative) is widely considered highly tolerant. In this study, an intergeneric F1 population of sweet orange and trifoliate orange was genotyped by Genotyping-by-Sequencing. The two separately constructed genetic maps for trifoliate orange and sweet orange exhibited high synteny and high coverage of the citrus genome. Progenies of the F1 population and their parents were planted in a field trial with eight biological replications, at Fort Pierce in 2011. The segregating population exhibited a wide range in severity of HLB foliar symptom and canopy damage. QTL mapping based on the two-year phenotypic data of foliar symptom and canopy damage identified three clusters of repeatable QTLs in trifoliate orange linkage groups LG- t6, LG-t8 and LG-t9. Identified QTLs for two traits were observed within all three co- localization regions. In sweet orange, one cluster of QTLs (linkage group LG-s7) was also detected. Most identified QTLs explained 18% to 30% of the phenotypic variation, indicating their major role in host responses to the disease. These results show a

quantitative genetic nature with major loci for the HLB tolerance in trifoliate orange and sweet orange.

Non-Technical Summary: With reduced cost of large numbers of SNP markers, we focus here on genetic mapping for HLB tolerance. Findings from this study should be very valuable and timely to researchers worldwide as they search for genetic solutions to the devastating HLB crisis through breeding, genetic engineering, or genome editing.

IRCHLB-P6-73

Using Agrobacterium-mediated transient expression of Cas9 and sgRNA to produce transgene-free, gene-edited citrus plants

Yi LI[1], Yanjun LI[1], Xiaojing WANG[1], Longmei ZHAI[1], Dan TANG[1], Richard MCAVOY[1], Fred. G. GMITTER[2] and Zhanao DENG[3] and Ziniu DENG[4]

[1]Department of Plant Science and Landscape Architecture, University of
Connecticut, Storrs, CT, USA

[2]Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA.

[3]Department of Environmental Horticulture, Gulf Coast Research and Education Center, IFAS, University of Florida, Wimauma, FL, USA.

[4]National Citrus Research Center, Hunan Agricultural University, Changsha, P. R. China.

Abstract: Methods to develop transgene-free, gene-edited citrus trees are highly desirable for the commercialization of HLB tolerant/resistant citrus trees. Using a model plant, we have demonstrated that Agrobacterium can be used to transiently express Cas9 and sgRNA. We have further shown the transient expression of CRISPR components is efficient for gene editing. We also developed fast screening methods for screening of transgene-free mutant plants. We have been working on adapting these methods and developing other new methods to create and screen for transgene-free, edited citrus plants. To speed up evaluation processes of edited citrus plants, we have also used mature tissues of citrus as explants. One issue we have observed is that a significant % of citrus shoots produced are mutant-wild type chimeras when attempting to produce transgene-free edited citrus plants. We will report our progresses and the challenges for using Agrobacterium-mediated transient expression of CRISPR components to produce edited, transgene-free citrus plants.

Non-Technical Summary: Genome editing technologies provides a powerful tool to create HLB tolerance/resistance. However, for commercial plantings, genome-edited citrus trees should be transgene-free. We will report our progresses for the development of methods of efficiently producing and expediently identifying gene-edited, transgene-free citrus plants.

IRCHLB-07: Chemical and Biological Vector Management Oral Presentations

IRCHLB-O4a-01

Addressing whether there is a need for continued vector management under HLB stress

Freddy IBANEZ[1], Gene ALBRIGO[1] and Lukasz STELINSKI[1]

[1] University of Florida, Citrus research and education center, Lake Alfred, USA

Abstract: In areas where huanglongbing is widespread, occurrence of disease can reach 90-100% within citrus groves. These groves also harbor Asian citrus psyllid (ACP), *Diaphorina citri*, which transmits the bacterium, '*Candidatus* Liberibacter asiaticus' (*C*Las), associated with

huanglongbing. Managing ACP populations is thought to benefit citrus yield in highly infected areas; however, insufficient scientific evidence is available. We are evaluating the relationship between the ACP abundance and *C*Las titer within citrus trees. The primary objective is to determine if vector suppression is a worthwhile investment under conditions of nearly complete tree inventory infection. Field experiments are located in two geographically distinct locations in central Florida. Two treatments are being evaluated among eight groves 50-100 acres in size. In the first treatment, insecticide treatments applied for ACP management are coordinated with bud break phenology to target newly breaking buds producing feather flush (feather flush management). In the second treatment, which serves as a *de facto* control, insecticide sprays are applied on a calendar basis without reference to tree phenology (conventional management). In both treatments, an approximately equivalent number of sprays are applied per annum. ACP populations were lower in groves treated with sprays coordinated with flush phenology than in groves where uncoordinated sprays were applied on a calendar basis. Likewise, *C*Las titers were lower in groves with coordinated sprays than conventional groves during June and July, but not throughout the entire season. In complementary growth chamber experiments, we are measuring citrus plant response to one-time inoculation, multiple inoculations by invading adult ACP (immigrating adults), and extended inoculations by continuously developing population of immature and adult ACP (resident population). Plants exposed continuously to *C*Las-infected psyllids express a higher rate of infection than the one- time or repeated, pulsed-inoculation treatments after 5 months. Thus far, our results support vector control even under conditions of 100% infection.

Non-Technical Summary: It is believed that psyllid management is important in areas in which HLB is widespread. However, the use of insecticides increases the cost of citrus production and growers have expressed concern regarding whether vector management should continue under circumstances of 100% HLB infection. We are investigating whether reduction of ACP in groves with 100% infection promotes tree health. Data from this investigation and others available to date support the hypothesis that ACP management is valuable even in areas with near complete endemic infection.

IRCHLB-O4a-02

Monitoring Susceptibility Of *Diaphorina citri* To Five Insecticides In Regional Control Areas Of Mexican States Growing Citrus

Francisco OSORIO-ACOSTA[1], Juan A. VILLANUEVA-JIMENEZ[1], Laura D. ORTEGA-ARENAS[2], Víctor H. GARCÍA-MÉNDEZ[2], Ulises A. DÍAZ-ZORRILLA[3], Rosaura JOSE-PABLO[1], Sherell ZAMORA-JUÁREZ[4], Jaqueline C. LUNA-OLIVARES[1], Gonzalo LUNA-OLIVARES[1]

[1]Colegio de Postgraduados, Campus Veracruz, MEXICO, [2]Colegio de Postgraduados, Campus Montecillo, MEXICO, [3]INIFAP, Campo Experimental Ixtacuaco, MEXICO. [4]Universidad Veracruzana, Peñuela.

Abstract: Asian citrus psyllid (*Diaphorina citri*) is the vector of *Candidatus* Liberibacter asiaticus, associated agent of Huanglongbing (Bove, 2006). Vector control is based on insecticide applications, although this has caused insect resistance to main molecules used (Tiwari *et al.*, 2011). Thus, there was a need to evaluate the susceptibility of *D. citri* to five insecticides in 24 states of Mexico where citrus are produced. For this purpose, a discriminating dose (DD: DL95 increased 3 times) was used for each insecticide. Previously, the reference dose (ng of active ingredient per insect) of each insecticide was obtained in a susceptible colony, which has

been maintained in a greenhouse since 2010 (García-Méndez *et al.*, 2018). Adult insects were collected from four to eight groves per state with an aspirator, and carried to an area under controlled conditions for insecticide application. Insects were anesthetized with CO2 and placed in a Petri dish containing 2 % agar and a piece of a clean and unsprayed- young tender leaf for insect feeding. Insecticides tested were solubilized in acetone. A drop of 0.2 µL was delivered in the pronotum of the insect with a Hamilton syringe, at doses of 5.68, 0.99, 7.50, 12 and 0.90 ng of chlorpyrifos, imidacloprid, dimethoate, abamectin and cypermethrin, respectively. Five replicates with 20 insects per insecticide dose were used; the control was inoculated with acetone; 24 h after insecticide application, mortality was evaluated. *D. citri* mortality below 95% was observed in 6, 5, 9, 12, and 5 out of 24 states for chlorpyrifos, imidacloprid, dimethoate, abamectin, and cypermethrin, respectively. These results indicate that insecticides widely used, such as avermectins (used for mite control) and organophosphates (long used products in citrus production) are showing signs of susceptibility lost in several states. However, most of them still can be used in regional control areas.

Non-Technical summary: *Diaphorina citri* control is mainly managed by insecticides in several countries, including Mexico. Our objective was to evaluate *D. citri* susceptibility to five insecticides in 24 citrus states of Mexico. These results are useful for planning insecticide use in regional control areas.

Citations:

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

García-Méndez, VH, Ortega-Arenas, LD, Villanueva-Jiménez, JA, Lara-Reyna, J, Lagunes-Tejeda, A, Sánchez-Arroyo, H. 2018. Dinámica de la resistencia de *Diaphorina citri* (Hemiptera: Liviidae) a insecticidas en áreas regionales de control en Veracruz, México. In: Vinay, VJC *et al.* (comps.). 2018. Avances en Investigación Agrícola, Pecuaria, Forestal, Acuícola, Pesquería, Desarrollo rural, Transferencia de tecnología, Biotecnología, Ambiente, Recursos naturales y Cambio climático. INIFAP, CP, UACH, INAPESCA, UV, TecNM. Medellín, Ver., México. Año 2, Núm. 1. pp. 1800-1810.

Tiwari, S, Mann, RS, Rogers, ME, Stelinski, LL. 2011. Insecticide resistance in field populations of Asian citrus psyllid in Florida. Pest Manag. Sci. 67:1258–68.

IRCHLB-O4a-03

Evaluation of early uptake of systemic pesticides on containerized citrus nursery stock for the control of Asian citrus psyllid

Frank BYRNE, Matthew DAUGHERTY, Elizabeth GRAFTON-CARDWELL

Department of Entomology, University of California, Riverside

Abstract: One of the primary responses to the detection of Asian citrus psyllid (ACP) in California was the implementation by the California Department of Food and Agriculture (CDFA) of quarantine restrictions on the movement of citrus nursery stock. In Florida, the unregulated movement of infested nursery stock is believed to have played a pivotal role in the spread of both ACP and HLB throughout the state, and has also been implicated in the interstate spread of ACP. In an attempt to minimize the risk of this occurring in California, quarantines were established by the CDFA in regions where the psyllid was detected. As part of the quarantine, production nurseries are required to treat all citrus nursery stock prior to shipment to retail outlets. With regard to shipments outside of the quarantine zone, including inter-state movement, nurseries must apply approved foliar and systemic insecticides no more than 90 days, and no less than 30 days, prior to shipment. The

delay in shipment imposed by this regulation results in a shortened window of protection for trees awaiting sale at retail outlets, and is particularly problematic because trees often reside at these locations well beyond the 90-day protection period afforded by the treatments. In a recent trial evaluating the uptake of imidacloprid into 4 citrus varieties, applications of label rates of imidacloprid to 1-year old potted citrus trees reached threshold levels of insecticide within as little as 3 days post-treatment. These data indicate that the current 30-day limit on shipping could be dramatically reduced, thereby prolonging the period of protection for trees awaiting sale at retail outlets.

Non-technical summary: In formulating strategies for protecting containerized citrus, the main concerns of regulators have been that the systemic insecticides have sufficient time to establish within the trees prior to shipment from the production nursery, and that the systemic insecticides remain effective during the transition period between leaving the production nursery and final sale. Our data address these concerns, and show that trees treated with imidacloprid could be shipped to retail outlets within three days of treatment, thereby prolonging the period of protection of trees awaiting sale at retail outlets.

IRCHLB-O4a-04

Evergreen Pressurized Spray for the Control of Asian Citrus Psyllid in Bulk Citrus

[1,2]**Sandipa GAUTAM**, [2]Nastaran TOFANGSAZI, [3]David SORENSON, [2,4]Elizabeth GRAFTON-CARDWELL, and [5]Spencer WALSE

[1,2]Kearney Agricultural Research and Extension Center, Parlier, CA; [2]University of California, Riverside, CA; [3]Citrus Consulting, Visalia, CA; [4]Lindcove Research and Extension Center; Exeter, CA, [5]USDA-ARS, Parlier, CA

Abstract: Asian Citrus Psyllid, ACP, *Diaphornia citri* Kuwayama) is a known vector of Huanglongbing (HLB) disease that is an imminent threat to the California citrus industry. At present, limiting the geographical distribution of ACP is a major goal of the California citrus industry to delay the spread of HLB, while disease management tools are developed (Grafton-Cardwell et al., 2013). ACP finds from trap catches in residential areas and at packinghouses and juice plants near transportation corridors suggest human- assisted transport signifying that insecticide treatments prior to harvest and tarping of the load are not fully effective in eliminating ACP from bulk citrus. While a variety of phytosanitary measures can be used to control adult ACP once fruit is at the packinghouse, a treatment must be developed to disinfest field- run fruit prior to exiting the grove to limit accidental transport of ACP in bulk citrus. High-pressure fogging with 1,100-liter of an aqueous mixture containing 0.2% Evergreen (6% pyrethrins & 60% piperonyl butoxide) and 0.5% (v/v) BreakThru (polysiloxane surfactant) was explored in laboratory-, pilot-, and commercial-scale trials as an approach to disinfest a 48-bin trailer load of fresh citrus. Laboratory-scale studies were conducted to quantify, and subsequently model, insecticidal coverage as a function of temperature, surface area, droplet size, and fog volume. Commercial scale trials showed that high pressure fogging resulted 99.78% mortality of caged adult ACP strategically placed in 140 different positions in a 48-bin trailer load of citrus. Results are discussed in the context of experimental variability across confirmatory trials and continued efforts to optimize the technical and economic feasibility of high-pressure spray as a postharvest control strategy.

Non-Technical Summary: A postharvest treatment to disinfest bulk citrus prior to exiting the grove is important to mitigate the spread of ACP and to limit accidental transport between quarantine zones. We evaluated the effects of a high-pressure spray using Evergreen to control adult ACP in bulk citrus.

Citations:

Grafton-Cardwell, E. E., L. L. Stelinksi, and P. A. Stansly. 2013. Biology and Management of Asian Citrus Psyllid, Vector of the Huanglongbing Pathogens. Annual Review of Entomology. 58: 413– 432.

IRCHLB-O4a-05

Biological Control of Asian Citrus Psyllid in Urban California – Release Strategies and Monitoring Results

David J. W. MORGAN, Alex MUNIZ, Rachael MASSIE

California Department of Food and Agriculture, Riverside, CA, USA

Abstract: Releases of biological control agents for the control of Asian citrus psyllid (ACP) in California started in 2011 with *Tamarixia radiata*. We developed three sequential release strategies to optimize biological control agent releases. The first, establishment, investigated agent survival and dispersal and lasted until 2014. The second, dispersal, ensured widespread release of agents throughout 4,000 square miles of residential Southern California from Imperial County to Santa Barbara County. The third, prioritization, started in 2017 and focused releases of agents in high-risk areas; around locations where diseased citrus plants where found, in locations where ACP were newly established, along the southern border of California, and along trade routes. In 2015 we initiated a monitoring program to track the population dynamics of ACP and its biological control agents. We report strong density dependence of *T. radiata* and a significant decline in ACP populations over the past four years. Transect monitoring has also shown that increasing release rates of *T. radiata* can be used to drive down local ACP populations in high-risk areas.

Non-Technical summary: Biological control has been used to target ACP in urban areas of Southern California since 2011. We show that ACP populations have declined since monitoring started in 2015. In addition to the proven value of beneficial insects as a classical biological control agent; we show that augmenting releases locally can drive down ACP populations in high-risk areas.

Citations:

California Department of Food and Agriculture (CDFA), 2018. CDFA ACP/HLB Regulation and Quarantine Boundaries.

Hoddle, M.S. and Hoddle, C.D., 2013. Classical biological control of Asian citrus psyllid with *Tamarixia radiata* in urban Southern California. Citrograph, 4: 52-58. Milosavljević I, Schall KA, Hoddle CD, Morgan DJW, Hoddle MS (2017) Biocontrol program targets Asian citrus psyllid in California's urban areas. California Agriculture 71, 169-177.

IRCHLB-O4a-06

Patterns of *Diaphoriana citri* (Hemiptera: Liviidae) parasitism by the nymphal parasitoid *Tamarixia radiata* (Hymenoptera: Eulophidae) on residential citrus

Mamoudou SÉTAMOU[1], Andrew CHOW[1], Jon DALE[2] and Teresa Feria ARROYO[3]

[1]Texas A&M University-Kingsville Citrus Center, Weslaco, TX, USA; [2]Texas Citrus Pest & Disease Management Corporation, Mission, TX, USA; [3]University of Texas Tio Grande Valley, Edinburg, TX, USA.

Abstract: The Asian citrus psyllid (ACP) is a pest of great concern in all citrus producing states

because it vectors *Candidatus* Liberibacter asiaticus (CLas), the Huanglongbing (HLB) associated bacterium. Vector control is a key component for reducing HLB incidence and spread. In areas where pesticide use is limited, biological control is essential for ACP population reductions. In South Texas, mass releases of *Tamarixia radiata* are ongoing to target ACP in urban areas, where on average ~4 citrus trees are present in 60-70% of residences. This study was conducted to quantify the spatiotemporal attributes of ACP suppression by *T. radiata* by assessing its host patch colonization and host use within patches. Percent ACP parasitism by *T radiata* varied with the time of the year from 3 to 23% but was unaffected by the citrus host on which ACP developed. About 40% of all citrus flush shoots infested by ACP harbored *T. radiata* parasitized nymphs, indicating the parasitoid's capacity to find its psyllid host in urban landscapes. Patch colonization increased with ACP numbers per flush shoot from 30% for the smallest patch size of ≤5 ACP nymphs per flush shoot to 70% for the largest patch size of 100-200 ACP nymphs per flush shoot. In contrast, the percentage of nymphs successfully parasitized within a patch (i.e. ACP-infested flush shoot) was inversely related to the patch size, rapidly decreasing from 48% for the smallest patch to 7% for the largest patch. The efficient dispersal and host finding ability of *T. radiata* explains the rapid and wide establishment of the parasitoid following releases into a new area. However, the relatively low levels of parasitism by *T. radiata* for higher ACP densities on citrus flush shoots suggests that effective suppression of psyllid populations will require augmentation by other natural enemies such as generalist predators.

Non-Technical summary: We observed seasonal variations in ACP parasitism levels by the nymphal parasitoid *Tamarixia*. Although the citrus host on which ACP developed did not affect *Tamarixia* parasitism levels, the number of ACP nymphs per flush shoot was an important factor because per- patch parasitism was inversely density dependent. For effective psyllid control in residential areas, *Tamarixia* should be combined with other natural enemies.

Citations:

Chen, X., and P. A. Stansly. 2014. Biology of *Tamarixia radiata* (Hymenoptera: Eulophidae), parasitoid of the citrus greening disease vector *Diaphorina citri* (Hemiptera: Psylloidea): A Mini Review. Fla. Entomol. 97: 1404-1413.

Flores, D., and M. Ciomperlik. 2017. Biological control using the ectoparasitoid, *Tamarixia radiata*, against the Asian citrus psyllid, *Diaphorina citri*, in the Lower Rio Grande Valley of Texas. Southwest. Entomol. 42: 49-59.

Kistner, E. J., R. Amrich, M. Castillo, V. Strode, and M. S. Hoddle. 2016b. Phenology of Asian citrus psyllid (Hemiptera: Liviidae), with special reference to biological control by *Tamarixia radiata*, in the residential landscape of Southern California. J. Econ. Entomol. 109: 1047-1057.

IRCHLB-07: Chemical and Biological Vector Management Poster Presentations

IRCHLB-P7-74

A novel methodology to produce and collect *Tamarixia radiata* and their release by Unmanned Aerial Vehicle or drones:

PhD. Maricarmen SANCHEZ [1] Rubén Leal MUBARQUI [1]**Roberto Angulo KLADT.**-

[1] Servicios Mubarqui Mexico.

Abstract:

The huánglóngbìng or yellow dragon disease, is a disease associated with the presence of the bacterium *Candidatus* Liberibacter asiaticus, in the citrus plant, and one of the main vectors, is the Asian citrus psyllid (ACP) *Diaphorina citri*. *Tamarixia radiata* is considered the best primary parasitoid to control populations of *D. citri* in the wild, so the aim of this document is to present the

methodology to produce in a mass rearing facility, the highest quality and the largest amounts of *T. radiata*. To begin with we need to obtain a good source of plants *Murraya paniculata* as a host for *D. citri*. The rotation of *M. paniculata* in different stages, allows us to obtain fresh flush in new branches. Once we have a good production of plants, the plants are moved to the next stage to be infested with a disease-free population of *D. citri*. The psyllids mate and lay eggs on new flush and through this process we obtain nymphs that are synchronized in age. The nymphs are exposed to oviposition by the adult colony of *T. radiata* in specially designed cages. After the parasitization of equal aged nymphs in the cage, we wait until new adults emerge from nymph mummies and the new generation is collected using the novel design of air flow panels and a light source (positive phototaxis) that attract the adult parasites. The entire procedure is conducted inside a high quality green-house avoiding escapes or introduction of unwanted organisms. After collection, *Tamarixia* are placed in a chilled room to immobilize them by causing them to become dormant and transported to specific polygons where they are released using a special device mounted in a drone with a flight path pre-designed for the specific needs of the field.

Non-Technical Summary:
Tamarixia radiata is a natural enemy for the juvenile stages of citrus psyllid, so a mass rearing and release for this parasite wasp, is a good method to control the population of the vector for the greening disease or HLB. The innovation in mass production for this parasite and the aerial vehicle to distribute them in the field, is important in the field for any project.

IRCHLB-P7-75

Advances in the evaluation of localized chemical control of *Diaphorina citri* on Valencia orange young trees in Mexico

Santos DÍAZ-MARTÍNEZ[1], **J. Isabel LÓPEZ-ARROYO[2]**

[1]Colegio de Postgraduados. Montecillo, Estado de Méx., Mexico, [2]INIFAP, Campo Experimental General Terán. Gral. Terán, N.L., Mex.

Abstract: The evaluation of localized, strategic sprays of insecticides for the control of *Diaphorina citri* Kuwayama (Hemiptera: Liviidae), the Asian citrus psyllid (ACP) seeks to increase the frequency of intervention with the use of agrochemical sprays to control the insect only on infested trees or against population outbreaks in the grove. The goal is to generate a strategy of minimum economic and environmental cost mainly for Mexican growers of low economic resources that are unable to perform pest management with high use of pesticides. The experiments were conducted in groves of Valencia orange young trees planted at 6x2 m. Treatments include a check without use of insecticides, complete coverage foliar sprays of insecticides (imidacloprid, abamectin+oil), soil drench insecticide application (imidacloprid, thiamethoxam), and three more strategies to control ACP on selected trees. We used 4 replicates/treatment in a randomized complete block design. Results obtained in the first year of evaluation in two years old trees of Valencia orange showed that the treatments where ACP was controlled by means of a total insecticide spray of the plot, or only against infested trees, maintained the lowest records of *D. citri* abundance; however, the control of ACP outbreaks in the plot showed the lowest level of infested trees (27%). In this treatment, after six insecticide sprays, only an average of 50% of the trees were treated. In the experiment with five years old orange trees, the abundance of the insect was 10 times lower than the above experiment and the alternative application strategies presented tree infestation in a range of 7-14%, while in the treatment that received total coverage such value was 15%; the check

presented 13% infestation and insect abundance that did not vary significantly with respect to the use of insecticides, this possibly associated with the activity of natural enemies.

Non-Technical summary: Our study is intended to develop a program of high frequency of ACP chemical control focused only on infested trees instead of complete groves. The goal is to give alternatives to the Mexican citrus growers in order that they could participate in the campaign for reduction of ACP populations in the country through pest chemical control. Preliminary results show that applying insecticides to only 50% of the trees presented similar results to the use of complete insecticide coverage. The strategies of reduced coverage involve a risk as only half of the plot is protected and relies in the abundance of natural enemies; however, such risk is lower than the situation where ACP management has been neglected. The study could support precision chemical control of ACP and viability of outbreak suppression in an area-wide integrated pest management program.

IRCHLB-P7-76

Assessing the impact of fungal pathogens for the integrated biological control of the Asian citrus psyllid in the Lower Rio Grande Valley of TX

Daniel FLORES[1], Justin WENDEL[2], Jonathan CISNEROS[2], Stefan JARONSKI[3], Christopher VITEK[2], Matt CIOMPERLIK[1]

[1]USDA APHIS PPQ S&T CPHST Mission Laboratory, Edinburg, TX; [2]University of Texas Rio Grande Valley, Edinburg, TX; [3]USDA ARS Northern Plains Agricultural Research Laboratory, Sidney, MT

Abstract: Fungal pathogens can serve as a management tool to help reduce Asian citrus psyllid (ACP) populations in urban environments of citrus growing areas of the Lower Rio Grande Valley (LRGV) of Texas. Methods were developed to determine the relative infectivity/virulence (= efficacy) of selected insect pathogenic fungi for ACP using spray exposure bioassays. Methods were also developed to collect, process, and screen indigenous fungal strains that naturally attack the ACP in the LRGV. Protocols developed assess the primary and secondary acquisition of fungal pathogens by adult ACP. Field protocols developed, thus far, yielded two isolates. Outcomes of this work are to enhance IPM strategies for the management of ACP populations in the LRGV.

Non-Technical Summary: Fungal pathogens were studied by screening domestic and international mycoinsecticides that are commercially available yet have never been tested against the ACP. Assessments of native fungal pathogens found on ACP populations of the LRGV were also conducted.

IRCHLB-P7-77

Biological control of Asian citrus psyllid: Conservation and augmentation for enhanced vector suppression

Jawwad A. QURESHI[1,2] and Philip A. STANSLY[1]

[1]University of Florida, Southwest Florida Research and Education Center, Immokalee, Florida, USA
[2]University of Florida, Indian River Research and Education Center, Fort Pierce, Florida, USA

Abstract: Huanglongbing (HLB) or citrus greening disease associated with the phloem-restricted bacteria *Candidatus* Liberibacter asiaticus (CLas), is transmitted by the Asian citrus psyllid (ACP) *Diaphorina citri*. Control of the ACP is critical for area-wide management of this vector-disease complex and sustainable

citrus production. Insecticidal control is most effective when ACP adults are few and other life stages largely absent due to lack of foliar flush, but repeated sprays of conventional insecticides are increasingly common. Biological control is an important component of ACP management and needs conservation and augmentation. Several naturally occurring predators in Florida and introduced parasitoid *Tamarixia radiata* attack ACP. From tracking 17 generations of ACP under untreated field environment, we observed more than 90% mortality in ACP populations mainly from ladybeetles and lacewings indicating significant role of biological control when present. However, foliar sprays of insecticides significantly reduce the populations of these and other species. In large-scale field studies, we showed that limiting the use of broad-spectrum insecticides such as organophosphates and pyrethroids to dormant winter months provides significant suppression in ACP populations lasting into growing season thus effective for integrated management of ACP. Lacewings, spiders, and ladybeetles were common during the growing season in blocks treated or not with broad- spectrum insecticide during dormant winter months. This suggest that reduced use of insecticides during growing season help conserve populations of beneficial insects whereas their use during dormant winter months is less damaging because most beneficial insects leave groves as most mature trees go through dormancy not producing the new growth thus limiting psyllid reproduction and ultimately prey for the natural enemies. We also observed that augmentation of *T. radiata* in commercial groves provide additional control of psyllid where conventional and especially organic insecticides are used. Studies have also shown good parasitism levels in urban environments.

Non-Technical summary: Biological control contribute to the reduction in psyllid populations both in the commercial and non-commercial environment. Sole reliance on anyone tactic is not enough against ACP-HLB complex. Chemical control also provides significant reductions in ACP populations and with negative effects on biological control and environment. Conservation and augmentation biological control helps with ACP-HLB suppression in all habitats thus useful for area-wide management of this vector disease complex.

Citations:

Qureshi, J. A., and P. A. Stansly. 2009. Exclusion techniques reveal significant biotic mortality suffered by Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) populations in Florida citrus. Biological Control. 50: 129-136.

Qureshi, J. A. and P. A. Stansly. 2010. Dormant season foliar sprays of broad pectrum insecticides: An effective component of integrated management for *Diaphorina citri* (Hemiptera: Psyllidae) in citrus orchards. Crop Protection. 29: 860-866.

Qureshi, J. **A.,** and P. A. Stansly. 2017. Compatibility of Organic and Conventional Insecticides to *Tamarixia radiata*. Citrograph. 8 (3): 66-70.

IRCHLB-P7-78

Biological Control of the Asian Citrus Psyllid, *Diaphorina citri*, in the Lower Rio Grande Valley of Texas Using the Ectoparasitoid, *Tamarixia radiata*

Daniel FLORES[1], Rupert SANTOS[1], Mayra REYES[1], Christopher VITEK[2], Matt CIOMPERLIK[1]

[1]USDA APHIS PPQ S&T CPHST Mission Laboratory, Edinburg, TX. [2]University of Texas Rio Grande Valley, Edinburg, TX

Abstract: *Tamarixia radiata* Waterston (Hymenoptera: Eulophidae), is a biological control agent of the Asian citrus psyllid (ACP), *Diaphorina citri* Kuwayama (Hemiptera:

Liviidae), that is being used as a tool to help reduce psyllid populations in urban environments of citrus growing areas in Texas. The USDA APHIS PPQ S&T CPHST Mission Laboratory has developed methods to produce large numbers of the beneficial insects for the biological control of ACP. Releases were made in citrus trees where plant tissue testing positive for HLB has been detected. In 2010, before we began our releases, we were detecting up 43 immature psyllids per flush in residential citrus. Since our biological control releases began, we have seen the populations gradually decline. The latest finds in 2018 indicate we are observing only <4 immature psyllids per flush. This is a reduction of over 90% of the psyllid population.

Non-Technical Summary: *Tamarixia radiata* is a biological control agent of the Asian citrus psyllid that is being used as a tool to help reduce psyllid populations in urban environments of citrus growing areas in Texas. The CPHST Mission Lab has mass produced and released thousands of *Tamarixia radiata* for the biological control of Asian citrus psyllid. A reduction of over 90% of the psyllid population is being presently reported.

IRCHLB-P7-79

Citrus psyllids around the world

Susan E. HALBERT[1] and Namgay OM[2]

[1]Florida Department of Agriculture and Consumer Services, Division of Plant Industry, Gainesville, Florida, USA, [2]National Plant Protection Centre, Department of Agriculture, Ministry of Agriculture and Forests, PO Box 670, Thimphu, Bhutan

Abstract: At least 16 species of psyllids have been reported on citrus around the world, and many others have been reported on related plants in the family Rutaceae. Some of the records do not include immature stages, suggesting that feeding is incidental on citrus. Some of the other species should be re- examined using modern taxonomic methodology, because they might be synonyms of previously described species. So far, the two common citrus-infesting species, *Diaphorina citri* Kuwayama and *Trioza erytreae* (del Guercio), have been shown to transmit *Candidatus* Liberibacter spp. to citrus. *Cacopsylla citrisuga* (Yang & Li) also apparently transmits *Candidatus* Liberibacter asiaticus (Cen et al. 2012). Two other species, *Cacopsylla heterogena* Li and *Diaphorina communis* Mathur, harbor *Candidatus* Liberibacter asiaticus but have not been shown to transmit the pathogens (Om 2017). More study is needed to determine the taxonomic status of *C. citrisuga* and *C. heterogena* (Om 2017). Little-known citrus- feeding psyllids may have a role in moving citrus pathogens to other crops or native plants. Plant trade around the world increases the possibility that some little-known species could become established in new places.

Non-Technical summary: Quite a few species of psyllids are reported to infest citrus around the world. Some of these little-known species have the potential to spread citrus pathogens to citrus, to other crops, or to native plants. Moreover, some little-known species might become established in new places.

Citations:

Cen Y, Gao J, Deng X, Xia Y, Chen, J, Zhang L, Guo J, Gao W, Zhou W, Wang Z, 2012a. A new insect vector of '*Candidatus* Liberibacter asiaticus', *Cacopsylla* (*Psylla*) *citrisuga* (Hemiptera: Psyllidae). Proc Intl Citrus Congress. Valencia, Spain, p. 194.

Om, N. (2017). The roles of psyllids, host plants and environment in the aetiology of huanglongbing in Bhutan. Ph.D. Dissertation. University of Western Sydney.

IRCHLB-P7-80

Comparison of the effectiveness of applying different pesticides to foliar spraying and soil-drench to control *Diaphorina citri* Kuwayama

ZhiChao YAO[1], ZhenYu ZHANG[1], XiaoQin CHENG[1], Zheng ZHAO [1], HongYu ZHANG[1]

1.Institute of Urban and Horticultural Pests, Huazhong Agricultural University, China

Abstract: Pesticides that have low environmental toxicity but are effective against *Diaphorina citri* Kuwayama were evaluated. We provide the theoretical basis for a comprehensive prevention and control program for citrus Huanglongbing (HLB). The toxicity and effectiveness of 12 pesticides were assessed using a laboratory membrane method and by field bioassays. The effectiveness and optimal concentrations of soft pesticides suitable for foliar sprays and soil application were determined. Of the 12 pesticides tested for laboratory efficacy, 48% clothianidin, 45% chlorpyrifos, 10% cyantraniliprole and 20% dinotefuran were the most toxic to adult *D. citri* with LC50s of 1.42, 1.48, 1.49, 1.60 mg/L, respectively. The results of foliar spray tests showed that applying 20% dinotefuran and 10% cyantraniliprole was 90% effective after 7 days with a better overall effect than that of the other 10 pesticides. The results of soil- drench tests indicated 30% thiamethoxam had a cumulative reduction rate of 100% of the population after 12 days, significantly higher than the other four pesticides tested. The residual effectiveness of applying 30% thiamethoxam to the soil at concentrations of 100 mg/L and 150 mg/L reached 60% after 30 days, and 100% after 7 days.

Non-Technical Summary: To prevent and control Asian citrus psyllids efficiently with less toxic pesticides, you should spray the foliage with 20% dinotefuran at 150 mg/ml or 10% cyantranil at 100 mg/mL, or apply 30% thiamethoxam at 100 mg/mL to the soil.

IRCHLB-P7-81

Effect of horticultural mineral oil on the acquisition of Candidatus *Liberibacter asiaticus* by Asian citrus psyllid from Citrus

YANG QIONGYU [1,2], Steven ARTHURS[3], QUAN JINCHENG[4], MAO RUNQIAN[2]

1. South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China; 2. Guangdong Key Laboratory of Animal Conservation and Resource Utilization, Guangdong Public Laboratory of Wild Animal Conservation and Utilization, Guangdong Institute of Applied Biological Resources, Guangzhou 510260, China; 3. Texas A&M University-Kingsville, Weslaco, TX 78596; 4.Guangxi Especial Crops Research Institute, Guilin, Guangxi, 54100,China)

Abstract: Citrus huanglongbing (HLB), previously called greening, is a serious citrus disease in Asia, eastern and southern Africa. It is associated with *Candidatus* Liberibacter asiaticus (CLas), a phloem-limited, transmitted by the Asian citrus psyllid (*Diaphorina citri*) (ACP) in Asia. Horticultural mineral oils (HMO) are commonly used as repellents for management of *D. citri*, however, the effects of HMO on the inhibition of CLas acquisition by ACP is poorly understood. The objective of this study was to quantify the effect of concentration and residual activity of HMO on the inhibition of CLas acquisition, that was measured by infection rates of ACP, which fed on citrus sprayed with either HMO or water as a control. In the greenhouse, CLas-citrus plants were sprayed with 0.1%, 0.5% and 2% HMO, then 25 ACP were transferred to each concentration treatment, and the CLas infection rates of ACP were tested with qPCR at 7 days after feeding. CLas-citrus were sprayed HMO with 0.5%, then ACP were transferred to CLas-citrus at 0, 4 and 7days after treatment (DAT), the infection rates of ACP were measured as above. The results indicated that HMO effectively decreased CLas acquisition by 85% after application of 0.5%, with no phytotoxicity. While the inhibition effect decreased with time from application, the residual activity of HMO \reduced HLB

infection rates by more than 58% for up to 4 DAT following an initial reduction of approximately 87%. Based on these results, this provides a strong evidence for the prevention HLB by horticultural mineral oil.

Non-Technical Summary: In the greenhouse, CLas-citrus plants were sprayed with varying concentrations of horticultural mineral oils, then ACP were transferred to each concentration treatment, and the CLas infection rates of ACP were tested with qPCR at 7 days after feeding. CLas-citrus were also sprayed with 0.5% of horticultural mineral oil, then ACP were transferred to CLas-citrus at 0, 4 and 7 days after treatment and the infection rates of ACP were measured. The results indicated that HMO effectively decreased CLas acquisition by 85% after application of 0.5%, with no phytotoxicity. Based on these results, this provides a strong evidence for the prevention HLB by horticultural mineral oil.

IRCHLB-P7-82

Entomopathogenic fungus *Isaria fumosorosea*, deploy an array of effector proteins during infection of *Candidatus* Liberibacter asiaticus infected-*Diaphorina citri.*

PITINO, Marco[1], Qiaolin ZHENG[1], Valentina CANDIAN[2], Qingchung SHI[3], Chang LIU[1], Surya SAHA[4], Prashant S. HOSMANI[4], David G. HALL[3], Rosemarie TEDESCHI[2], Pasco B. AVERY[5], Ronald D. CAVE[5], Ed STOVER[3], Robert G. SHATTERS[3], Liliana M. CANO[1]

[1]Department of Plant Pathology, University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, Fort Pierce, Florida. [2]University of Torino, Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA). [3]United States Department of Agriculture Agricultural Research Service, Fort Pierce, Florida. [4]Boyce Thompson Institute for Plant Research, Ithaca, New York. [5]University of Florida, UF-IFAS, Department of Entomology and Nematology, Indian River Research and Education Center IRREC, Fort Pierce, Florida.

Abstract: The Asian citrus psyllid (ACP), *Diaphorina citri*, is a critical insect pest that affects citrus production as the vector of the Huanglongbing (HLB) associated pathogen, "*Candidatus* Liberibacter asiaticus (CLas)." HLB is one of the most destructive diseases of citrus, with high economic costs to the worldwide citrus industry, and currently there is no cure for the disease. ACPs insert their stylets into the phloem of citrus plants to feed, thus transmitting the pathogen. HLB disease management tactics include intensive applications of insecticides to control ACP. To control arthropod pests, one of the most promising alternatives to pesticides is biological control using entomopathogenic fungi. These fungi evade host immunity by secreting effector proteins that counteract host defenses such as chitin-binding proteases, cell wall degrading enzymes and other small- secreted proteins that contribute to fungal virulence. The entomopathogenic fungus *Isaria fumosorosea* was assayed for efficacy against ACP causing 88% mortality after 6 days, a parallel experiment for RNAseq was conducted in tandem with the mortality test. Using a combination of bioinformatic tools, we analyzed the secretome of *I. fumosorosea* to identify candidate effectors that are secreted during early ACP infection and compared their expression levels in a time course analysis for 5 days. We also cross analyzed levels of effector proteins produced during both spore germination and mycelium growth on *I. fumosorosea* and on the artificial medium respectively.

Non-Technical Summary: Biological control of arthropod pests using entomopathogenic fungi is compatible with beneficial insects and more environmentally friendly, offering an alternative to broad- spectrum pesticides. The fungus *Isaria fumosorosea* attacks many insects and has been demonstrated to infect the Asian citrus psyllid (ACP) which vectors the huanglongbing pathogen in citrus. Using bioinformatics tools, we analyzed the genome *I. fumosorosea* to identify genes that are expressed during infection of ACP and contrasted expression levels during growth on ACP versus artificial medium.

IRCHLB-P7-83

Frequency of processed kaolin applications to prevent *Diaphorina citri* infestation in flushing citrus trees

Arthur F TOMASETO[1]; Odimar Z ZANARDI[1]; Haroldo XL VOLPE[1]; Luciano BACHMANN[2] and Marcelo P MIRANDA[1]

[1]Fund for Citrus Protection, Araraquara, SP, Brazil, [2]Faculty of Philosophy, Sciences and Literature of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil

Abstract: Processed kaolin interferes negatively in different aspects of the host plant finding ability of *Diaphorina citri*. Recent studies have demonstrated that *Candidatus* Liberibacter asiaticus transmission rates by *D. citri* adults were higher in the flushing than in the non-flushing citrus plants. Thus, an effective protection of young shoots from psyllids is important to mitigate the risk of Huanglongbing spread. However, the frequency of kaolin application needed to prevent psyllid infestations in flushing citrus trees is unknown. In this study, two frequencies of Kaolin 2% (Surround WP®; 99.4% active ingredient; Tessenderlo Kerley Inc.) application were tested under field conditions. *D. citri* settlement and dispersal were assessed after weekly releases of marked psyllids in a flushing citrus orchard. Additionally, spectral reflectance assessments were performed on leaf samples. In general, processed kaolin at 2% reduced *D. citri* infestation by 88% until 21 days after first psyllid release regardless the frequency of kaolin application. Leaf spectral assessments indicated higher light reflectance of kaolin-treated leaves in comparison to control, reinforcing the kaolin repellency role on *D. citri* host plant finding ability. Results suggest that a 14-day frequency of kaolin application is sufficient to prevent *D. citri* infestation on citrus orchards during winter (low amounts of rainfall). Nevertheless, processed kaolin should be sprayed at a 7-day frequency at conditions of frequent rainfall (spring).

Non-Technical summary: Processed kaolin is a product that when sprayed on citrus trees forms a white cover that camouflages the plant from Asian citrus psyllid. Here, we studied the frequency of kaolin application to prevent psyllid infestation in a flushing citrus orchard. Results indicated that applications at intervals of 7 to 14 days were enough to reduce psyllid infestation during rainfall and dry season respectively. We also observed that citrus leaves treated with kaolin reflect the light more than untreated leaves. These findings may explain the psyllid "blindness" to find kaolin-treated citrus trees.

IRCHLB-P7-84

Implementing a resistance monitoring program for ACP in California citrus

Frank BYRNE

Department of Entomology, University of California, Riverside

Abstract: All segments of the California citrus industry (commercial production, nursery, residential) have been impacted by the introduction of the Asian citrus psyllid into California. With the current number of residential trees infected with the citrus greening pathogen now approaching one thousand, insecticides are playing an increasingly important role in the management of the pest/pathogen complex. Recently, the citrus industry implemented an area-wide management program with the establishment of psyllid management areas (PMAs). Specific treatment protocols have been designed for the different citrus-growing regions within California, and the goal of this study was to determine whether insecticides used within that program were being impacted by resistance. Neonicotinoids and pyrethroids are two of the most important insecticide classes being used within the area-wide treatment program. As with any system that relies on the use of insecticides, there is a risk that insects will develop resistance. Regular monitoring of populations can safeguard against this development. Thus, a resistance-monitoring program for California citrus was conducted based around the framework of the PMA system. Routine monitoring will enable program managers to detect shifts in tolerance to chemicals and, if such changes are detected, apply remedial measures that include alternating the insecticide with a different class. With the assistance of ACP scouts, grower liaisons, pest control advisors (PCAs), and growers, we sampled insects from multiple PMAs in Riverside, San Bernardino and Ventura counties, and tested their responses to imidacloprid and fenpropathrin in topical application bioassays. All field populations tested during the 2018 monitoring effort showed similar levels of susceptibility to the reference susceptible strain that is maintained in the UCR insectary.

Non-technical summary: Resistance to insecticides can seriously hamper ACP control efforts, increasing the risk of HLB spread in commercial citrus. In our first year of resistance monitoring in field populations of ACP, all populations that were tested were susceptible to the insecticides that growers rely on for successful ACP management within the area-wide treatment program. The success of the monitoring effort lends itself to the use of a robust bioassay method, as well as excellent cooperation between researchers and field personnel, including growers, ACP scouts, PCAs and grower liaisons. The data are encouraging and indicate that there is no evidence of insecticide resistance.

IRCHLB-P7-85

Integrated management of Asian citrus psyllid, vector of the associated pathogens of HLB

Jawwad A. QURESHI[1,2] and Philip A. STANSLY[1]
[1]University of Florida, Southwest Florida Research and Education Center, Immokalee, Florida, USA
[2]University of Florida, Indian River Research and Education Center, Fort Pierce, Florida, USA

Abstract: The Asian citrus psyllid (ACP), *Diaphorina citri* (Hemiptera: Liviidae), vector pathogens of huanglongbing (HLB) or citrus greening disease. Currently, there is no cure for HLB and management of ACP focuses on conventional practices. Nevertheless, HLB continues to spread. Limited effectiveness of conventional chemical control, negative effects on biological control and environment, secondary pest outbreaks, increased production costs and ACP resistance warrant integrative and sustainable strategies for psyllid control. We conducted a four-year study to investigate the potential of organic and conventional insecticide spray programs to manage ACP and their impact on its natural enemies. Experiments were conducted in two mature blocks of oranges 1) 15-acre block of ‘Valencia’ and 2) 10-acre block of ‘Hamlin’. Three organic programs, one conventional program and an untreated control were replicated four times. The program using conventional insecticides provided the most reduction in ACP followed by programs using organic insecticides plus horticultural mineral oil (435 oil) or insecticidal soap (M-pede). Parasitoid *T. radiata* released in all programs was recovered more from untreated control and organic programs than conventional program. Consistent mass releases of this parasitoid may

provide useful additional control where conventional and especially organic insecticides are used to control ACP. Predators such as lacewings, spiders and ladybeetles were observed in all programs that also may have contributed to ACP reduction. Yields were not different between program using organic insecticides with 435 oil or program using conventional insecticides. Further understanding of ACP-HLB management is being developed through programs integrating biological control, organic insecticides and conventional insecticides.

Non-Technical summary: Integrated pest management will be useful toward developing sustainable and area-wide management of ACP-HLB complex. Through integrated programs grower will be able to conserve biological control and currently effective mode of action insecticides and control ACP in all habitats. Reduced use of conventional insecticides will help with conservation and augmentation biological control, reduced secondary pest outbreaks, resistance management and better environment. Biological control and organic insecticides will also help with vector management in organic production systems and urban areas where us of conventional insecticides is not allowed or common.

Citations:

Qureshi, J. A., and P. A. Stansly. 2009. Exclusion techniques reveal significant biotic mortality suffered by Asian citrus psyllid *Diaphorina citri* (Hemiptera: Psyllidae) populations in Florida citrus. Biological Control. 50: 129-136.

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.

Qureshi, J. A., and P. A. Stansly. 2016. Development of an Asian citrus psyllid management plan for organic citrus. Citrograph. 7 (2): 52-58.

IRCHLB-P7-86

Regional Successes in Managing Asian Citrus Psyllid in Southern California

Elizabeth GRAFTON-CARDWELL[*1], Sandra ZWAAL[2], Joanne O'SULLIVAN[1], Tobias MOYNEUR[1], Geoff HOLLENBECK[1] and Kevin GONZALEZ[1]

[1]Dept of Entomology, University of California, Riverside CA 92521; [2]Consultant, Orange Co. CA

Abstract: The goal of this project is to determine the impact of grower pesticide treatments on population densities of Asian citrus psyllid in four regions of southern California (Ventura, Riverside/San Bernardino, Temecula/San Diego and Coachella/Imperial). Data on the number of psyllid nymphs and adults and the levels of leaf flush are collected by four scouts from >170 grower orchards every two weeks. Periodically, samples of nymphs are collected into alcohol for testing for HLB. Data show that growers in the hot dry climates of the desert Valleys are able to suppress psyllids below detectable levels most of the year. In contrast, in Ventura, San Bernardino and the city area of Riverside, frequent flushing of trees creates an environment in which it is more difficult to control ACP with insecticides. In these regions, populations of psyllids reach very high numbers in the fall and a greater number of treatments and longer residual insecticides are needed. Recommendations for improving management of psyllids to protect against HLB incursion are provided. This research is supported by USDA Multi-Agency Coordinated Funds.

Non-technical summary: Monitoring Asian citrus psyllid populations in 170 commercial citrus groves in southern California reveals which insecticides suppress populations most effectively. These surveys also demonstrate that desert conditions suppress psyllids and the cooler coastal and inland areas support very high populations. This information is used to develop management programs tailored for the various regions of California.

IRCHLB-P7-87

Surveys for Asian citrus psyllid (*Diaphorina citri*)-infecting viruses and their influence on *Candidatus* Liberibacter asiaticus (*C*Las) dispersal in Florida citrus groves

Kellee BRITT[1], Samantha GEBBEN[1], Amit LEVY[2], and Ozgur BATUMAN[1]

[1]*Southwest Florida Research and Education Center, University of Florida, Immokalee, FL, USA;* [2]*Citrus Research and Education Center, University of Florida, Lake Alfred, FL, USA*

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is the bacteria associated with the world's worst disease in citrus, known as Huanglongbing (HLB), and continues to decimate the Florida citrus industry and the USA. This pathogen is vectored by the Asian citrus psyllid (*Diaphorina citri*; ACP), and collectively demonstrates the ability to overcome all current forms of HLB disease control. Challenges with isolating CLas from the ACP or citrus host has steered research efforts toward analyzing its conspicuous and intricate dependence on the insect. Our primary goal of this study aims to unravel the virome of the ACP and how we can utilize such information to control the dispersal of CLas by the ACP. This project identified and monitored the prevalence of several ACP-infecting viruses throughout Florida citrus groves: the *Diaphorina citri-associated C virus* (DcACV), *D. citri flavi*-like *virus* (DcFLV), *D. citri densovirus* (DcDNV), *D. citri reovirus* (DcRV), *D. citri picorna*-like virus (DcPLV) and *D. citri bunyavirus* (DcBV). Every month over an ongoing span of two years, ACP populations were sampled from 20 different citrus producing counties in Florida. Total nucleic acids were extracted from ACP samples and polymerase chain reactions (PCRs) and reverse-transcriptase PCRs (RT-PCRs) were optimized for detection of these DNA and RNA viruses, respectively. Quantitative PCR (qPCR) was also used to track CLas titers in these ACP samples and to observe any temporal interactions between CLas populations and viral prevalence. Preliminary results have shown that the DcACV is the most prevalent virus in Florida ACP populations each month (>60%), with varying incidences throughout the year. Using this data, scientists may be able to enhance the specificity and deployment of future biotechnological control strategies of the ACP and HLB in Florida citrus groves.

Non-technical summary: In order to understand and manipulate the relationship between the psyllid vector and the citrus bacterial pathogen associated with HLB, we are trying to track the prevalence of psyllid-infecting viruses throughout Florida citrus groves. We are also trying to see how these viruses influence the bacterial populations in the psyllid and whether it could aid in constructing more specific biotechnological controls of HLB in Florida.

IRCHLB-P7-88

The Greening Management Program at Cambuhy

Cláudia FERRACINI[1], Alexandre TACHIBANA[1], Ivan BRANDIMARTE[1]

[1]Cambuhy Agricultural, São Paulo, BRAZIL

Abstract: Greening (HLB) is a major citrus disease in São Paulo State (SPS) Brazil, therefore internal and external management is essential to control the rates of infection and disease spread. Cambuhy Agricultural, located in the center of SPS has a total area of 14,123 hectares, which the main crops are orange and sugarcane. Cambuhy is the model for management of Greening in SPS., soon after the first detection of the disease in 2004, internal

management was started with inspections and eradications of symptomatic plants, vector monitoring and insecticide applications. However, internal management was not enough to slow the rate of disease incidence. In 2011, after the studies carried out by Fundecitrus, showing the importance of regional management outside of the property, a large effort was initiated that focused on the neighbors. The program began with the identification of the properties with citrus up to a radius of 4 km from the border of Cambuhy farm. Then, frequency of insecticide sprays inside the farm was adjusted according to the neighbors' proximity. The external measures adopted were: i) elimination of Citrus and Murraya plants in exchange for other non-citrus fruit plants; (ii) monthly spraying in commercial orchards and backyards; (iii) eradication of abandoned orchards; iv) aerial spraying. As a result of this effort, annual disease incidence has been reduced from 3.9% in the 2010/2011 crop to 1.19% in the 2017/2018 harvest. Consequently, fruit drop due to Greening is practically 0%, while the level for state of São Paulo is 4.06%. Finally, due to excellent prevention of primary infection of trees by maintenance of low inoculum levels in proximity to the farm, average production has risen to around 1,200 boxes per hectare.

Non-Technical Summary: Greening has no cure and in the absence of aggressive control measures for inoculum in the form of infected trees and the psyllid vector has caused major loss in citrus production in the state of São Paulo. The psyllid responsible for the transmission of this disease can travel long distances, for this reason the insect vector and infected plants outside the farm is essential for inoculum control.

IRCHLB-08:
Antimicrobials Oral Presentations

IRCHLB-O4b-01

Needle-Assisted Trunk Infusion (NATI): An alternative method for delivering therapeutic materials into the citrus vasculature

Ozgur BATUMAN[1], Kellee BRITT[1], Ana REDONDO[1], Shahzrad BODAGHI[1], Samantha GEBBEN, Bo MEYERING[1], Fernando ALFEREZ[1], Ute ALBRECHT[1], and Yiannis AMPATZIDIS[1]

[1]Southwest Florida Research and Education Center, University of Florida, Immokalee, FL, USA

Abstract: With the devastating impacts of HLB on the citrus industry and no “silver bullet” in sight, growers are seeking alternative solutions, such as bactericides, to reduce CLas titer levels in trees and prevent disease-induced decline. However, limited success in delivery of bactericides through the leaves has rendered these strategies ineffective. Thus, there is a critical need to develop immediate and sustainable alternative measures for controlling this disease and its vector. We developed a new delivery method as an alternative to foliar application and trunk injection that are less efficient or potentially harmful to the tree. This method involves the infusion of therapeutic materials through numerous punctures produced with small needles on the tree trunk. We used dyes to track the success of this method in two-year old greenhouse-grown citrus trees and monitored transport and distribution throughout the tree by comparing with other delivery methods. Among the methods compared, our novel method, Needle-Assisted Trunk Infusion (NATI), was superior in delivering the dye to leaves and roots compared with delivery by foliar spray, soil drenching, and trunk injection. The occurrence of dye in the roots only a few hours after NATI application demonstrated that materials, although initially delivered into and transported along the transpiration stream in the xylem, moved into the phloem where they would come in direct contact with CLas. Plants did not show any sign of stress following delivery by NATI, and openings at the trunk infusion site were completely healed. In contrast to trunk

injection, no phytotoxicity was observed on plants treated with NATI. Together, our results indicate that NATI is a viable method for delivery of liquid materials with therapeutic properties into the citrus vasculature without causing a detrimental effect to plants. The automatization of NATI for large-scale treatment of HLB-affected trees is currently being explored.

Non-Technical Summary: We have developed a novel method to deliver liquid materials into the citrus vascular system through small openings on the tree trunk. The novel method, Needle-Assisted Trunk Infusion (NATI), delivered liquid materials more effectively and without any adverse effects on plant growth and health compared with single (large)-needle trunk injection, root drenching, or foliar spray. If NATI is automated, it may be used for delivering not only bactericides to control HLB, but also other pesticides into citrus for combating a wide range of pests and diseases.

IRCHLB-O4b-03

Optimizing uptake and performance of bactericides in citrus

J. Kent MORGAN[2], Randall P. NIEDZ[1], Kimberly AT. WOOD[2], Mark TRIMMER[2], Taw RICHARDSON[2], **Robert G. SHATTERS, Jr.[1]**

[1]US Department of Agriculture, Agricultural Research Service, US Horticultural Research Laboratory, Fort Pierce, FL, USA
[2]AgroSource, Inc., Tequesta, FL, USA

Abstract: The bacteria associated with Huanglongbing, *Candidatus* Liberibacter asiaticus (CLas), resides in the phloem of the tree, which could reduce effectiveness of surface treatment applications. Specific adjuvants are known to facilitate the penetration of the active ingredients of FireLine™ 17 WP (oxytetracycline hydrochloride) and FireWall™ 50 WP (streptomycin sulfate) into the phloem and subsequent circulation throughout the tree. Initial recommendations for applications of these bactericides were as a stand-alone treatment using these specific adjuvants. However it is not always economical for growers to apply bactericides as a stand-alone treatment, so common practice is to apply bactericides with other tank mix products, e.g. insecticides, fertilizers/ nutritionals, etc. Preliminary evidence indicates different tank mix components applied to citrus trees simultaneously with FireLine™ 17 WP and FireWall™ 50 WP can facilitate or inhibit uptake of oxytetracycline and streptomycin, respectively. Certain fertilizers and nutritionals were found to facilitate uptake, while other components were found to be antagonistic to uptake. A resolution V fractionated factorial experimental design was used to determine the individual and interaction effects of eight tank mix components on the uptake of oxytetracycline and streptomycin. This experiment will determine which tank components result in the largest uptake of oxytetracycline and streptomycin; this information should improve their effectiveness against CLas.

Non-Technical Summary: Bactericides are only effective against HLB when they are inside the tree, where the causal agent of HLB resides. This can be accomplished by the use of specific adjuvants, however to save money, time and labor, growers usually apply many products at the same time. This research investigates how various products affect the uptake of the active ingredients of FireLine™ 17 WP and FireWall™ 50 WP by citrus trees.

IRCHLB-O4b-04

Zinkicide^{TM}: how effective are zinc oxide nanoparticles for HLB control?

Mayara M. MURATA[1], Swadeshmukul SANTRA[2] and **Evan G. JOHNSON[1]**

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, Florida, United States

[2]University of Central Florida, Orlando, Florida, United States

Abstract: The tremendous economic losses in citrus caused by Huanglongbing (HLB) in recent years requires the development of affordable and sustainable methods for HLB management. Just a few bactericides are currently available, but their efficacy is limited. We aim to test the antimicrobial activity of a nano-zinc oxide material, Zinkicide™, against *Candidatus* Liberibacter asiaticus (CLas). This product is a plant nutrient-based nanoparticle with bactericidal activity that translocates systemically after spray or soil drench application. HLB data from citrus canker and HLB field trials suggested efficacy correlated with application interval rather than number of sprays, indicating a need for optimized rate and application schedule. Phytotoxicity of weekly Zinkicide application on Sour orange seedlings guided rate and timing studies. Acute toxicity was observed at Zinkicide™ rates above 3,000 ppm and cumulative toxicity was observed at low Zinkicide™ rates (400 ppm) after 4 months of weekly drench applications. Following acute phytotoxicity results, five-year old graft-inoculated CLas- positive (for 3 years) 'Hamlin' sweet orange plants in the first experiment or 2 year old 'Pineapple' sweet orange in the second experiment were treated with Zinkicide™ at 1,600 ppm every 7, 14, and 28 days under greenhouse conditions. Efficacy against CLas was tested using DNA and RNA in leaves for titer and activity. Zinkicide™ spray and drench applied biweekly significantly reduced CLas DNA titer in plants treated for 20 weeks. CLas RNA activity decreased in new flush treated weekly or biweekly after 5 weeks of treatment in the first experiment, but was correlated to DNA in new flush in the second experiment. After 24 weeks of treatment, Hamlin plants treated weekly at 1,600 ppm showed phytotoxicity symptoms and treatment was stopped. Field testing of pulses of clustered applications need to be done to improve field efficacy of Zinkicide for HLB.

Non-Technical Summary: A couple of years ago, a group of chemists and plant pathologists from Florida came together to look for practical solution to control Huanglongbing (HLB). This led to the design and synthesis of a new bactericide called Zinkicide™. After good results observed in Zinkicide™ field trials with citrus canker, these are the first results of Zinkicide™ efficacy against HLB. Greenhouse studies are important to determine the best concentration and frequency application of new products in the field. Our results showed that frequent application of Zinkicide™ at 1,600 ppm successfully reduced bacterial population in HLB-infected plants but overtime it caused phytotoxicity. Therefore, field application should be tested every 14 days in seasonal pulses for better efficacy and to avoid phytotoxicity.

IRCHLB-O4b-05

ACCEL ELISA to Monitor Oxytetracycline Hydrochloride and Streptomycin Sulfate Movement in Citrus Trees.

JeongSoon KIM[1,3], J. Kent MORGAN[1], Mark TRIMMER[1], Taw RICHARDSON[1], Teri J. SLACK[3], Robert G. SHATTERS, Jr.[2]

[1]Agrosource, Inc., Tequesta, FL; [2]U.S. Department of Agriculture, Agricultural Research Service, U. S. Horticultural Research Laboratory, Fort Pierce, USA; [3]PlexenseBio, Inc., Davis, CA.

Abstract: Citrus Huanglongbing (HLB) or 'greening' is a disease of citrus trees associated with the phloem-residing bacteria '*Candidatus*

Liberibacter asiaticus' (CLas). To combat CLas and HLB's effects, AgroSource bactericides FireLine™ 17 WP (active ingredient oxytetracycline hydrochloride) and FireWall™ 50 WP (active ingredient streptomycin sulfate) have been approved for use in Florida and California under EPA issued Section 18 emergency exemptions. As CLas resides within the citrus tree, research was performed by AgroSource together with the USDA-ARS-USHRL in Fort Pierce, Florida, to identify specific adjuvants and/or nutritionals to aid the movement of each of the bactericide's active ingredients (AIs) from the leaf surface, into, and then throughout the citrus tree. To track the effectiveness of uptake and distribution of the AIs in citrus, AI specific detection methods were needed. Initial methods were developed for HPLC-MS/MS and bacterial growth inhibition bio-assays to detect both AIs in citrus; however, both methods have limitations. HPLC-MS/MS is highly sensitive, but time requirements for extraction and purification, along with associated expenses and technical requirements, make it a highly specialized tool to be used judiciously. Bio-assays, though easy to perform and provide a 'yes/no' level of detection, lack sensitivity needed to accurately quantify AI levels. To bridge the gap between these methods, and in response to grower requests for alternate methods, we developed methods using the competitive ACCEL ELISA system from PlexenseBio, Inc. to detect oxytetracycline and streptomycin in citrus trees. The ACCEL ELISA methods for oxytetracycline and streptomycin are rapid (45-minutes), sensitive (limits of detection for oxytetracycline: 2-50 ppb and for streptomycin: 0.2-12.5 ppb) and do not require enrichment protocols. In addition, the methods allow for large numbers of samples to be processed and tested. These methods can be used to help citrus growers and researchers guide decisions regarding HLB management with these quick and cost-effective methods.

Non-Technical Summary:

The ACCEL ELISA platform was used to develop methods for detection of oxytetracycline and streptomycin sulfate in extracts of citrus leaves, roots and fruit. This method is highly sensitive, rapid, and cost- effective for growers and researchers to monitor uptake and distribution of oxytetracycline and streptomycin in HLB infected citrus trees. This method is readily adaptable for both highly sensitive laboratory analyses and for in-field rapid analysis.

IRCHLB-O4b-06

High throughput sequencing to determine levels of spontaneous streptomycin resistance in agricultural soils

Joseph R. PETRONE[1], Alam MUNOZ-BERISTAIN[1], and Eric W. TRIPLETT[1]

[1]University of Florida, IFAS, Microbiology and Cell Science Department, Gainesville, USA

Abstract: Annually since 2016, 173,000 hectares of citrus groves in Florida are being treated with oxytetracycline and/or streptomycin for the control of citrus greening disease. The effect of these antibiotic treatments on selection for spontaneous resistance in non-target soil bacteria is unknown. To rapidly assess changes in streptomycin resistance and composition of soil communities in citrus groves, a high throughput, barcoded sequencing approach was developed for the conserved rpsL gene, which encodes for an essential protein in the small subunit of the ribosome. Single base, missense mutations in this gene are known to confer spontaneous streptomycin resistance in many bacterial species. Primers were designed to amplify a 380bp region of the rpsL gene containing the sites known to confer spontaneous streptomycin resistance. Given that rpsL is not as highly conserved as the 16S rRNA gene, a set of primers is required for each bacterial phylum or sub-phylum. DNA from 20 citrus grove soil samples was isolated and amplified using the primer set targeted for Alphaproteobacteria. A

protein database containing rpsL protein sequences in NCBI was constructed to translate and classify the sequences. Over 16.5 million reads were obtained from the soil. Single base mutations in codons 88 and 43 were identified in 13.84% and 0.31% of reads, respectively. These mutations are known to confer streptomycin resistance. These samples were taken from groves that had not yet been sprayed with streptomycin, giving us a baseline level of around 14% rpsL streptomycin resistance mutations prior to selection pressure being applied. These results show that streptomycin resistance conferred by rpsL can be relatively quantified and taxonomically classified, making it easier to assess the effects of streptomycin applications on non-target bacteria.

Non-technical summary: Growers have been spraying citrus crops with streptomycin and/or oxytetracycline and there is not much known on the effects of this on the non-target soil microbes. We have developed a method to detect, taxonomically classify, and quantify the levels of spontaneous streptomycin resistance by high throughput rpsL sequencing.

IRCHLB-08:
Antimicrobials Poster Presentations

IRCHLB-P8-89

An *in vitro* protocol for rapidly assessing the effects of antimicrobial compounds on *Candidatus* Liberibacter asiaticus.

Joseph KRYSTEL[1], Qingchun SHI[1], Jefferson SHAW[1], Goutam GUPTA[2], David HALL[1], and Ed STOVER[1]

[1]Subtropical Insect and Horticulture Research Unit, US Horticultural Research Laboratory, 2001 S. Rock Rd, Ft. Pierce, Fl 34945, USA. [2]New Mexico Consortium, 100 Entrada Dr, Los Alamos, NM, USA

Abstract: Huanglongbing (Greening disease, HLB) has emerged as the greatest threat to citrus production of this century. Commercial *Citrus* scion cultivars appear to lack meaningful resistance to the disease. No effective treatment for HLB has been found to date. Once infected, the trees decline in productivity and in many cultivars, fruit quality is compromised. The pathogen associated with HLB, *Candidatus* Liberibacter asiaticus (CLas), cannot be cultured; severely limiting research options. We have developed and optimized an *in vitro* protocol for testing CLas-killing therapeutic agents without the need for a pure culture. The assay recovers intact bacterial cells from CLas-infected *Diaphorina citri* (Asian citrus psyllid, ACP), the insect host and vector for CLas. ACP are homogenized in a buffered saline solution and the cells recovered through spin filtration. Cell membrane integrity is measured with propidium monoazide (PMA) and qPCR analysis. The conditions needed for PMA to distinguish non-intact CLas cells were determined and confirmed using multiple bactericidal treatments. The non- ionic detergent Triton-X 100 at 0.1% v/v was used as a positive control for cell lysis. The mature assay was conducted on a panel of anti-microbial peptides and their effectiveness in disrupting CLas cells was determined. This assay allowed for rapid assessment of CLas- killing therapeutics, serving as a substitute for culture based assays. It provides greater confidence when investing the cost and time for testing treatments using *in planta* experiments.

Non-technical summary: Research into Huanglongbing (Greening) disease in citrus is severely limited due to the unculturable nature of the causal pathogen, *Candidatus* Liberibacter asiaticus (CLas). We have developed an in vitro assay to test CLas- killing therapeutic agents providing a substitute for culture based assays. This assay uses CLas cells in a homogenate of the insect vector, *Diaphorina citri* (Asian citrus psyllid, ACP), and represents a significant savings in cost and time when compared to other available methods. Using this assay, we screened a selection of peptides currently under study for effectiveness against CLas and identified the most promising candidates.

IRCHLB-P8-90

Bacteriophage communities associated with citrus roots and rhizosphere

Jennie FAGEN[1], Kevin LINCE[2], Janetta HARKOVIRTA[1], Chase BEISEL[1,3], Evan BRASWELL[2]

[1]North Carolina State University, Raleigh NC, USA [2]U.S. Department of Agriculture, Edinburg TX, USA [3]Hemholtz Institute for RNA-based Infection Research, Wurzburg, Germany

Abstract: The associated agent of citrus greening, *Candidatus* Liberibacter asiaticus, is phylogenetically related to *Rhizobiacea* species that are commonly found in soil and often associate closely with plant roots as pathogens or nitrogen-fixing commensals. These soil microbes also support a reservoir of bacteriophage that are well positioned for uptake and subsequent transport through plant vascular tissues, xylem and phloem. Bacteriophages are common facilitators of horizontal gene transfer and have the ability to impact evolution of the otherwise isolated Liberibacter pathogens *in planta*. Conversely, these same phage represent a potential source of bio-control of Liberibacter asiaticus owing to their ability to infect and lyse closely- related bacteria. This study aims to identify phage both as sources of new genetic material to phloem/xylem limited citrus pathogens as well as naturally-occurring phage with the potential to infect Liberibacter species. Our group probed the phage within these niches using both data-mining and primary metagenomic sequencing. The primary free phage identified in this work were members of the *Microviridae* family of ssDNA phages. Such ssDNA phage is a promising engineering platform for development of both investigative and therapeutic bio-techniques within Citrus.

Non-technical Summary: This work characterizes the viruses of bacteria that are present in and around citrus tree roots. The primary goal of this investigation was to find potential sources of new genetic material for Liberibacter as well as the use of these viruses as a biocontrol measure for citrus greening.

IRCHLB-P8-91

Demonstrating Topical Delivery of Antimicrobial Products as a Means of Controlling Citrus Greening.

Robert G., SHATTERS[1], Jr., Marco PITINO[2], Lilian CANO[2], Kasie STURGEON[1], J. Kent MORGAN[3], Mark TRIMMER[3], Taw RICHARDSON[3]

[1]US Department of Agriculture, Agricultural Research Service, US Horticultural Research Laboratory, Fort Pierce, FL, USA[2]University of Florida, UF-IFAS, Department of Plant Pathology, Indian River Research and Education Center IRREC, Fort Pierce, Florida.[3]AgroSource, Inc., Tequesta, FL, USA

Abstract: Application of bactericide molecules to control citrus greening or Huanglongbing (HLB) is challenging because the associated bacterium, *Candidatus* Liberibacter asiaticus (CLas), resides in the phloem of the tree. Uptake of direct foliar or soil applied bactericides results in inefficient uptake because of biological barriers that have to be crossed for molecules to move from the environment into the citrus vascular tissue. We have designed a research program to develop alternative application methods that will allow more efficient molecule uptake with potential for use in commercial groves. As part of this work, we designed 3-D

printed devices that are applied directly to young trees and facilitate delivery of molecules into vascular tissue of small trees. Systemic movement of applied molecules was demonstrated for: 1) a phloem mobile fluorescent dye (MW 557 Da), 2) a fluorescent protein (green fluorescent protein, GFP MW 27kDa); and, 3) antimicrobial compounds including two antimicrobial peptides as well as both oxytetracycline and streptomycin. Using the fluoresce dye and GFP, each were shown to move systemically when applied to trees using this device, with movement throughout the canopy and roots detected. Results from delivery of antimicrobial compounds will be presented showing effectiveness of this strategy on controlling CLas infections. Using direct delivery into the citrus vascular tissue, such an application strategy can improve the efficacy of currently available antimicrobials and also expand the range of antimicrobials that can be used to fight citrus greening.

Non-Technical Summary: Bactericides are only effective against HLB when they are inside the tree where the associated agent of HLB resides. In this work we have used 3-D printing to construct devices that deliver molecules of up to ~27 kD in size into the citrus vascular tissue. These devices allow delivery of antimicrobials that control citrus greening without the loss of product associated with foliar or soil drench treatments (essentially 100% of the active ingredient is delivered to the citrus vascular tissue). Although the printed devices are acceptable for delivery of compounds to small trees, we are currently using this strategy to develop devices based on the similar concepts that work on larger trees in productive citrus groves.

IRCHLB-P8-92

Develop effective protectants and therapies to manage citrus HLB using a novel class of citrus-derived antimicrobial peptides

Chienyu HUANG[a], DongDong NIU[ae], Gregory KUND[c], Mike JONES[c], Kris GODFREY[d], John TRUMBLE[c], Ute Albrecht[f], Kim Bowman[f], Chandrika Rammadugu[g] and Hailing JIN[a,b]

[a] Department of Plant Pathology and Microbiology, UCR, CA 92521, USA

[b] Institute for Integrative Genome Biology, UCR, CA 92521, USA

[c] Department of Entomology, UCR, CA 92521, USA

[d] Contained Research Facility, UCD, CA 95618, USA

[e] Department of Plant Protection, Nanjing Agriculture University, Nanjing 210095, China

[f] US Horticultural Research Laboratory, USDA, Agricultural Research Service, 2001 South Rock Road, Fort Pierce, FL 34945, USA

[g] Department of Botany and Plant Science, UCR, CA 92521, USA

Abstract: Huanglongbing (HLB) currently is the most devastating citrus disease associated with phloem-limiting bacteria pathogen *Candidatus* Liberibacter asiaticus (CLas) vectored by the Asian citrus psyllid (ACP). Despite extensive efforts and research, there is still no cure for HLB. It is urgent to develop therapeutic cure strategies to ensure the survival of the citrus industry. Small RNAs is one of the regulators of reprogramming gene expression in plant defense response as well in citrus (Zhao et al., 2013). We use small RNA as a tool to identify the important regulatory components of host natural defense against citrus HLB. By comparative analysis between HLB-sensitive cultivars and HLB-resistant/tolerant citrus hybrids and relatives, such as *Eremocitrus glauca*, *Microcitrus australiasica*, and *Poncirus trifoliata*, we identified and cloned a novel class of antimicrobial peptides (AMPs), SAMP. SAMP is highly stable and can stand high temperature

and proteolysis. These plant endogenous SAMPs from HLB- tolerant/resistant citrus or close relatives have dual functions, which can effectively inhibit/kill the bacterium CLas in HLB- positive citrus trees, and also induce citrus defense responses and enhance host immunity against CLas infection. SAMP has potential to be developed as HLB management tool.

Non-Technical summary: We proved the novel class of antimicrobial peptides, SAMP, has good potential to be used as HLB management tool. The advantage of using SAMP to develop the tool for HLB management includes: 1. SAMP can suppress/kill CLas in HLB diseased trees; 2. SAMP have priming effect for host protection;
3. SAMP can be easily synthesized which could avoid genetically modified approaches;
4. SAMP is safe to human consumption because SAMP is sensitive to human stomach pepsin, and existed in fruits of citrus-hybrids and close relatives. 5. SAMP is stable in high-temperature situation and citrus cell lysate.

Citations:

ZHAO, H., SUN, R., ALBRECHT, U., PADMANABHAN, C., WANG, A., COFFEY, M. D., GIRKE, T., WANG, Z., CLOSE, T. J., ROOSE, M., YOKOMI, R. K., FOLIMONOVA, S., VIDALAKIS, G., ROUSE, R., BOWMAN, K. D. & JIN, H. 2013. Small RNA profiling reveals phosphorus deficiency as a contributing factor in symptom expression for citrus huanglongbing disease. *Mol. Plant*, 6**,** 301-310.

IRCHLB-P8-93

Evaluation of the *in planta* minimum inhibitory concentration of oxytetracycline against *Candidatus* Liberibacter asiaticus

Jinyun LI and Nian WANG

University of Florida, Lake Alfred, FL, USA

Abstract: Oxytetracycline (OTC) has been employed to control citrus Huanglongbing (HLB) in Florida, but there is no information about its antibacterial activity against the pathogen *Candidatus* Liberibacter asiaticus (CLas) due to the inability to culture the bacteria. Here, we evaluated the inhibitory activity of OTC against CLas in greenhouse and field experiments. Citrus trees were trunk- injected with OTC, and leaves were inspected for CLas populations and OTC residues using qPCR and HPLC assays respectively, at various times after OTC treatment. In greenhouse, OTC injection at 0.25 g/tree resulted in ≥ 95% reduction (i.e. ≥ 1.5 Log 10 reduction) of CLas populations between 7 and 30 days post injection (DPI), and, the residues of OTC in leaves were about 0.4 ppm. In field, OTC injection at 2.0 g/tree eliminated or suppressed CLas, resulting in a ≥ 98% reduction (i.e. ≥ 1.7 log 10 reduction) of CLas populations between 7 and 30 DPI, which occurred with ≥ 0.8 ppm OTC residue levels in leaves. In both greenhouse and field experiments, OTC residue levels in leaf tissues (midribs) were positively associated with initial application concentrations of OTC. Reduction of CLas populations by OTC was positively associated with OTC residue levels in leaves. These results suggest that there is a minimum concentration required for OTC to eliminate the pathogen from CLas- infected trees. The information presented here may help citrus growers in decision-making regarding developing an effective HLB management program using OTC.

Non-Technical summary: The antibiotic oxytetracycline has been used to control HLB but there is no information on its inhibitory activity against CLas due to the difficulty to culture the bacteria. We are investigating the *in planta* activity of oxytetracycline for a minimum inhibitory concentration to eliminate or repress the pathogen in HLB diseased trees, with aim at helping citrus growers in decision-making regarding using oxytetracycline for effective management of citrus HLB.

IRCHLB-P8-94

Expression of snakin-1 antimicrobial peptide as a strategy for HLB resistance in citrus rootstocks

U GARDELLA [1], G. CONTI [2], G. JORIS[5], C. HAUTEVILLE[5], L. BURDYN[5], A.M. GOCHEZ[4], C.A. GOMEZ[5], N. ALMASIA[2], V. NAHIRÑAK[2], C. VÁZQUEZ-ROVERE[2], K. KOBAYASHI[3], B.I. CANTEROS[4] M.L. GARCÍA[1], E. HOPP[2], C.A. REYES[1]

[1]Instituto de Biotecnología y Biología Molecular, CCT-La Plata, CONICET— UNLP, La Plata, Buenos Aires, Argentina; [2]Instituto de Biotecnología, CICVyA-INTA, Hurlingham, Buenos Aires, Argentina; [3]Laboratorio de Agrobiotecnología, Instituto de Biodiversidad y Biología Experimental Aplicada (IBBEA-CONICET-UBA),
Departamento de Fisiología, Biología Molecular y Celular, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina;; [4]Citrus Pathology, EEA INTA Bella Vista, Corrientes, Argentina; [5]Estación Experimental Agropecuaria, INTA, Concordia, Entre Ríos, Argentina.

Abstract: The disease Huanglongbing (HLB) was first detected in Argentina in Misiones Province in 2012, and currently exceed 300 positive cases including several provinces. Since 2010 there is a National HLB prevention program (http://www.senasa.gob.ar/hlb) mainly aimed to the phytosanitary control and monitoring tasks as well as diagnostic services. Among other control strategies for HLB disease, genetic engineering techniques appear as very promising. Citrus plants are mainly propagated by grafting commercial varieties onto rootstocks. The present work shows the generation of transgenic plants of one of the most common rootstocks in Argentina, Citrange troyer [(C. sinensis (L.) Osbeck x Poncirus trifoliata (L.) Raf.] using a construct for the expression of the antimicrobial peptide snakin-1 (Segura et al., 1999). This peptide was previously demonstrated to exert antimicrobial effects in potato and lettuce plants (Almasia et al., 2008; Darqui et al., 2018). We generated twelve *C.* troyer transgenic lines that are being rusticated in the greenhouse. Three of the lines were already characterized by molecular analysis and were positive for *Snakin-1* expression by RT-PCR and Western blot. Preliminary challenge assays conducted in detached leaves against *Xanthomonas* sp, revealed a tolerance behavior of the transgenic lines. These lines will be then propagated and challenged against *Candidatus* Liberibacter. Commercial, non-GMO sweet orange scions will be grafted onto resistant/tolerant transgenic lines for field-testing to determine if the improved tolerance can also be transferred to the scion.

Non-technical summary: Snakin-1 is an antimicrobial peptide, which has been proven effective against different bacterial and fungal infections in potato and lettuce. We generated transgenic Citrange troyer citrus rootstocks expressing this peptide and plants were challenged against *Xanthomonas* sp. Lines will be tested against *Candidatus* Liberibacter aiming to analyze acquired resistance to HLB.

Citations:

Segura, A et al. (1999). Snakin-1, a Peptide from Potato That Is Active against Plant Pathogens. Molecular plant-microbe interactions (MPMI) 12(1): 16–23.

Almasia, N.I,, Bazzini, A.A., Hopp, H.E., Vazquez-Rovere, C. (2008). Overexpression of snakin-1 gene enhances resistance to Rhizoctonia solani and Erwinia carotovora in transgenic potato plants. Mol Plant Pathol. 9(3):329–38.

Darqui, F., Radonic, L., Trotz, P., López, N., Vázquez Rovere, C., Hopp, H. and López Bilbao, M. (2018). Potato snakin-1 gene enhances tolerance to Rhizoctonia solani and

Sclerotinia sclerotiorum in transgenic lettuce plants. Journal of Biotechnology, 283: 62-69.

IRCHLB-P8-95

Identification, assessment and delivery of antimicrobial compounds for the management of citrus HLB

Yongping DUAN[1], Hong LIN[3], Muqing ZHANG[1,2], Cheryl ARMSTRONG[1], Chuanyu YANG[2], Charles A. POWEL[2], Veronica ANCONA[4], Fatta B. GURUNG[4], Marc GIULIANOTTI[5], Ute ALBRECHT[6], Liz BALDWIN[1], Jinhe BAI[1], Stephen H. FUTCH[7] and Zhengfei GUAN[8]

[1]USHRL-USDA-ARS, 2011 S. Rock Rd, Fort Pierce, FL 34945, USA, [2]IRREC-IFAS, University of Florida, 2199 S. Rock Rd, Fort Pierce, FL 34945, USA,[3]San Joaquin Valley Agricultural Sciences Center, USDA-ARS, PARLIER, CA 93648,[4]Texas A&M University-Kingsville Citrus Center, 312 N. International Blvd., Weslaco TX 78599,[5]Torrey Pines Institute for Molecular Studies, 11350 Village Pkwy Dr, Port St. Lucie, FL 34987,[6]SWFREC, University of Florida/IFAS, 2685 SR 29 N, Immokalee, FL 34142,[7]CREC-IFAS, University of Florida, Lake Alfred, FL 33850, [8]GCREC, University of Florida/IFAS, 14625 County Road 672, Wimauma, FL 33598

Abstract: Citrus huanglongbing (HLB), associated with three species of fastidious, phloem-limited '*Candidatus* Liberibacter', is the most devastating citrus disease worldwide. Elimination of the bacterium via chemotherapy is an important approach for combating HLB because unlike other plant bacterial diseases, no resistant citrus cultivars are currently available. After much effort, we have developed and optimized a couple of systems for rapidly screening chemical compounds for *Candidatus* Liberibacter asiaticus (CLas) control using both cultured Liberibacter species and HLB-affected citrus. In addition, we used *Xylella fastidiosa* (*Xf*), as surrogate bacterium for functional analysis and interference of virulence genes by substituting *Xf* genes with those from Las including *pilG, popP* and *rpfA* and *mopB* through cross-over chromosome recombination. Altogether, we have screened millions of compounds from two small molecule libraries to date and have uncovered a number of antimicrobial compounds that are effective in suppressing or eliminating Las in these systems under laboratory and greenhouse conditions. These compounds include several small molecules and agro- antibiotic compounds, which have shown high efficacy and low phytotoxicity. Furthermore, the results from a three-year field trial in Fort Pierce, Florida indicated that the use of the three non-medical antimicrobial compounds, Aliette, Carvacrol, and Validoxyamine A can decrease Las bacterial titers and disease severity in affected plants compared to water controls. These compounds also show some positive effects in the ongoing grapefruit and Navel Orange trials in Texas.

Non-Technical Summary: Chemical control of citrus HLB is one of several essential techniques for saving the Florida citrus Industry. We have developed a number of rapid screening and evaluation systems in the pipeline to identify, assess and deliver antimicrobial compounds for the control of citrus HLB.

IRCHLB-P8-96

In vitro antimicrobial activity and mode of action of Zinckicide^{TM} against *Liberibacter crescens*, a surrogate of '*Candidatus* Liberibacter asiaticus'

Eber NARANJO[1], Swadeshmukul SANTRA[2,3,4,5], Mikaeel YOUNG[2,5], Parthiban RAJASEKARAN[2], Evan JOHNSON[6], Leonardo DE LA FUENTE[1]

[1]Department of Entomology and Plant Pathology, Auburn University. [2]NanoScience Technology Center, University of Central Florida. [2]NanoScience Technology Center, [3]Department of Chemistry, [4]Department of Materials Science and Engineering & [5]Burnett School of Biomedical Sciences, University of Central Florida,[6]Citrus Research and Education Center, University of Florida.

Abstract: The in vitro mode of action of the commercial formulation of Zinkicide™, a zinc oxide-based nanoparticle compound, was evaluated using *Liberibacter crescens* (Lcr). Lcr is the only culturable species of the genus and has been used as a biological model for ´Candidatus *Liberibacter* spp.´ The minimum inhibitory concentration in microtiter assays was 125 ppm, while the minimum bactericidal concentration was 150 ppm. Lcr biofilm formation was inhibited with concentrations above 125 ppm. Interestingly, Lcr biofilm/planktonic ratios increased significantly from 25 ppm to 100 ppm, probably as an Lcr stress response mechanism against Zinkicide™. In microfluidic chambers (MC), Zinkicide™ inhibited biofilm formation at 125 ppm but did not affect Lcr cell-surface attachment. The use of the Live/Dead BactLight cell viability kit inside the MC revealed that Zinkicide™ killed the remaining attached cells in the treated channel. Zinkicide™ did not disrupt Lcr preformed biofilms in batch assays or MC at concentrations as high as 300 ppm, however cell viability assessment revealed that sessile cells in Zinkicide™-treated channels were also killed by the compound. To better resemble the expected uneven distribution of this compound and ´*Candidatus* Liberibacter asiaticus´ in mature citrus plants, Zinkicide™ was applied at sub-lethal concentrations in MC for both biofilm inhibition and biofilm disruption assays, and visually assessed for cell viability. Results showed Zinkicide™ bactericidal effect varied according to structural features of the MC. Observation of Zinkicide™ effects in cell shape by transmission electron microscopy showed that when used at 125 ppm this compound caused cell deformation, abnormal cell budding, and cell lysis at 24 hours after inoculation. Ongoing work is focused in assessing the transcriptional response of Lcr against sub- lethal concentrations of Zinkicide™ by combined approaches of RT-qPCR and RNAseq.

Non-technical summary: The phloem- limited, insect-vectored and unculturable nature of HLB associated agent, '*Candidatus* Liberibacter asiaticus' (CLas), makes this disease hard to study as well as hard to assess possible control methods. In this work, the in vitro antimicrobial activity and mode of action of Zinkicide™, a novel ZnO-based nano- formulation, was evaluated in batch cultures and under flow conditions, using *Liberibacter crescens* (Lcr) as a biological model for CLas. Zinkicide™ was effective at killing Lcr at low concentrations.

IRCHLB-P8-97

Novel antimicrobial peptides against Huanglongbing (HLB)

Jeanette. C. VELASQUEZ GUZMAN[1], Supratim BASU[1], Roel RABARA[1], Loan HUYNH[1], Hau NGUYEN[1], Shujian ZHANG[1], Jefferson SHAW[2], Qingchun SHI[2], Ed STOVER[2] and Goutam GUPTA[1].

[1]New Mexico Consortium, Los Alamos, NM. U.S.A.

[2]U.S. Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL, U.S.A

Abstract: Effective treatment of infected citrus would be invaluable in HLB management. Current use of streptomycin and oxytetracycline by foliar spray appears to be insufficient in suppressing *Candidatus* Liberibacter asiaticus (CLas) to subclinical levels. In addition, the potential evolution of antibiotic resistance and passage into humans is a concern.

Here, we report the development of an alternative strategy that relies on designing helix-turn-helix (HTH) peptides based upon citrus 10-18 amino acids long antimicrobial helical peptides. These endogenous antimicrobial helical peptides are subcomponents of longer citrus proteins and show antimicrobial activity against gram-negative bacteria such as CLas. However, the endogenous helices show poor bactericidal activity, host and non-host toxicity, and susceptibility to bacterial resistance. We conducted the following steps to design the HTH peptides as CLas killers. *First*, we have performed *in vitro* evolution of bacterial resistance to the endogenous helices and comparative genomics of susceptible and resistant strains to show that resistance is generated by gene and intergenic mutations that lead to decrease in attachment, insertion, or rupture of the bacterial membrane by the endogenous peptide. *Second*, we have developed a molecular dynamics (MD) based algorithm to design several helix-turn-helix (HTH) peptides based on the endogenous helices that more efficient in membrane attachment, insertion, and rupture than the endogenous helical peptides. *Third*, we performed bactericidal assays to show that not only are the designed HTH peptides are more active than the endogenous helices but also are less susceptible to bacterial resistance. *Fourth*, we carried out *in vitro* analyses to demonstrate that the designed HTH peptides are non-toxic to human and plant cells.

Finally, one such citrus derived HTH peptide, cysP26, appears to be a prominent candidate for HLB treatment by topical delivery. In summary, the citrus-derived HTH peptides may provide an efficacious and a practical therapy for HLB treatment.

Non-Technical Summary: Effective HLB treatment is urgently needed to increase the productive years of already infected citrus. In this study, we describe the design of a novel class of citrus-derived peptides, demonstrate their efficacy as CLas killers, and propose their use in HLB treatment by topical delivery.

IRCHLB-P8-98

Physiological and mechanical constraints on bacteriophage therapy for the *in planta* treatment of Liberibacter asiaticus.

Kevin LINCE[1], **Jennie FAGEN**[1], Janetta HARKOVIRTA[2], Evan BRASWELL[2], Chase BEISEL[1,3]

[1]North Carolina State University, Raleigh NC, USA [2]U.S. Department of Agriculture, Edinburg TX, USA

Abstract: Huanglongbing is an economically important disease that causes substantial damage to the citrus industry worldwide. While chemical, cultural, and biological control methods have had success with controlling the insect vector *Diaphorina citri*, elimination of the associated bacterial pathogen *Candidatus* Liberibacter asiaticus has not been possible. Here we investigate the potential of various natural predators of bacteria known as bacteriophage to control Liberibacter and other phloem/xylem-limited bacterial plant pathogens. Little research has been done to investigate the impact of bacteriophage phenotypes on invasion, transport, and sustained infectivity within citrus tissues. Such understanding will greatly benefit the rapid search for and validation of potential biological control phage. For this work we introduce a mixture of canonical bacteriophage representing a range of morphologies in the Caudovirales, Inoviridae, Leviviridae to citrus

tissues using standard inoculation methods. These phages were then tracked and quantified within the plant tissues using qPCR, and their continued viability was determined through subsequent infection on their preferred bacterial host. We further investigate the bacteriophage delivery through the root and soil inoculations as well as the foliar application of the bacteriophage in an attempt to explain the constraints on phage movement within citrus plants. By first establishing the constraints of phage delivery, movement, and in planta infection a deeper focus can be placed on refining phage-based control methods for *Candidatus* Liberibacter asiaticus. Such phage may additionally serve as mediators of CRISPR antimicrobials therapy, Liberibacter genome modification, or RNAi. The end goal of this study is to establish effective and reproducible phage delivery methods to be used in conjunction with other bio control methods to eliminate and manipulate the associated bacterial pathogen of HLB, *Candidatus* Liberibacter asisaticus

Non-Technical summary: We are taking a look at the efficiency of using bacteriophage, the natural predators of bacteria, as a method for controlling plant pathogens like *Candidatus* Liberibacter asiaticus. More specifically, we are establishing phage delivery methods *in planta* to better grasp the usefulness of bacteriophage in plant pathogen control.

IRCHLB-P8-99

Uptake, translocation and stability of the antibiotics, streptomycin and oxytetracycline in citrus trees

Nabil KILLINY[1], Fuad ALRIMAWI[1], Faraj HIJAZ[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: Huanglongbing (HLB), also known as citrus greening disease, is the most damaging disease to the world citrus industry. It is associated with the fastidious α-proteobacterium, *Candidatus* Liberibacter asiaticus (CLas) and is transmitted by the Asian citrus psyllid, *Diaphorina citri*. Currently, there is no effective cure for HLB and the management depends mainly on the control of the insect vector. Recent studies suggested that antibiotics can inhibit the growth of the pathogen *in planta*. CLas growth can be reduced in infected plants using antibiotics such as streptomycin and oxytetracycline. In the current study, we investigated the uptake and translocation of these two antibiotics in citrus seedlings Antibiotics were delivered via root drench and their levels in various plant tissues were monitored using enzyme-linked immunosorbent assays and high-performance liquid chromatography. After drenching roots with 20-200 ppm antibiotic solutions, 16 hours later, antibiotic levels were assessed. For both streptomycin and oxytetracycline, the highest level of antibiotics was found in tissues as follows: roots>xylem>phloem,> leaves. Although the ratio for both antibiotics in xylem tissue compared to phloem tissues was three-to-one, oxytetracycline was five times higher than streptomycin. This finding suggests that citrus trees take up oxytetracycline more effectively than streptomycin and that it translocates more readily. Additionally, the antibiotics were recovered from *D. citri* fed on treated plants and their survival was assessed. We found that both antibiotics had a negative effect on the fitness and lifespan of psyllids. Gathering information about the dose, distribution and stability of antibiotics in citrus trees is essential for designing an effective program to control the disease.

Non-technical summary: Understanding the uptake and translocation of antibiotics within citrus trees will provide important information that may lead to effective control of Huanglongbing by inhibiting bacterial growth of the pathogen within the trees.

IRCHLB-P8-100

Zinkicide improved yield and fruit size on younger Huanglongbing-affected trees

Megan M. DEWDNEY[1], Diane B. BRIGHT[1],Kayla M.GERBERICH[1] Swadeshmukul SANTRA[2], James H. GRAHAM[3], Evan G. JOHNSON[1]

[1]Plant Pathology, Citrus Research and Education Center, Lake Alfred, FL USA [2]University of Central Florida, Orlando, FL USA[3]Soil and Water Sciences, Citrus Research and Education Center, Lake Alfred, FL USA

Abstract:

Huanglongbing (HLB) is a phloem-limited bacterial disease, associated with *Candidatus* Liberibacter asiaticus, that has devastated citrus production in many regions including Florida. A major impediment to HLB management is getting compounds into phloem cost effectively. One solution is to create molecules small enough to pass into phloem like the nanoscale Zinkicide, a zinc oxide-based compound. A fixed Zinkicide formulation was developed from agricultural- grade chemicals and used in field trials with 5- year-old Ruby Ray grapefruit and 20-year-old Valencia sweet orange in the 2017 and 2018 seasons. The grapefruit trial was a completely randomized design with five tree plots with the outer two trees acting as guard trees. The treatments were an untreated control (UTC), 0.14, 0.28, or 0.56 kg/ha of foliar, soil, or foliar and soil (F&S) applications. The Valencia trial was conducted similarly although it was a complete randomized block design with five blocks and five treatments. Each treatment had five trees and they were separated by a guard tree. The treatments were a UTC, 1.12 and 2.24 kg/ha foliar application, 2.24 kg/ha soil application, and 2.24 kg/ha F&S application. Higher rates were used because of tree volume. Zinkicide treatments were applied monthly. In the 2017 grapefruit trial, there was significant yield ($P < 0.05$) increase (kg fruit/tree) over the UTC for all treatments and a dose response for the foliar and F&S applications. The proportion of medium and large fruit increased in the F&S treatments compared to the UTC. Similar results were observed in 2018. Results were less clear in the Valencia for the 2017-2018 season as no treatment had significantly greater yield than the UTC but the F&S application was better than either alone ($P < 0.05$). There was a shift of small fruit to medium and large fruit for F&S compared to the UTC.

Non-technical summary:

Zinkicide is a zinc oxide-based nanoparticle designed to enter the citrus plant vascular system, where the bacterium associated with HLB is located, from conventional foliar or soil drench applications. The greatest effect of Zinkicide was when the foliar and soil drench applications were combined. In our young tree grapefruit trial, yield and fruit size improved in the foliar and soil drench treatment compared to the untreated control in both years. In the older tree Valencia trial, the results were not so clear and no treatment had greater yield than the untreated control but there was a shift in fruit size.

IRCHLB-09: Molecular and Cultural Vector Management Oral Presentations

IRCHLB-O5a-01

Effect of Huanglongbing Antimicrobial Therapies on *Candidatus* Liberibacter asiaticus, and Its Vector, the Asian Citrus Psyllid, *Diaphorina citri* Kuwayama (Hemiptera: Liviidae)

Kirsten PELZ-STELINSKI and Eliott SMITH

University of Florida, Lake Alfred, USA

Abstract: The bacterial pathogen, *Candidatus* Liberibacter asiaticus (CLas), and its vector, *Diaphorina citri*, are global pests of citrus production. It is unknown whether antibiotics

used for management of CLas infection in *Citrus* plants, oxytetracycline and streptomycin, will also contribute to management of *D. citri*. The purpose of this study was to investigate the effect of these antimicrobial compounds on plant infection and on the fitness, behavior, and transmission capacity of *D. citri*. Oxytetracycline and streptomycin were administered orally to *D. citri* through foliar application on plants and in artificial diet bioassays to assess their effects on endosymbiont densities, reproductive output, longevity, feeding behavior, host selection, and the inoculation capacity of *D. citri*. We determined that oxytetracycline elicited the greatest reduction in endosymbiont titer and *D. citri* fitness parameters compared with streptomycin and control treatments. Deterrent effects were observed in response to oxytetracycline as compared with control treatments. Cumulatively, these data suggest that antimicrobials used for CLas management, particularly oxytetracycline, elicit lethal and sublethal effects in *D. citri*. Further investigations are needed to confirm the efficacy of these compounds against *D. citri* in the field and to determine whether applications of higher antimicrobial doses will reduce pathogen transmission.

Non-Technical summary: We evaluated the efficacy of antibiotics for management of plant CLas infections in greenhouse assays and tested whether antibiotics would also reduce titers of psyllid CLas infection and psyllid endosymbionts. Feeding activity and survival was reduced among psyllids exposed to antibiotic treatments. Additionally, we evaluated the capacity of the antibiotics to translocate from the roots to the leaves and subsequently be detected in psyllids. CLas reduction among infected psyllids may indicate that antibiotics can be used to reduce the pathogen to concentrations that are insufficient to cause an infection in plants.

IRCHLB-O5a-02

Bt toxins for management of Asian citrus psyllid (*Diaphorina citri*, Hemiptera)

Pavan KUMAR[1], M. Teresa FERNANDEZ-LUNA[2], David HALL[3], Michael BLACKBURN[4], and Bryony C. BONNING[1, 2]

[1]Department of Entomology and Nematology, University of Florida, Gainesville, FL, [2]Department of Entomology, Iowa State University, Ames, IA, [3]USDA, ARS, Ft. Pierce, FL, [4]Invasive Insect Biocontrol and Behavior Laboratory, USDA, Agricultural Research Service, Baltimore, MD

Abstract: The bacterium *Candidatus* Liberibacter asiaticus (CLas), the presumed causative agent of citrus greening, is vectored by the Asian citrus psyllid (ACP). Management of ACP is a primary focus for limiting disease spread, but with increasing incidence of resistance to insecticides, management options are limited. While toxins derived from the bacterium, *Bacillus thuringiensis* (Bt) may provide a safe and effective alternative for ACP management, few toxins with activity against hemipteran insects have been identified. To identify ACP- active toxins, bioassays were conducted with toxin mixtures derived from individual Bt isolates. Eight strains that show basal toxicity on feeding of adult ACP at 500µg/ml of proteolytically activated toxin were identified, and the trypsin proteolytic profile of the toxic strains characterized. Peptide sequencing and genomic analyses were then used to identify individual toxins produced by isolates with activity against ACP. These results identified Cry1Ab and Cry1Ba1 as candidate toxins for screening against ACP. Bioassays with these individual toxins, along with Cry51Aa1 previously shown to exhibit toxicity against hemipteran species, showed that all three toxins had comparable LC50 values of ~120 to 190 ppm. Based on demonstration that addition of a gut binding peptide can increase efficacy of

Bt toxins against hemipteran pests (N.P. Chougule et al., 2013), optimization of these toxins is underway. Multiple delivery systems are also under investigation for delivery of Bt toxins for management of ACP including via transgenic citrus and transgenic trap plants (*Murraya koenigii*).

Non-Technical summary: A bacterium commonly known as Bt produces insect-specific, pesticidal proteins. We have identified three Bt proteins with toxicity against the Asian citrus psyllid, which plays an important role in the spread of citrus greening. We are working on optimization of these proteins for increased insecticidal activity, and testing various approaches for delivery of the proteins for suppression of psyllid populations. In the long term, these pesticidal proteins derived from Bt may provide additional tools to help prevent citrus greening.

Citation:

Nanasaheb P. Chougule, Huarong Li, Sijun Liu, Lucas B. Linz, Kenneth E. Narva, Thomas Meade, and Bryony C. Bonning (2013). Retargeting of the Bacillus thuringiensis toxin Cyt2Aa against hemipteran insect pest Proc. Natl. Acad. Sci. USA 21: 110 (21) 8465-8470.

IRCHLB-O5a-03

BAPC-assisted-CRISPR-Cas9 Delivery into Nymphs and Adults for Heritable Gene Editing (Hemiptera)

Wayne B. HUNTER 1., John TOMICH 2.

1. USDA, ARS, 2001 South Rock Road, Fort Pierce, FL, USA; 2. Kansas State University, Dept. Physics, Manhattan, KS, USA.

Abstract: A new method called BAPC-assisted-CRISPR-Delivery System, for heritable gene editing by treating nymphs and adult Insects, Hemiptera, was demonstrated. Addition of Branched Amphiphilic Peptide Capsules, BAPC™, (Phoreus™ Biotechnology, Inc) improves delivery of CRISPR components, plasmids, and dsRNAfor heritable gene editing and gene targeting or expression, in insect nymphs and adults (Asian Citrus Psyllid-Diaphorina citri). The method bypasses traditional microinjection of eggs. This reports the first heritable gene knock-outs, KO, using BAPC-assisted- CRISPR-Cas9, produced G2 mutants from injected adult psyllid females. The KO target was the *thioredoxin* gene, resulting in 550 nt deletion in gDNA of target insects. In psyllids the *thioredoxin* gene, TXT, and *Vermillion*, Vm, knock outs produced changes in physiology and eye color, respectively. Strategies to alter the psyllid vector into a non-vector of the associated bacterium, *Candidatus* Liberibacter asiaticus (CLas). One function of TXT is to promote development. When knocked out development is slower, reduced survival, and reduced adult lifespan and fecundity. Reduced fecundity and slow development traits would reduce insect populations by increasing exposure time to parasitism and predation with more time, extra 2 to 3 weeks longer to attack nymphal stages. Emerging adults only lived one-third as long. Classical CRISPR/Cas9 injections of nymphs and pupae produced *Vm*-KO, white eye phenotypes. The *BAPC™-assisted* delivery system advances gene editing across all hemipterans and insects by permitting the use of nymphs and adults. The technology opens strategies to protect citrus and food crops from insect vectors and pathogens [citrusgreening.org]. Funding in part: USDA-NIFA 2014-70016-23028. Developing an Infrastructure and Product Test Pipeline to Deliver Novel Therapies for Citrus Greening Disease.

Non-Technical Summary: A new method represents a major breakthrough in management of insect vectors. Using gene targeting permits safer, more specific, management methods to reduce insect pests and pathogens. The new method, called *BAPC-assisted-Delivery*, improved gene knockouts in adult Asian Citrus Psyllid, *Diaphorina citri*. The method permits researchers to rapidly test potential gene targets. Using the method evaluations identified a suitable gene that may be used in reducing psyllid populations, and thereby reduce the spread of Huanglongbing.

Citations:

Avila, L.A., Chandrasekar, R., Wilkinson, K., J.M. Reeck, G.R. 2018. Delivery of lethal dsRNAs in insect diets by Branched Amphiphilic Peptide Capsules. J. Control. Release. 273:139-146.

Hunter, W.B., Gonzalez, M.T., Tomich, J. 2018. BAPC-assisted CRISPR/Cas9 System: Targeted Delivery into Adult Ovaries for Heritable Germline Gene Editing (Arthropoda: Hemiptera). bioRxiv 2018. http://dx.doi.org/10.1101/478743.

Hunter, W.B., Sinisterra-Hunter, X. 2018. Emerging RNA Suppression Technologies to Protect Citrus Trees from Citrus Greening Disease Bacteria. Advances Insect Physiol. 55:163-199.

IRCHLB-O5a-04

On the use of psyllids for directed delivery of therapeutics for HLB.

W. Evan BRASWELL[1], Kuzy ZARZOSA[2], Hisham ABDOU[2], and Yuanbing MAO[2]

[1]USDA APHIS PPQ CPHST, Mission Laboratory, Edinburg, TX 78541 [2]Department of Chemistry, University of Texas – Rio Grande Valley, Edinburg, TX 78539

Abstract: Huanglongbing disease poses significant economic, regulatory, and scientific challenges. These challenges are exacerbated by the lack of tolerant or resistant Citrus genotypes as well as the lack of traditional therapeutics that can cure infected trees. Spurred by these challenges and the desire to save trees already in the ground, researchers are working to develop new types of therapeutics. Many of these are based on viruses, such as phage therapy or engineered antimicrobials, and/or small molecules, such as bacteriocins or oligonucleotides. Delivery of such therapeutics is a challenge, however. Environmental exposure to UV radiation and desiccation during delivery often damage such therapeutics, reducing or eliminating effectiveness. Since transcuticular delivery is generally passive, it can be a challenge to deliver efficacious doses of un-damaged therapeutics to large field trees. To circumvent these challenges, we evaluated the potential of using the disease vector to delivery therapeutics directly into the plant. Specifically, we generated nanoparticles, fed them to psyllids, confirmed psyllids consumed the nanoparticles, and then asked whether psyllids could transmit them to Citrus plants. Our efforts focus on nanoparticles due to their ease of detection and their potential to be functionalized as carriers of therapeutics. This approach may offer unique advantages in that therapeutic delivery will occur exactly where it’s needed most – where the pathogen invades the plant.

Non-Technical Summary: A number of laboratories are developing new types of treatments for Huanglongbing. Many of these treatments are small molecules that are easily destroyed by sunlight or by drying out, making delivery difficult. We tested whether psyllids could transmit these treatments, just as they transmit the pathogen, thereby avoiding environmental stressors.

IRCHLB-O5a-05

Trap crop as a potential strategy to control *Diaphorina citri*

Arthur F TOMASETO[1], Rodrigo N MARQUES[2], Alberto FERERES[3], Odimar Z ZANARDI[1], Haroldo XL VOLPE[1], Berta ALQUÉZAR[1,4], Leandro PEÑA[1,4] and **Marcelo P MIRANDA**[1]

[1]Fund for Citrus Protection, Araraquara, SP,

Brazil, [2] Federal University of São Carlos, Buri, SP, Brazil, [3] Instituto de Ciencias Agrarias, Consejo Superior de Investigaciones Científicas, Madrid, Spain, [4]Instituto de Biología Molecular y Celular de Plantas, Consejo Superior de Investigaciones Científicas, Valencia, Spain.

Abstract: Trap cropping may be a potential strategy to reduce the migration of *Diaphorina citri* into commercial citrus orchard and to improve the current Huanglongbing (HLB) management. In this study, laboratory and field experiments were performed to assess the effect of orange jasmine (*Murraya paniculata*) as a trap crop to control *D. citri*. Planting orange jasmine as a border trap crop on newly established commercial citrus orchards reduced by 40% the captures of *D. citri* populations in yellow stick traps and by 80% the number of marked psyllids that settled on the experimental citrus orchard. Furthermore, in the commercial citrus orchard, HLB incidence was 43% lower on the presence of the trap crop than on fallow field plot. Olfactometer experiments showed that orange jasmine plays an attractive role on psyllid behavior and that this attractiveness may be associated with differences in the volatile profiles emitted by orange jasmine in comparison with sweet orange. Our findings indicated that *M. paniculata* treated with insecticide may act as a "pull" and "kill" strategy to intercept and control *D. citri* before they settled on the edges of citrus orchards, contributing to the reduction of HLB primary spread.

Non-Technical summary: We demonstrated that orange jasmine plants, treated with insecticides, attracted and killed psyllids before they landed on the border of citrus orchard. In addition, we also observed that orange jasmine plants have a better odor for psyllids than orange trees. Consequently, trap cropping may be a valuable strategy to reduce the psyllid migration into commercial citrus orchard.

IRCHLB-O5a-06

Field implementation of a multimodal attract-and-kill device for Asian citrus psyllids

Justin GEORGE[1], Monique RIVERA[2], Lukasz STELINSKI[1]
and Stephen LAPOINTE[3]

[1]University of Florida, CREC, Lake Alfred, FL, USA
[2]University of California, Riverside, CA, USA
[3]US Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL, USA

Abstract: Phytophagous insects including Asian citrus psyllids (*Diaphorina citri* Kuwayama) (ACP) use multiple sensory modalities [vision, olfaction, contact chemoreception, gustation (taste), perception of auditory or vibrational stimuli] to locate host plants or conspecifics. Many studies have reported that ACP is strongly attracted to bright yellow and light green. George et al. (2016) found that acetic and formic acids were highly excitatory to antennae of male and female adult ACP. We have developed an optimal 3-component blend (3.5:1.6:1 blend of formic acid: acetic acid: p-cymene) for maximal probing by ACPs. This blend acts as a phagostimulant by increasing the number and length of probes into a wax substrate containing the blend (Lapointe et al. 2016). Our studies have also shown that incorporation of magnesium oxide (MgO) as a UV reflectant increases the attraction of ACP towards the SPLAT surface. We have incorporated these sensory cues into a cylindrical trap device in which Color, Attractant, Phagostimulant, UV reflectant and Toxicant (CAPUT trap) are incorporated into a wax (SPLAT) medium. In this attract-and- kill device, adult psyllids are attracted to the AKD by color and odor. After alighting, psyllids are induced to probe and attempt to feed by the presence of phagostimulants in a wax matrix attached to the surface in the interior of the device. Psyllids probing the wax matrix containing the insecticide

are killed upon contact and during attempted ingestion from the wax. Dead psyllids then fall off from the trap surface thereby retaining complete trap activity over long periods (months). Results from cage assays and preliminary field experiments will be discussed. The goal of this project is to develop an economical and environmentally appropriate management strategy for ACP that exploits the psyllid behavior including its response to visual, olfactory and gustatory stimuli.

Non-Technical summary: Asian citrus psyllids (ACP) transmit the pathogen responsible for citrus greening disease. Psyllids use color, smell, taste and vibrational cues to identify their host plants and conspecifics. The main goal of this project is to develop an attract-and-kill device strategy that will exploit the psyllid's responses to these sensory cues. Adult psyllids will be attracted to the trap by color and odor. After alighting, psyllids are induced to probe and attempt to feed by the presence of phagostimulants (compounds that induce ACP probing and feeding) in a wax matrix attached to the surface of the device. Psyllids may then be killed by a contact insecticide or other agent contained in the wax matrix.

Citations:

George, J., P.S. Robbins, R.T. Alessandro, L.L. Stelinski and S.L. Lapointe. 2016. Formic and acetic acids in degradation products of plant volatiles elicit olfactory and behavioral responses from an insect vector. Chem. Senses 41 (4): 325-338.

Lapointe, S.L., D.G. Hall and J. George. 2016. A phagostimulant blend for the Asian citrus psyllid. J. Chem. Ecol. doi. 10.1007/s10886-016-0745-4.

IRCHLB-09: Molecular and Cultural Vector Management Poster Presentations

IRCHLB-P9-101

A high-quality reference genome for *Diaphorina citri* with manually curated genes in developmental, structural and immune related pathways

Prashant S. HOSMANI[1], Mirella FLORES-GONZALEZ[1], Sherry MILLER[2], Teresa SHIPPY[2], Wayne B. HUNTER[3], Tom D'ELIA[4], Susan BROWN[2], Lukas A. MUELLER[1] and Surya SAHA[1]

[1]Boyce Thompson Institute, Ithaca, NY 14853; [2]Division of Biology, Kansas State University, Manhattan, KS 66506; [3]USDA- ARS, U.S. Horticultural Research Laboratory, Fort Pierce, FL 34945; [4]Indian River State College, Fort Pierce, FL 34945;

Abstract: The vector, *Diaphorina citri* (Asian citrus psyllid) is the primary target of approaches to stop the spread of the pathogen *Candidatus* Liberibacter asiaticus (CLas) associated with Huanglongbing, citrus greening disease. The previous psyllid genome (v1.1) in NCBI was missing 25% of the single copy markers conserved in other Hemipterans. Manual genome annotations were able to identify a significant number of genome anomalies, misassemblies and missing genes. Quality of the assembly was improved using a *de novo* assembly with PacBio long reads followed by Dovetail Chicago-based and Hi-C scaffolding (Diaci v3). Full-length cDNA transcripts from diseased and healthy tissue from multiple life stages were sequenced with Pacbio IsoSeq technology. Combined with other Illumina RNAseq datasets the Official Gene Set (OGS v3) was generated, with ~20,000 full-length protein coding genes. Details about a genome independent transcriptome with a comprehensive catalog of all genes in the psyllid are described. Gene-targeting technologies like RNAi, antisense oligos and CRISPR require accurate annotation of genes. The quality of automatically predicted gene models depends on knowledge transfer from well characterized model organisms such as *Drosophila*. This can

be challenging due the complexity and diversity of insect genomes. A high-quality manually curated gene set for developmental, structural and immune pathways is presented. Gene sets involved in chitin metabolism, cuticle, Hox/Hox cofactors, segmentation, chromatin remodeling complexes, circadian rhythm, carbohydrate metabolism, iron metabolism and endocytosis were produced. Immune related pathways include pathogen recognition molecules, signaling cascades associated with pathogenesis (Toll, IMD and JAK-STAT pathways) and response to pathogens. These curated gene models are in better accord with expression and homology based evidences without assembly errors. All resources are available on https://citrusgreening.org/ a portal for all omics resources for the citrus greening disease research community.

Non-technical summary: The Asian Citrus Psyllid genome is an important resource for studying the biology of the insect that transmits Huanglongbing, citrus greening disease. We have significantly improved the genome and made it freely available to the research community, and public. The improved psyllid genome, Diaci_v3, was generated using the latest technologies. A large community based manual annotation effort resulted in the improved structural and functional characterization of the genes involved in the developmental, structural and immune related pathways. The improved accuracy and knowledge will enable development of gene based targeting products to reduce psyllid populations thereby slowing pathogen spread.

IRCHLB-P9-102

Attributes of color that affect attraction of the Asian Citrus Psyllid

Sandra A. ALLAN

U.S. Department of Agriculture, Gainesville, FL, USA

Abstract: The Asian citrus psyllid, *Diaphorina citri*, the vector of the pathogen causing the devastating citrus disease, huanglongbing, primarily visual cues for host plant location. Previous electrophysiological studies have determined the presence of different photopigments contributing to color vision and laboratory and field studies have revealed colors eliciting the strongest responses. Two series of laboratory assays were undertaken in this study. One was to evaluate whether attributes of reflected color could be manipulated to enhance attraction compared to standard yellow traps and this included use of novel paints that enhanced ultraviolet (UV) reflection or included fluorescent pigments. The second set of assays studied their responses to defined colors compared to gray sticky traps matching the intensity of reflected light. A commercial yellow paint providing moderate UV reflectance was very attractive. Addition of UV reflecting pigments enhanced attraction to certain wavelengths (yellow) but not others. Fluorescent or neon pigments were not more attractive than standard yellow sticky traps. Strong attraction of psyllids to green or yellow but not blue traps compared to paired equal intensity gray traps indicated that hue rather than intensity was responsible for attraction. These results provide a better understanding into attributes of color that are attractive to the Asian citrus psyllid which can potentially enhance

Non-Technical summary: The Asian citrus psyllid utilizes visual cures, particularly color, for attraction to potential host trees. Based on this attraction, use of colored sticky traps have become a standard for surveillance. In this study, we are investigating if attraction to the basic yellow sticky trap can be enhanced by varying attributes of the color such as with neon, fluorescent or UV-reflecting pigments. Results from this study may potentially enhance surveillance or attract-and-kill strategies.

IRCHLB-P9-103

Capability of 'Photonic Fence' technology to detect, track, and control Asian citrus psyllid

Joseph PATT[1], Arty MAKAGON[2] and Jeremy SALESIN[2]

[1]USDA-Agricultural Research Service, Fort Pierce, FL, [2]Photonic Sentry, Bellevue, WA

Abstract: The 'Photonic Fence' is a novel technology that detects and excludes flying insects from protected areas such as citrus propagation structures, grove blocks, and packing houses. It works by first generating a silhouette of an insect as it flies across a plane of infrared light and then profiling the silhouette's shape, velocity, and wingbeat frequency. This enables the system to distinguish targeted from innocuous species within milliseconds. A low-energy, eye-safe laser can then be engaged to intercept and kill targeted insects if desired. Under development for over 10 years, tests began with the first large-scale prototype of the Photonic Fence at the USDA-ARS research grove in Fort Pierce, FL in October 2017. The prototype extended along the centerline of a screenhouse and had an operational area that was 30m long and 3m high. The effectiveness of the system against *Diaphorina citri* was evaluated with release-recovery tests. Release and recovery sides of the screenhouse were designated, separated by the Photonic Fence. 600 psyllids were discharged from cages positioned on the release side. Twenty-four hours after release, final counts were made of psyllids captured in yellow 3D traps positioned on both sides of the screenhouse and of individuals that alighted on the screenhouse walls. Tests were performed in one of two modes: monitoring only, with the interdicting laser disengaged, and lethal, with both monitoring and laser modes enabled. In an initial monitoring test with the lethal laser disengaged, 18.7% of the released psyllids were found on the screenhouse walls or recovered in traps. In subsequent tests with the lethal laser engaged, 3.7% and 2.3% of the released psyllids were recovered or counted. Concurrent tests with the mosquito *Aedes aegypti* showed that the system could detect 1500 insects per minute. A next generation prototype is being developed for testing in citrus groves.

Non-Technical summary: We are testing the capability of Photonic Fence technology to exclude flying Asian citrus psyllids from citrus production areas. This technology uses a combination of infrared light, eye-safe lasers, sensitive optical detectors and sophisticated software to detect, track, identify, and kill targeted insect species as they cross a photonic 'fence line'. Initial tests showed that the system could rapidly reduce psyllid populations in a screenhouse setting. Optimization of the system is underway to produce a second generation prototype capable of operating in citrus groves.

IRCHLB-P9-104

Could Brazilian native rutaceous support the Asian Citrus Psyllid?

Gustavo R. ALVES[1], Alexandre J. F. DINIZ[1], Adriano G. GARCIA[1], José R. P. PARRA[1]

[1]University of São Paulo, Department and Entomology and Acarology, USP/ESALQ, Brazil

Abstract: The Asian citrus psyllid (ACP) *Diaphorina citri*, has a range of hosts of the Rutaceae family, especially of the genus *Citrus*, although little is known about non- citrus species. The objective of this work were to evaluate the effect of six brazilian rutaceous, *Balfourodendron riedelianum* (BR), *Dictyoloma vendellianum* (DV), *Esembeckia leiocarpa* (EL), *Helietta apiculata* (HA), *Metrodorea stipularis,* (MS) and *Zanthoxylum rhoifolium* (ZR) besides citrus (CR) and orange jasmine (OJ) (as control), on ACP biology (fertility and nymphal development). The fertility was evaluated by counting the number of eggs in each plant in

confinement tests. In cages, three couples were confined to the plants for 72 hours. After this, the insects were removed and the eggs counted. For the biology test, on each plant, a couple was confined for 24 h. After this period, the pair was removed and the eggs were counted. Egg hatching and nymph mortality were daily observed until the adults emergence. The highest oviposition was obtained in OJ, with an average of 38 eggs/plant followed by citrus and HA (28 and 22 eggs/plants, respectively). No egg was observed on EL, and values less than one egg/plant was observed on DV and MS. The highest values of total viability were observed in OJ and citrus, with average of 83 and 78%, respectively. Among the native species, only HA allowed the complete development of the ACP, with a little more than 12% survival. The result obtained for HA characterizes this plant species as a new host for ACP, being important for the management and epidemiology of HLB.

Non-Technical Summary: The importance of non-citrus hosts for *D. citri* biology and survival is little known. Investigating this parameters we found a new host (*Helietta apiculata)* for the pest. This information could improve the IPM (Integrated Pest Management) strategies for psyllid control in *Citrus*.

IRCHLB-P9-105

Developing field detection systems and characterizing other Liberibacters associated with citrus HLB.

Chandrika RAMADUGU[1], James SNYDER[2], Stefanie DOWLING[2], Susan HALBERT[2], Eric ROHRIG[2], Madhurababu KUNTA[3], Olufemi ALABI[3], Mamoudou SETAMOU[3], Ryo KUBOTA[4], Daniel JENKINS[5] and Mikeal ROOSE[1]

[1] University of California, Riverside, CA. [2] Division of Plant Industry, Gainesville, FL. [3] Texas A&M, Weslaco, TX. [4] Diagenetix, Honolulu, HI. [5] University of Hawaii, Honolulu, HI.

Abstract: Management of citrus huanglongbing (HLB) will require large scale surveys in regions where the disease is getting established. Yellow sticky traps used routinely are not suitable for recovering psyllids with intact DNA for pathogen testing. Since the HLB pathogen can be detected in the psyllid vector long before symptom development, we have developed 3D printed traps from which psyllids can be tested. After designing and evaluating about 25 iterations, we now have traps that can capture about 50% of what is captured on a yellow sticky trap, deemed sufficient for adequate testing. We have evaluated different lures and incorporated LED lights into the 3D traps. Another aspect of the project is to facilitate the ability to conduct testing for the pathogen in the field situation. We have developed loop-mediated amplification based methods for field testing of psyllids. The BioRanger units designed for this are affordable, easy to use, portable and can complete a test in 30 minutes. Liberibacter variants are prevalent in nature and may affect disease dynamics significantly. We have developed a detection system capable of detecting all known Liberibacters. We are characterizing a new species of Liberibacter, *Ca*. L. caribbeanus (CLca) from Colombia. This bacterium colonizes psyllid hosts to a very high titer (Ct 15) yet, in citrus hosts the titer remains low (>Ct 36). We have sequenced regions of CLca using metagenomics approaches and conducted comparative analysis of Liberibacter genomes. The closest known Liberibacter for CLca appears to be *Liberibacter crescens*. Whole genome sequencing and *de novo* assembly of CLca are in progress. Understanding the biology of this Liberibacter variant may provide novel solutions to the HLB situation. The psyllid traps will be available to growers shortly and the testing units are available through Diagnetix, HI. Training to interested growers will be provided

by our team.

Non-technical summary: Effective disease management strategies for HLB include ability to capture testable psyllids and development of tools to conduct field testing for the HLB pathogen. Our 3D printed traps can capture psyllids that can be preserved and are suitable for testing. The testing device that is now available will facilitate field testing in a 30-minute test. We are studying Liberibacter variants that may have significant implications in understanding psyllid/citrus – pathogen interactions. We have also developed a detection system capable of testing all known forms of Liberibacter bacteria.

IRCHLB-P9-106

Effect of organic fertilizer on the biological characteristics of Asian citrus psyllid *Diaphorina citr* Kuwayama

Yijing CEN, Changbao XU, Lei TAO, Jifeng WANG, Zhe LIU, Xin XU, Xuying ZHANG, Xiaoling DENG

Citrus Huanglongbing Research Laboratory, South China Agricultural University, Guangzhou 510642, CHINA.

Abstract: Fertilization may affect the occurrence of pests by changing the host plant condition. It was found that number of the vector of huanglongbing, Asian citrus psyllid *Diaphorina citri* Kuwayama occurring on the citrus trees applied with organic fertilizers was significantly fewer than that of chemical fertilizers in the orchard. In this study, the biological characteristics of *D. citri* feeding on *Citrus reticulata* cultivated by organic fertilizers (OF), chemical fertilizers (CF), and mixed fertilizers (mixture of organic and chemical fertilizer, MF) were compared with a life table method. The results showed that the durations of the 3rd, 4th, 5th instar nymphs and the total immature stage in the OF treatment were significantly shorter than other treatments. Although there was no significant difference of the survival rates of all developmental stages, female's pre- oviposition period, fecundity, oviposition period and longevity among the three treatments, the maximum breeding, shortest pre-oviposition duration and longest lifespan of adults were all on the trees applied with organic fertilizers. In addition, the net reproduction rate (R0), intrinsic rate of increase (rm), finite rate of increase (λ), and index of population trend (I) were highest on the OF treatment, followed by the MF treatment. The preference of *D. citri* adults to different fertilizer-cultured citrus was also observed. It was found that the number of released adults settling on the CF treated trees was significantly higher than other treatments during 2 days.

Non-Technical Summary: Host plants treated with organic fertilizers were less attractive to *D. citri* adults, but were more beneficial to the population growth of *D. citri* than chemical fertilizers.

IRCHLB-P9-107

Evaluating lighting preferences to enhance trapping efficacy of Asian Citrus Psyllid.

Daniel JENKINS[1],
Chandrika RAMADUGU[2], James SNYDER[3], Stefanie DOWLING[3], Susan HALBERT[3], Eric
ROHRIG[3], Madhurababu KUNTA[4], Olufemi ALABI[4], Mamoudou SETAMOU[4], Ryo KUBOTA[5] and Mikeal ROOSE[2]

[1] University of Hawaii, Honolulu, HI. [2] University of California, Riverside, CA. [3] Division of Plant Industry, Gainesville, FL. [4] Texas A&M, Weslaco, TX. [5] Diagenetix, Honolulu, HI.

Abstract: Combating newly introduced pest species is one of the most important biosecurity challenges facing agriculture today. Effective surveillance is critical for controlling these pests and spread of associated diseases. For example, the bacterial pathogen causing citrus greening is vectored by the Asian Citrus Psyllid (ACP; *Diaphorina citri*), and efforts to control the spread of disease in California and other citrus producing states include testing of psyllids caught in the field. To test lighting preferences to potentially improve the efficacy of trapping and surveillance efforts for these insects, we have developed a miniature LED-based lighting circuit. The circuit can be programmed wirelessly through a custom Android application to turn on any of 6 LEDs (UV, blue, green, yellow, amber, and/or red), with different modulation schemes and diurnal cycles based on ambient light. We used these lighting circuits to test lighting perception / preferences of ACP. In preliminary forced choice experiments we found that illumination of custom traps for ACP with yellow resulted in the best trap yield compared to other human visible wavelengths, but surprisingly ACP was most highly attracted to UV light (385 nm). Modulation of light at 1.4 Hz, chosen to mimic the predominant frequency of light to be reflected from a citrus canopy under light breeze, was observed to strongly deter ACP from traps. While several colors of artificial light were observed to improve trap catch significantly under forced choice experiments, lighting did not have a significant effect on trapping in more natural conditions with established ACP colonies on citrus trees. Results suggest that including lights in customized ACP traps is not a cost-effective approach for improving catch, especially as it may result in greater likelihood of theft.

Non-technical summary: Trapping of psyllids in the field is a critical step for surveillance and early detection / control of Huanglongbing (HLB). We tested a variety of artificial lighting conditions to incorporate into custom 3D printed traps used to capture psyllids that could be easily tested for HLB. While UV and yellow significantly enhanced catches of ACP when forced to choose between lighted and unlit traps, lights were not shown to be effective at enhancing catch under more natural conditions with established ACP colonies, suggesting that incorporation of lights is an unnecessary expense.

IRCHLB-P9-108

Field Assays of 3D Printed Asian Citrus Psyllid (*Diaphorina citri*) Trapping Systems

James SNYDER[1], Amy C. HOWE[1], Stefanie DOWLING[1], **Eric ROHRIG[1]**, Chandrika RAMADUGU[2], Russell MIZELL[3], Gregory SIMMONS[4], Ruth HENDERSON[5], and Susan HALBERT[1]

[1]Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL
[2]University of California, Riverside, CA
[3]University of Florida
[4]USDA-APHIS, CA
[5]Citrus Research Board, CA

Abstract: Insect traps monitor populations responsible for spreading vectored diseases; however, the early detection of infected psyllids (ACP) responsible for citrus greening remains a challenge, especially in regions where these HLB-associated Liberibacters are not yet widespread (California). Using 3D print-deposition technology, our researchers have designed and tested preliminary ACP traps using different attractive shapes, colors and photometrics. Traditional sticky card traps are messy, capture specimens indiscriminately, and make tissue analysis for Liberibacter nearly impossible. Our printed traps are ACP-specific, reducing nontarget bycatch, and, most

importantly, preserve specimens for molecular tissue analysis. All traps were created using Computer Aided Design (CAD) software, exported as stereo lithography (STL) files, and printed on Cartesian Fused Depositional Modeling (FDM) 3D printers using polylactic acid (PLA) filaments. Evaluations were conducted in the Florida laboratory using cage trials and in field psyllid cages maintained in Pomona, CA. The traps having the highest success rates were then used for field trials. To determine trap efficacy, field assays were conducted at two locations in central Florida during 2018. A randomized block design was used to divide each testing area into eight equidistant blocks. Two different 3D printed traps and one yellow ACP trap sticky card (Alpha Scents, Inc.) were deployed per block, with samples collected weekly from May 02 through July 18, 2018. Trap positions were rotated every week within their block, allowing researchers to sample large areas while adjusting for the natural variation in psyllid populations related to the new flush in plant growth. The number of psyllids caught per trap style were compared with those detected on sticky cards as a standard of measure. Field tests demonstrated trap catch rates suitable for tissue recovery and analysis.

Non-technical Summary: Asian citrus psyllid (ACP) traps were created using 3D print technology to improve ACP-target catch, eliminate sticky card mess, and preserve ACP. These are suitable for tissue identification and qPCR testing for citrus greening related pathogens. Field tests also demonstrate trap catch rates that is about 50% of the capture rate recorded from disposable sticky card counterparts; this number will be adequate for monitoring the HLB pathogens.

IRCHLB-P9-109

Genetic manipulation of Asian Citrus Psyllid (ACP), *Diaphorina citri*, by CRISPR/Cas9 technology to mitigate the effects of Huanglongbing in Florida citrus

Nabil KILLINY[1], Yulica SANTOS-ORTEGA[1], Siddarame GOWDA[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: Experiments to genetically modify Asian citrus psyllid (ACP) genome were carried out using CRISPR/Cas9 technology towards debilitating Asian citrus psyllid (ACP) as the vector of *Candidatus* Liberibacter asiaticus (CLas), and the putative associated agent of citrus Huanglongbing (HLB). Initially, we targeted the genes involved in the development of adult ACP wings, abnormal wing disc (*Awd*) and the muscle protein, troponin (*Tro*) involved in the development of the sucking apparatus in the mouth. Protospacer sequences were identified for *Awd* and *Tro* genes of ACP based on the gRNA design tool (PNA Bio), adjacent to the Protospacer adjacent motif (PAM). The selected sequences were uniquely representative in the ACP genome without nonspecific interactions. The protospacer was positioned with dU6-2 promoter towards the 5’ end and the tracRNA to the 3’ end to generate the desired gRNA. Additionally, the 3’ of this region contains the Cas9 coding sequence flanked by the SV40 nuclear localization signals. The delivery of the gRNA vector DNA was achieved via soaking of the ACP eggs. The eggs were transferred to an artificial media recently developed in our laboratory. After hatching in the artificial media, nymphs were transferred to young *Citrus macrophylla* plants with new flushes. In the experiments with *Tro* gRNA/Cas9 vector, there was considerably less honeydew produced by nymphs compared to control (treated with empty vector) suggesting impaired muscles of the sucking apparatus. *Awd* gRNA/Cas9 vector caused wing malformation. Additionally, we will target the genes in the ACP genome involved in the acquisition of CLas, ACP survival, insecticide resistance, fecundity, flight, and sex ratios. The successful transformation events potentially will contribute to mitigating the effects of Huanglongbing in

Florida citrus.

Non-technical summary:
New biotechnological techniques may provide an out-of-the-box solution to control Huanglongbing. We used CRISPR/Cas9 technology to genetically edit the Asian citrus psyllid genome to interfere with the transmission of *Candidatus* Liberibacter asiaticus, the associated agent of Huanglongbing.

IRCHLB-P9-110

Host plant selection in the Asian citrus psyllid is affected by salinity stress in citrus plants

Marilene FANCELLI[1], Maria Ângela Cruz Macêdo dos SANTOS[2], Mauricio Antonio COELHO FILHO[1]

[1]Embrapa Mandioca e Fruticultura, Brazil, [2]Universidade Federal do Recôncavo da Bahia, Brazil

Abstract: The Asian citrus psyllid (ACP), *Diaphorina citri* (ACP), is a vector of Huanglongbing. Young shoots provide a better oviposition substrate for ACP adults. Considering that the presence of new shoots in citrus plants depends on the availability of water, the manipulation of abiotic stress can influence ACP´s behavior. This study aimed to evaluate the effects of saline stress in Rangpur lime plants on the host-plant selection behavior of *D. citri*. Two contrasting salinity levels were investigated: control (1.7 dS m^{-1}) and stressed (10 dS m^{-1}). The experiments were carried out under controlled conditions and consisted in: study of non- preference for feeding or oviposition of ACP adults on citrus plants (5 replicates) and behavioral responses of ACP adults to volatiles of citrus plants submitted to contrasting levels of salinity (30 responsive females). The seedlings were transplanted to plastic cups containing washed and sterilized sand and kept in trays with solution fertilizer control. For the non-preference studies under free choice conditions, 30 ACP adults without sex determination were released in a cage containing plants of each treatment evaluated. For tests conducted without choice, a plastic cage involved each plant and five *D. citri* pairs were introduced through a small hole placed in the upper part of the cage. In behavioral bioassays, a female starved for 1 h was used. For each replicate, a fresh insect was introduced and the olfactometer was turned 90º to minimize the bias. The insect behavior was observed for 10 min. Stressed citrus plants (10 dS m-1) presented differential physiological responses 15 days after saline stress imposition. ACP adults were less attracted to stressed plants and the attractiveness might be related to chemical and visual cues. Also, stressed plants were less oviposited by *D. citri*.

Non-Technical summary: Knowledge about interactions between ACP and salinity may be useful to support development of management strategies under field conditions, preventing or minimizing insect settlement and hence reducing the chances of HLB dissemination.

IRCHLB-P9-111

Impact of transgenic grapefruit and orange plants expressing a *Bt toxin* gene on the Asian citrus psyllid

Vladimir ORBOVIĆ[1], Seyed Ali RAVANFAR[1], Xuedong CHEN[1], Yuting CHEN[3], Bryony BONNING[2] and Lukasz STELINSKI[1]
Citrus Research and Education Center, University of Florida/IFAS, Lake Alfred, FL[1], Entomology and Nematology Department, University of Florida, Gainesville, FL[2], Present address: Department of Entomology, North

Carolina State University, Raleigh, NC[3]

Abstract: Efficient management of Asian citrus psyllid (ACP) populations can lead to a reduction in the spread of HLB-inducing bacteria in newly planted citrus groves in Florida. Bt toxins produced by the bacterium *Bacillus thuringiensis* are natural insecticides that could be delivered to ACP *via* transgenic citrus plants. We produced transgenic plants of Duncan grapefruit and Valencia sweet orange expressing a *Bt toxin* gene (Cry1Ba) [1]. For both Duncan and Valencia, transformation rate was below 1%. Two transgenic grapefruit plants exhibited low relative *cry1Ba* transcript levels (3-4 times higher than in wild-type-WT) while the other 12 as well as seven orange plants had relative transcript levels of between 25 and 820. All transgenic plants appeared phenotypically similar to their WT counterparts. The results of detached leaf bioassays to determine possible insecticidal activity of the Bt toxin on ACP will be presented.

Non-Technical summary: Delivery of a Bt toxin to Asian Citrus psyllids was attempted via transgenic grapefruit and orange plants. Within the laboratory, insects were allowed to feed on leaves cut from transgenic plants. The results of these bioassays will be reported.

Citations:

Orbovic V, Grosser JW (2015). Citrus transformation using juvenile tissue explants. In Agrobacterium protocols-Methods in Molecular Biology, W. K, ed. (USA: Humana Press Inc.), pp. 245-257.

IRCHLB-P9-112

Lower Asian Citrus Psyllid populations by timing sprays based on flush development

L. Gene ALBRIGO and Lukasz L. STELINSKI

CREC, IFAS, UF, 700 Experiment Station Rd., Lake Alfred, FL

Abstract: The citrus disease huanglongbing (HLB) is endemic in Florida. It is presumably caused by the *Candidatus* Liberibacter asiaticus (CLas) phloem limited bacteria and is vectored from tree to tree by the Asian citrus psyllid, *Diaphorina citri* Kuwayama, (ACP). It is a major factor in the approximate 70 % reduction in Florida citrus production since 2003 (NASS, 2018). Even though HLB is present in most citrus trees in Florida, many people believe that continued control of the ACP population is beneficial in minimizing the level of bacteria in trees that are already infected with CLas. Adult ACP require immature leaves to lay eggs on and for nymph stages to develop. If adult ACP are very low when a new flush occurs then few eggs canbe laid and development of a new population is minimal. Spraying for ACP adults at budbreak of a new flush, before feather flush is available, should minimize ACP population development in a new season. Spraying at budbreak of each new flush in the growing season should continue to minimize ACP population development. In 2017 blocks of round oranges were sprayed at budbreak in the spring and again when adult ACP started to increase. These two sprays maintained low ACP populations until after flowering, eliminating the need to spray during the bee active period. In 2018, sprays were applied during budbreak of later flushes also maintaining lower ACP populations. This was accomplished with 5 or 6 sprays during this growing season. This spray timing method should minimize psyllids for HLB spread in new areas as well.

Non-Technical Summary: The Asian citrus psyllid (ACP) vectors Liberibacter asiaticus bacteria associated with huanglongbing (HLB) disease of citrus. Adult ACP need immature flush to lay eggs and have nymph stages develop into adults which vector the disease. Spraying adults at flush budbreak and once more near leaf full expansion minimized population development in the 2017 and 2018 growing seasons.

Citations:

NASS, 2018. Citrus Summaries https://www.nass.usda.gov/Statistics_by_State/Florida/Publications/Citrus/Citrus_Summar y/index.php

IRCHLB-P9-113

Production of genetically modified *Murraya koenigii* plants expressing a *Bt toxin* gene

Seyed Ali RAVANFAR[1], Yuting CHEN[3], Bryony BONNING[2], **Vladimir ORBOVIĆ[1]**

Citrus Research and Education Center, University of Florida/IFAS, Lake Alfred, FL[1], Entomology and Nematology Department, University of Florida, Gainesville, FL[2], Present address: Department of Entomology, North Carolina State University, Raleigh, NC[3]

Abstract: The introduction of Bt toxin genes into transgenic plants for control of insect pests has been successfully employed in multiple crops for more than two decades [1-2]. For Asian citrus psyllid (ACP) management in citrus groves, delivery of Bt toxin to ACP can be attempted *via* transgenic citrus plants or *via* alternative ACP host plants. The production and commercialization of citrus trees expressing a Bt toxin may face resistance from both the general public and regulatory agencies. For this reason, production of plants other than citrus that can be grown in citrus groves and attract ACP would be preferable. *Murraya koenigii* is a good candidate alternative host for ACP as the psyllids are attracted to the young shoots of this plant [3]. We have produced transgenic plants of *M. koenigii* expressing a *Bt toxin* gene (Cry1Ba). The transformation success rate was low at 0.7%. The percentage of shoots that grew roots after incubation on different rooting media was also low (0.00 - 0.94%). Because of poor rooting, some shoots were micrografted onto Carrizo rootstock. When compared to wild-type (WT), the transgenic plants did not exhibit any significant morphological phenotypic changes except for a lower growth rate. The relative transcription level of *cry1Ba* in five transgenic, soil-adapted lines ranged from 30 to1900. Determination of Bt toxin levels in leaf tissue of these plants is in progress. When transgenic plants develop multiple, fully grown leaves, they will be used in “detached leaf bioassays” to estimate the effect of Bt toxin on ACP mortality.

Non-Technical summary: Genetically modified Indian curry leaf plants were produced as a tool for ACP management in citrus groves. A gene encoding a Bt toxin was introduced into this species which attracts ACP more than citrus. After feeding on young leaves of transgenic Indian curry leaf trees planted around citrus groves and ingesting the Bt toxin, ACP may die due to toxin-mediated disruption of the digestive tract.

Citations:

Calles-Torrez V, et al. (2017) Transgenic Bt corn, soil insecticide, and insecticidal seed treatment effects on corn rootworm (Coleoptera: Chrysomelidae) beetle emergence, larval feeding injury, and corn yield in North Dakota. J. Econ. Entom. 111:348-360.

Li YH, et al (2017) Bt rice in China - focusing the non-target risk assessment. Plant Biotech. J. 15:1340-1345.

Martini X, Kuhns EH, Hoyte A, Stelinski LL (2014) Plant volatiles and density-dependent conspecific female odors are used by Asian citrus psyllid to evaluate host suitability on a spatial scale. Arthropod-Plant Interact.8:453-460.

IRCHLB-P9-114

RNA interference of candidate effector genes promotes reduction of *Diaphorina citri* feeding

Inaiara de Souza PACHECO[1,2], Diogo Manzano GALDEANO[1], João Roberto Spotti LOPES[3], **Marcos Antonio MACHADO**[1,2]

[1]Centro de Citricultura Sylvio Moreira- Instituto

Agronômico de Campinas, [2]Universidade Estadual de Campinas, [3] Escola Superior de Agricultura Luiz de Queiroz-Universidade de São Paulo

Abstract: Insect effectors are molecules secreted mainly by salivary glands that acts modulating plant physiology and/or favoring establishment and transmission of phytopathogens. Feeding is the principal vehicle of acquisition and transmission of *Candidatus* Liberibacter asiaticus (*Ca*. Las) by *D. citri*. The aim of this study was predict putative candidate effectors that may act on Huanglongbing pathosystem. After an extensive bioinformatic analysis, followed by gene expression investigations, we selected three *D. citri* candidate effectors genes. These candidates are small secreted proteins exclusive of *D. citri*. Moreover, these genes were over expression in the head of insect compared to body and modulated by the presence of *Ca*. Las. In order to evaluate the action of candidate effectors on *D. citri* feeding, double-stranded RNAs (dsRNAs) of target genes were delivered to *D. citri* adults via artificial diet for five days. The mortality was evaluated daily and feeding capability were evaluated by the production of honeydewdroplets. *In vitro* bioassays showed increase of mortality (nearly to 45%) on effector-silenced insects and decrease of 65% on honeydew production. Moreover, after five days of *D. citri* feeding on artificial diet containing dsRNA of target genes, the live insects were transferred to young leaves of Cravo Lime seedlings and observed for five days. After this period, the reduction of honeydew production was 65% and increased the mortality to 40%. These preliminary results indicate that gene silencing of *D. citri* effectors may promote alterations on *D. citri* feeding behaviour, potentially acting on insect control and/or disease spreading.

Non-Technical summary: We are looking for proteins that are present on *D. citri* saliva and could promote alterations in *D. citri* feeding behavior. Three proteins were selected by bioinformatics and gene expression analysis. To prove the action of these proteins on *D. citri* feeding, gene silencing weas performed. Mortality of *D. citri* and reduction of honeydew production were observed as a result of gene silencing, indicating that selected proteins are important for *D. citri* feeding.

IRCHLB-P9-115

RNA interference of Ras-like family small GTPases genes of Asian citrus psyllid, *Diaphorina citri* (Kuwayama), vector of citrus Huanglongbing

Marcos A. MACHADO[1], Diogo M. GALDEANO[1] and Vitória B. RODRIGUES[1,2]

[1]Centro de Citricultura 'Sylvio Moreira', Instituto Agronômico de Campinas, Cordeirópolis-SP, Brazil, [2]Centro Universitário Hermínio Ometto – FHO, Araras-SP, Brazil

Abstract: The Asian citrus psyllid (ACP), *Diaphorina citri* (Hemiptera: Liviidae) has become one of the most important pest in citrus in the recent years, due to the increasing damage caused to plants by presumed bacteria transmission, *Candidatus* Liberibacter americanus (CLam) and *Candidatus* Liberibacter asiaticus (CLas), associated agents of Huanglongbing (HLB). The gene knockdown by RNA interference (RNAi) is one of the promises of new technologies used in the pest control. The main challenge of using RNAi is the choice of the target gene and the stage of pest development that can trigger a lethal RNAi response. Ras-like family small GTPases (RSGs) are proteins of signaling pathways that link extracellular signals via trans-membrane receptors to cytoplasm, acting in growth, cell division and trafficking through the golgi apparatus, nucleus and endosomes. The aim of this study was evaluate the expression profile of RSGs genes at different stages development of *D. citri* and delivery of double strand RNAs (dsRNAs) to the insect. Firstly, RSGs genes were identified in *D. citri* transcriptome data from

NCBI and primers were designed to dsRNAs synthesis and qRT-PCR. The expression levels of four RSGs genes (*ran*, *rab-2*, *rab-6* and *rab7a*) were evaluated in all *D. citri* stages (first, second, third, fourth and fifth nymphal instars, female and male). Differences in the expression levels of these genes were observed along the development of *D. citri*. For instance, *ran* was up-regulated in fourth and fifth nymphal instars and *rab-6* was up-regulated in third nymphal instar. The *ran* dsRNAs (500 ng.µL^{-1} and 1000 ng.µL^{-1}) were delivery by topical application in nymphs. qRT-PCR analysis verified the down-regulation of the target gene and the mortality of *D. citri* increased over time. Thus, *ran* may used as a candidate target for dsRNA-based pesticide for ACP control.

Non-Technical summary: The management of HLB is based on the use of healthy young plants from protected nurseries, eradication of infected plants and chemical control of the vector. RNAi have been used as new approaches to the control of pests and vectors. Here we investigated the expression levels candidate targets genes of *D. citri* and one gene was knockdown, increasing the mortality of *D. citri*. Thus, the RNAi may used to control *D. citri*.

IRCHLB-P9-116

Silencing genes implicated in osmoregulation as a potential control in *Diaphorina citri*

Nabil KILLINY[1] and Yulica SANTOS-ORTEGA[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: *Diaphorina citri* feeds exclusively on citrus phloem sap (sucrose-rich fluid). Thus, it faces a constant challenge of maintaining the osmotic potential gradient between the gut and hemolymph, which can increase up to five-fold after each feeding. Sucrose cannot traverse cell membranes and thus is not absorbed by midgut cells unless it is broken down into monomers. In Hemiptera, three mechanisms contribute to regulating the osmotic potential: 1) hydrolysis of sucrose to fructose and glucose; 2) transglycosidation of sucrose into oligosaccharides (honeydew secretion); and 3) dilution by water either by feeding on xylem sap or by transferring water from the hindgut. In this study, a sucrose hydrolase homolog (*DcSuh*) and Aquaporin (*DcAqp*) were targeted by RNA-interference (RNAi). The highest gene expression levels of both genes was found in the 4th and 5th instar nymphs. dsRNA-mediated RNAi was achieved through topical feeding. The reduction in gene expression for both genes caused nymph mortality and reduced the lifespan of adults. For *DcSuh*, some emerged adults from treated nymphs showed a swollen abdomen phenotype, indicating that these insects were under osmotic stress. Metabolomic analyses using GC-MS showed an accumulation of sucrose and a reduction in fructose, glucose and trehalose in treated nymphs, confirming the inhibition of sucrose hydrolase activity. Additionally, most of the secondary metabolites were reduced in the treated nymphs, indicating a reduction in the biological activities in *D. citri* and that they were under stress. For *DcAqp*, a decrease in the body length and body weight of the emerged adults was observed. In addition, accumulation of uric acid was found using GC-MS. Our findings indicate that osmoregulation might be a potential target for effective RNAi control of *D. citri*.

Non-technical summary: New biotechnological techniques may provide an out-of-the-box solution to control the Huanglongbing. We used RNAi technology to target the osmoregulation process in *Diaphorina citri* as a new control strategy for Huanglongbing

IRCHLB-P9-117

Trapping of male *Diaphorina citri* with combinations of vibrational and light stimuli

Richard MANKIN[1] and Maximilian KIEFER[2]

[1]USDA ARS Center for Medical, Agricultural, and Veterinary Entomology, Gainesville, FL USA, [2]Carnegie Mellon University, Pittsburgh, PA, USA

Abstract: Efficient trapping of *Diaphorina citri* (ACP) can assist the timing and distribution of management treatments against this important pest which vectors bacteria causing a deadly citrus greening disease. Based on previous studies that demonstrated attraction of males to mimics of female vibrational signals (Lujo et al. 2016) and attraction of both males and females to ultraviolet and other light frequencies (Paris et al. 2017), traps have been constructed that incorporate both types of stimuli, and testing of trapping efficacy is in progress. In preliminary bioassays, the light stimulus increases attraction of flying ACP to the tree, and once on the tree, males walking on the branches are attracted to the vibrational signals. Additional studies are planned with the goal to increase the attractivity of the vibrational and light signals, decrease the (battery) power usage, and decrease the overall cost of the trap. Ultimately the management of ACP in enclosed screened areas or greenhouses with high-value citrus and low ACP populations can benefit from the use of such traps, so long as they maintain high trapping efficiency at moderate cost.

Non-Technical summary: Efficient trapping of ACP can improve pest management of this important vector of bacteria causing deadly citrus greening disease. One trapping system under study combines vibrational and light stimuli, each of which has been demonstrated to be attractive to males when presented alone in a trap, and attraction significantly increases when the two stimuli are presented together. Efforts are in progress to increase the attractiveness of the stimuli, decrease power usage, and decrease overall trap cost; the management of ACP in enclosed areas can benefit from traps like these with potential to maintain high trapping efficiency at moderate cost.

Citations:

Lujo S, Hartman E, Norton K, Pregmon EA, Rohde BB, and Mankin RW. 2016. Disrupting mating behavior of *Diaphorina citri* (Liviidae). J. Econ. Entomol. 109: 2373-2379.

Paris TM, Allan SA, Udell BJ, Stansly PA. 2017. Evidence of behavior-based utilization by the Asian citrus psyllid of a combination of UV and green or yellow wavelengths. PLoS One 12: eo189228.

IRCHLB-P9-118

Use of 3D Technology for Asian Citrus Psyllid (*Diaphorina citri*) Trapping Systems

James SNYDER[1], Amy C. HOWE[1], Stefanie DOWLING[1], **Eric ROHRIG[1]**, Chandrika RAMADUGU[2], Russell MIZELL[3], Gregory SIMMONS[4], Ruth HENDERSON[5], and Susan Halbert[1]

[1]Division of Plant Industry, Florida Department of Agriculture and Consumer Services, Gainesville, FL
[2]University of California, Riverside, CA
[3]University of Florida, FL
[4]USDA-APHIS, CA
[5]Citrus Research Board, CA

Abstract: Effective disease management of HLB requires large scale testing of the psyllid vectors. The currently used yellow sticky card traps are excellent for monitoring populations required for decisions regarding treatment options. To contain the spread of HLB, it is essential to conduct testing of the psyllids for the presence of *Candidtatus* Liberibacter asiaticus (CLas) associated pathogen. To facilitate this, we have designed psyllid traps that can preferentially capture Asian citrus psyllids and preserve them so that they will be suitable for disease monitoring and spread. Using Computer Aided Design (CAD) software and Cartesian

Fused Depositional Modeling (FDM) 3D printers, traps have been produced that are ACP-specific. These traps attract psyllids, reduce nontarget bycatch, and preserve specimens for molecular tissue analysis. Traps were designed using Rhinoceros 5 (CAD) software, exported as stereolithography (STL) files and printed using standard polylactic acid (PLA) filaments. Laboratory cage trials were conducted to measure average catch rates of new trap designs. Models having the highest success rates were chosen for field testing throughout central Florida. The use of 3D technology is a valuable tool in the development of early pest detection methods. This poster presentation highlights the design features of our 3D printed traps that make them optimal for ACP detection and screening for citrus greening disease associated pathogen.

Non-technical Summary: Asian citrus psyllid (ACP) traps were created using 3D print technology to improve ACP-target catch, eliminate sticky card mess, and preserve ACP for identification and qPCR testing for HLB associated pathogens. Print technology allows researchers to quickly create prototypes that incorporate the latest research into their design schemes. Developments in trapping design have improved success rates in trapping this vector of citrus greening disease.

IRCHLB-10: Regulatory and Programs Updates Oral Presentations

IRCHLB-O5b-01

Introduction of Florida citrus varieties into California. Project status and regulatory developments in citrus germplasm movement.

Irene LAVAGI[1], Greg GREER[1], Jude GROSSER[2], Frederick GMITTER[2], Kim D. BOWMAN[3], Ed STOVER[3], Greg McCOLLUM[3], Ben ROSSON[4], MaryLou POLEK[5], Robert KRUEGER[5], Melinda KLEIN[6], and Georgios VIDALAKIS[1]

[1]University of California, Riverside, USA; [2]University of Florida, Lake Alfred, USA; [3]USDA-ARS, Ft. Pierce, USA; [4]Florida Department of Agricultural and Consumer Services, Chiefland, USA; [5]USDA-ARS, Riverside, USA; [6]Citrus Research Board, Visalia, USA

Abstract: Our Citrus Research Board (CRB, California) and USDA-APHIS Huanglongbing (HLB) Multi-Agency Coordination (MAC) funded projects, have been transforming citrus germplasm movement in the USA since October 2014. By February 2016, the first protocol (SOPs, facilities standards, permits and material transfer agreements) for the introduction of varieties from Florida into California was developed. This first-generation protocol allowed for a single introductory pathway of accessions that had completed therapy and testing at the Bureau of Citrus Budwood Registration in Florida, to the Citrus Clonal Protection Program (CCPP) in California. In February 2018, scientists, regulators and stakeholders at a national meeting, acknowledged that this single pathway was not sufficient to support current HLB research efforts. In response, the CCPP adjusted its SOPs for introductions from HLB areas, and in May 2018, a USDA/CDFA permit not limited to Florida tested germplasm was issued. This permit, combined with the international citrus introduction services (USDA-APHIS, Beltsville, Maryland), allows for any citrus propagative material to be introduced into the CCPP, and after quarantine release to become available to all of the USA via the National Clean Plant Network (NCPN). Despite this positive development however, and because of the lengthy introduction time (~24 months), the need for flexible and efficient citrus germplasm movement protocols persisted. Therefore, in October 2018, a

regulatory summit laid the foundation for the future development of risk assessment based protocols for the interstate movement of citrus propagative materials (pollen, seed, and budwood) for HLB research and ultimately for commercial use. To date, over 80 citrus varieties have been introduced into the CCPP under the current protocols and 6 have been released from quarantine. We expect private entities as well as the Florida Foundation Seed Producers (FFSP) and the New Varieties Development and Management Corporation (NVDMC) to start coordinating the use of these varieties soon.

Non-Technical summary: CRB and HLB-MAC funded projects for the introduction of citrus germplasm from Florida into California have become a valuable platform for scientific collaboration and regulatory debate for the development of novel risk-based citrus germplasm movement protocols for HLB research and ultimately commercial use.

IRCHLB-O5b-02

Methods for Legal Interstate Movement of Regulatory Articles from Quarantine Areas in the US for Citrus Greening and Citrus Canker Research

Zhaowei LIU, Colin STEWART, Angela McMELLEN BRANNIGAN

USDA-APHIS-PPQ, Riverdale, Maryland, USA.

Abstract: Citrus production is over a $3 billion part of U.S. agriculture. The introduction and spread of Huanglongbing (citrus greening) and citrus canker (*Xanthamonus* spp.) have resulted in increased production costs and decreased production in areas in which they are found. To protect uninfected areas, interstate movement of citrus propagative materials is only allowed from APHIS-approved exclusionary facilities that meet regular testing and inspection requirements. Citrus materials that do not originate from these structures and are potentially infected with the disease-causing bacteria are prohibited from moving interstate from a quarantined areas unless the movement of the material is for research purposes. The U.S. Department of Agriculture, Animal and Plant Health Inspection Service, Plant Protection and Quarantine (USDA-APHIS- PPQ) regulates plant pests and biological control organisms. PPQ issues permits to allow for the interstate movement of regulated plant parts for research on citrus greening and citrus canker. These permits may be used for diagnostic, laboratory, growth chamber, and/or greenhouse research. Working with our state counterparts, we develop permit conditions to mitigate the risk of moving disease between states. We generally require that research with these materials/organisms be performed in a USDA APHIS-inspected containment facility and end with the destruction of the materials at the conclusion of the research. Citrus seeds from certified trees may be moved interstate for field research if seeds are pre-treated with APHIS- designated chemical disinfectants. Permit holders are also required to fulfill the requirements of state regulatory authorities that regulate other citrus pests. To facilitate access of the citrus research community to necessary materials to advance research, PPQ strives to ensure that permitting decisions are grounded in science, timely, consistent, risk-based and as transparent as possible.

Non-technical Summary: USDA-APHIS works with researchers around the country to safely move research materials between states to facilitate the advancement of pathosystems that are of utmost concern to the U.S. citrus industry. APHIS works with state departments of agriculture to determine ways to mitigate risk of disease spread while allowing research to continue.

IRCHLB-O5b-03

A qualitative model to estimate the risk associated with the movement of bulk citrus between the regional quarantine zones in

California.

Sara GARCIA-FIGUERA, Neil MCROBERTS.

Department of Plant Pathology, University of California, Davis, CA.

Abstract: When fresh market citrus fruit is harvested, it is loaded in bins on trailers for transportation to the packinghouses. Bacteriliferous psyllids can be moved with the fruit on trailers and transportation corridors can thus be a pathway for Huanglongbing (HLB) dispersal. Because of this, the state of California was divided into seven regional quarantine zones. Movement of bulk citrus between any two zones is prohibited unless it complies with uniform risk mitigation requirements. Since the quarantine regulation was implemented, the citrus industry has asked for more flexible mitigation requirements in response to evidence-based assessment of risk. To address this issue, we developed a model based on the international framework for plant pest risk analysis that provides a qualitative estimation of the risk. A series of risk factors are assigned an ordinal qualitative rating through expert opinion and available data. The model is built as a tree in which the combination of ratings for risk factors on each node of the tree is computed using the software DEXi, generating a risk matrix with zones of origin (harvest) as columns and zones of destination (packing) as rows. The model is being used by a joint industry/regulatory agency working group to review the quarantine regulation.

Non-Technical summary: Following a request from the citrus industry, we developed a model to estimate the risk of spreading HLB when transporting bulk citrus fruit between regional quarantine zones in California. This model assigns a risk rating of *Negligible*, *Low*, *Medium* or *High* to any transportation route between two zones and could be used to adjust mitigation requirements to the level of risk.

IRCHLB-O5b-04

Citrus Genome Database resources for HLB and *Citrus* genomics, genetics, and breeding research

Jodi HUMANN[1], Morgan FRANK[1], Sook JUNG[1], Chun-Huai CHENG[1], Taein LEE[1], Heidi HOUGH[1], Katheryn BUBLE[1], Ping ZHENG[1], Kristin SCOTT[1], Julia PIASKOWSKI[1], Mirella FLORES[2], Surya SAHA[2], Lukas A. MULLER[2], Fred GMITTER[3], Albert ABBOTT[4], Dorrie MAIN[1]

[1]Department of Horticulture, Washington State University, Pullman, WA; [2]Boyce Thompson Institute, Cornell University, Ithaca, NY; [3]Horticultural Sciences, University of Florida, Gainesville, FL; [4]Forest Health Research & Education Center, University of Kentucky, Lexington, KY

Abstract: The Citrus Genome Database (CGD, www.citrusgenomedb.org) is a resource for HLB and citrus researchers and citrus breeding programs. CGD has tools for citrus and *Ca.* Liberibacter genomics research such as the JBrowse genome browser, a BLAST tool to search genome sequences, and a genome synteny viewer to compare analyzed genomes. Currently, CGD has genomes of six different *Citrus* species and the complete genomes of *Ca*. Liberibacter asiaticus, americanus, africanus, and solanacearum, as well as the complete genome of *Liberibacter crescens*. CitrusCyc, which is a tool to explore the metabolic maps of the JGI produced *C. clementina* and *C. sinensis* genomes, is also available. CGD has a functionally annotated reference transcriptome (RefTrans) generated from the assembly of published RNA-Seq and EST datasets from *C. sinensis*. For citrus, genetic marker, genetic map, and QTL data, current with published papers, is viewable and searchable in the database. Genetic maps can be viewed and compared using

MapViewer, allowing export of map images, dot plots, and correspondence matrices for use in presentations and publications. For citrus breeding programs, CGD has the Breeding Information Management System (BIMS) which is an online system to manage and analyze private breeding data. BIMS works with the Android app called Field Book, which is used to collect the field data. Public citrus phenotype data from the USDA-GRIN database is available to explore with BIMS by all users. CGD is being developed based on user feedback and needs from the scientific community to ensure it provides integrated data and tools that enable citrus discovery and crop improvement. CGD is supported by USDA-NRSP10, NSF-PGRP, USDA-SCRI
and US Land Grant Universities.

Non-technical summary:
The Citrus GenomeDatabase (CGD, www.citrusgenomedb.org) is an integrated community resource for citrus genetics, genomics, and breeding research. The database has tools for citrus breeding management as well as tools for basic and applied research of the host and HLB pathogen.

IRCHLB-O5b-04

Citrusgreening.org: A Public Repository of Systems Biology Resources for the Huanglongbing (HLB) Disease Complex

Mirella FLORES-GONZALEZ[1], Prashant S HOSMANI[1], Justin GEORGE[2,3], Marina MANN[4], John S. RAMSEY[5], Angela KRUSE[4], Denis WILLETT[6], Lukasz STELINSKI[3], Susan BROWN[7], Stephen L. LAPOINTE[2], Michelle HECK[1,4,5], Lukas MULLER[1] and **Surya SAHA**[1]

[1] Boyce Thompson Institute, 533 Tower Road, Ithaca, NY 14853. [2] United States Department of Agriculture, Agricultural Research Service, 2001 South Rock Road, Fort Pierce, FL 34945. [3] University of Florida, Entomology and Nematology Department, Citrus Research and Education Center, 700 Experiment Station Rd., Lake Alfred, FL 33850. [4] Plant Pathology and Plant Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY. [5] USDA-ARS Emerging Pests and Pathogens Research Unit, Robert W. Holley Center for Agriculture and Health, Ithaca, NY 14853. [6] Cornell University, Department of Entomology, 630 West North Street, Barton Lab, Geneva, NY 14456. [7] Kansas State University, KSU Bioinformatics Center, Division of Biology, Manhattan, KS 66502

Abstract: We have created an open access and public web portal with pathosystem-wide resources and bioinformatics tools for citrus, the vector Asian citrus psyllid (ACP) and multiple pathogens including *Candidatus* Liberibacter asiaticus (CLas). This is the first example of a database to use the pathosystem as a more holistic framework to understand insect biology and disease transmission. The endeavor will connect and mine data sets generated by the community to study the citrus greening disease complex. We welcome submission of relevant data sets to enable sharing and allow the community to analyze data in an integrated system. The portal contains a variety of tools for omics data. DiaphorinaCyc and CitrusCyc pathway databases can be used to analyze transcriptomics and proteomics results to identify pathways with differentially regulated genes. Psyllid Expression Network (PEN) contains profiles of ACP genes from multiple life stages, conditions and hosts. Citrus Expression Network (CEN) contains public expression data for citrus from NCBI. All tools like JBrowse, Biocyc, Blast, CEN and PEN connect to a central database containing gene models for citrus, ACP and multiple Liberibacter pathogens. The portal also includes raw and annotated data from electrical penetration graph (EPG) recordings of ACP feeding on citrus and near relatives from

published experiments. EPG datasets are extensive and only partially analyzed depending on particular questions of interest. Full datasets are released to encourage data mining and analysis by researchers interested in addressing questions related to ACP feeding behavior not addressed in our original publications. The portal includes user-friendly manual curation tools to allow the research community to continuously improve this knowledge base as more experimental research is published. Bulk downloads are available for all genome and annotation datasets from the FTP site (ftp://ftp.citrusgreening.org). The portal can be accessed at https://citrusgreening.org/.

Non-technical Summary:
Citrusgreening.org is an open access and public web portal with information and omics resources for the host citrus, the vector psyllid and multiple pathogens including CLas. This data and resource hub is designed as a central repository for all data related to the citrusgreening disease complex. Scientists are encouraged to submit their data sets to the portal for sharing with the grower and research community.

IRCHLB-O5b-05

Progress in breeding rootstocks to prevent or mitigate HLB in commercial trees

GROSSER, J.W[1], F. G. GMITTER[1], W.S. CASTLE[1], A.A. OMAR[1] and L.M. CANO[2].

[1] University of Florida, IFAS, Citrus Research and Education Center, Lake Alfred, FL USA
[2]University of Florida, IFAS, Indian River Research and Education Center, Fort Pierce, FL, USA.

Abstract: Genetic variability for HLB tolerance/resistance has been identified (grafted trees) in existing experimental rootstock germplasm planted throughout Florida. New rootstocks are being identified in these trials that show a reduced infection frequency, less severe symptoms once infected, as compared to commercial rootstocks. Many such rootstocks are also showing a faster and stronger recovery following infection in commercial trials. For the past 7 years, we have developed and utilized our high-throughput 'Gauntlet' screening program that focuses directly on the ability of a rootstock candidate to mitigate HLB in trees grafted with *Candidatus* Liberibacter asiaticus (CLas)-infected Valencia scion. To date, several thousand individual hybrids have been screened. The oldest 'Gauntlet' trees have now been in the field for over 5 years, and several promising new rootstocks have been identified. Although mostly PCR+, individual trees appear to have a reduced bacterial titer and are showing normal healthy growth and fruiting patterns. A few trees have tested negative for CLas in the scion, and several are negative for CLas in the roots. Seed trees of two of the most promising selections are producing abundant polyembryonic seed, and propagations for large-scale field trials are underway. Alternative means of propagation are being pursued for candidates where viable seed trees are not available. Patterns are emerging regarding successful genetic combinations, and this will be discussed in detail. Our goal is to develop rootstocks that will facilitate sustainable and profitable citriculture in an HLB-endemic Florida, eliminating the need for costly and unsustainable psyllid control.

Non-Technical Summary: New rootstocks have been identified in field trials that show a reduced HLB-infection frequency, and stronger recovery once initial infection, as compared to commercial rootstocks. Several of the most promising rootstock selections are being propagated from seed, tissue culture and/or cuttings for large-scale commercial evaluations with multiple scions. If successful, this approach would allow for sustainable, affordable production and marketing of all successful US scion cultivars currently in the marketplace.

IRCHLB-O5b-06

USDA Efforts to Develop Resistance and Tolerance to Huanglongbing in Citrus Scions

Ed STOVER[1], Randall DRIGGERS[1], Joseph KRYSTEL[1], Guixia HAO[1], David HALL[1], Goutam GUPTA[2], John HARTUNG[3], Elizabeth BALDWIN[1], Jinhe BAI[1], Anne PLOTTO[1], John MANTHEY[1].

[1]USDA/ARS, Ft. Pierce, FL, USA, [2]New Mexico Consortium, Los Alamos, NM, USA, [3]USDA/ARS, Beltsville, MD, USA.

Abstract: HLB-resistant or -tolerant cultivars will be essential to sustain the Florida citrus industry, with solutions needed in the short and long term. Potentially useful tolerance to HLB is present in cultivated citrus and greater resistance is apparent in more distant members of the gene pool. Several mandarin hybrids, including some released cultivars, display sustained growth and canopy health despite prolonged HLB infection. In a replicated trial of 50 selections and cultivars, decline in canopy growth appears to be a marked indicator of HLB-susceptibility even while trees are superficially healthy. This was not evident until after four years of field growth, even though trees were infected pre-planting. Several genotypes with *Poncirus* in the pedigree continue to show strong HLB-tolerance but not resistance. A *Poncirus*-derived scion cultivar, US SunDragon was released with this attribute, is being used in breeding, and some progeny are now fruiting. Hybridization is ongoing to combine HLB-tolerance from *Poncirus* and mandarin sources. Juice quality assessments are underway to explore juice blends from HLB-tolerant material. Several transgenics are showing reductions of *Candidatus* Liberibacter asiaticus (CLas) titer by 100-800 X and are now in field trials with the promise of greater HLB control than is seen in conventional material. The most successful transgenics to date are expressers of: a modified plant thionin; chimeras derived from the citrus proteome with membrane binding and lytic peptides connected by a linker; and ScFv directed at a CLas membrane protein or a protein secreted by CLas. It must be noted that pre-HLB Florida citrus plantings typically did not provide a return on investment until 7-8 years after planting. Validation of economic tolerance to HLB will likely require ten or more years of replicated field trials in multiple locations. Our best conventional and transgenic material has or is undergoing cleanup at FDACS DPI to facilitate such trials.

Non-Technical summary: Tolerance to HLB is present in some cultivated citrus and greater resistance may be drawn from more distant members of the gene pool. Strong resistance and even immunity should be possible using genetic engineering. Efforts are underway to produce HLB-resistant or tolerant scions in the USDA citrus breeding program.

Citations:

Hao, G., E. Stover, and G. Gupta, 2016. Overexpression of a modified plant thionin enhances disease resistance to citrus canker and Huanglongbing (HLB). Frontiers Plant Sci. 7 doi: 10.3389/fpls.2016.01078

Ramadugu, C., M. Keremane, S. Halbert, Y-P. Duan, M.L. Roose, E. Stover, R. Lee. 2016. Long term field evaluation reveals HLB resistance in *Citrus* relatives. Plant Disease 100:1858-1869.

Stover, E., S. Inch, M. Richardson, and D.G. Hall. 2016. Conventional citrus of some scion/rootstock combinations show field tolerance under severe huanglongbing disease pressure. HortScience 51:127-132

IRCHLB-10: Regulatory and Programs Updates Poster Presentations

IRCHLB-P10-119

A summary of HLB testing in California from 2008 – 2018 by the California Department of Food and Agriculture

Lucita KUMAGAI[1], Monica NEGRETE[1], Joe VIGLIENZONI[1], Magally Luque-WILLIAMS[1], Lydia RODRIGUEZ[1], Maribel DELTORO[1], Tina GALINDO[1], Qijun XIANG[2], Judith BROWN[3], Ying GUO[1], Patrick WOODS[1], Israfiel MOHAMMED[1], Katie POSIS[1], Lainey PHAN[1], Victoria HORNBAKER[1], Cheryl BLOMQUIST[1]

[1]California Department of Food and Agriculture (CDFA), Sacramento, CA, USA; [2]Jerry Dimitman Citrus Research Board/Citrus Pest Disease Prevention Program Lab, Riverside, CA, USA; [3]University of Arizona, Tucson, AZ, USA

Abstract: Huanglongbing (HLB) is a fatal disease of citrus associated with the bacterium, *Candidatus* Liberibacter asiaticus (CLas) and vectored by the Asian citrus psyllid (ACP). In 2008, ACP was first detected in San Diego, California, which triggered a statewide risk-based survey for HLB. In 2012, CLas was detected in ACP collected from a symptomatic residential tree in Los Angeles (LA) County. This tree was the first HLB-infected tree reported in California. Since then, 967 trees have been detected, expanding the HLB quarantine zone to 886 square miles, encompassing areas in 20 cities within LA, Orange, and Riverside counties. Trees adjacent to HLB infected trees have been intensively sampled by testing leaves from four quadrants per tree which has resulted in increased detection and tree removal. Lime, orange, and lemon were the most common citrus hosts reported. Symptoms have ranged from classic blotchy mottling and yellowing of leaves, to reduced leaf size and absence of fibrous roots with no visible leaf symptoms, even in leaves with high bacterial titer. Preliminary genotyping data on California CLas isolates show distinct groupings suggesting different origins of introduction. In the CDFA laboratory from 2012 to 2018, the average number of ACP samples increased per year from 33K to 68K and plant samples from 15K to 88K, an increase of 106% and 487% respectively. Statewide field sampling occurs uniformly throughout the year, with samples coming primarily from LA, Orange, Riverside, and San Diego counties. To date, HLB has only been detected in landscapes, not in commercial groves, with detections showing no seasonal pattern. The timely detection and immediate removal of HLB infected trees, in conjunction with ACP control, are key elements to lowering the inoculum level in the field, slowing the spread of HLB, and protecting California's commercial production of citrus.

Non-technical summary: 967 HLB positive trees have been confirmed in California. The detections have expanded the HLB quarantined zones to 886 square miles encompassing areas in 20 cities within the counties of LA, Orange, and Riverside. Statewide field sampling occurs uniformly throughout the year, with detections showing no seasonal pattern. To date, HLB has only been detected in landscapes, not in commercial groves. The CDFA's timely detection and immediate removal of HLB infected trees, in conjunction with ACP control, are key elements to lowering the inoculum level in the field, slowing the spread of HLB, and protecting California's commercial production of citrus.

Citations:

Zonghe, Y., B. Kasiborski, L. Kumagai, M. Nakhla, J. Rascoe, 2019. Development of routine multi-locus genotyping methods to characterize Huanglongbing (HLB) findings in California. In: 6th International Conference on Huanglongbing (IRCHLB VI) 2019, Riverside, CA.

Dai, Z., F. Wu, Z. Zheng, R. Yokomi, L. Kumagai, W. Cai, J. Rascoe, M. Polek, J. Chen, X. Deng 2018. Prophage Diversity if *Candidatus* Liberibacter asiaticus Strains in California. In: In: APS/ICPP Abstract/Poster of Presentation. Boston, MA. July 2018.

Kumagai, L.B., C.S. LeVesque, C.L. Blomquist, K. Madishetty, Y. Guo, P.W. Woods, and S. Rooney-Latham, J. Rascoe,

T. Gallindo, D. Schnabel, and M. Polek 2013. First Report of *Candidatus* Liberibacter asiaticus Associated with Citrus Huanglongbing in California. Plant Disease 97(2): 283.1.

IRCHLB-P10-120

A tissue and host-specific expression atlas for *Citrus sinensis* and *Diaphorina citri* for exploring the citrusgreening disease complex

Mirella FLORES-GONZALEZ[1], Noe FERNANDEZ-POZO[1,5], Marina MANN[4], Prashant S HOSMANI[1], Susan BROWN[2], Michelle HECK[1,3,4], Lukas A. MUELLER[1] and Surya SAHA[1]

(1) Boyce Thompson Institute, Ithaca, NY, (2) KSU Bioinformatics Center, Division of Biology, Kansas State University, Manhattan, KS, (3) USDA-ARS Emerging Pests and Pathogens Research Unit, Robert W. Holley Center for Agriculture and Health, Ithaca, NY (4) Plant Pathology and Plant Microbe Biology Section, School of Integrative Plant Science, Cornell University, Ithaca, NY, (5) Plant Cell Biology, Faculty of Biology, University of Marburg, Marburg, Germany

Abstract: We have created an open access and public eb portal (https://citrusgreening.org/) for the citrus greening community. It contains omics resources for the host citrus, the vector psyllid and multiple pathogens including *Ca.* Liberibacter asiaticus (CLas). The portal has tools such as a genome browser, metabolic pathway databases, blast search and expression atlas. Summarized research updates are presented using a blog for consumers and growers. Citrus Expression Network (CEN) contains public expression data from RNAseq resources from NCBI Sequence Read Archive (SRA). We have created an expression atlas for *C. sinensis* and *C. clementina*. It includes data from different stages, tissues, organs and infection states. The vector is the primary target of approaches to manage the disease. Psyllid Expression Network (PEN) is an open-access, interactive and user friendly web tool with proteomics and RNAseq resources for the psyllid. PEN contains high-resolution expression data from CLas infected and healthy individuals across multiple life stages, tissues and sexes. Insects were collected from a variety of hosts (*C. reticulata*, *C. macrophylla*, *C. sinensis*, *C. medica* and *C.clementina*). The expression patterns were determined with transcripts per million reads (TPM) and only genes with more than 1 TPM in at least one tissue were retained. Gene transcription and translation patterns are critical for understanding specific phenotypes at key developmental and infection stages. CEN and PEN allow visualization of the spatiotemporal context of gene expression and helps elucidate function. This facilitates effective data analysis by enabling simultaneous visualization of correlated genes to develop novel hypothesis in addition to candidate gene identification.

Non-technical summary: Citrusgreening.org is an open access and public web portal with information and omics resources for the host citrus, the vector psyllid and multiple pathogens including CLas for use by consumers, growers and scientists. We present a comprehensive gene expression atlas for the host citrus (CEN) and the vector psyllid (PEN). This user-friendly web based tool facilitates big data analysis by enabling simultaneous visualization of correlated genes to develop novel hypothesis and understand gene function.

IRCHLB-P10-121

Case study of Huanglongbing development of a single grapefruit tree in Texas, a decade of incubation

J. V. DA GRAÇA[1], M. SÉTAMOU[1], M. KUNTA[1], and M. KEREMANE[2]

[1]Texas A&M University-Kingsville Citrus Center, Weslaco TX 78599, USA. [2]USDA-ARS, Riverside CA 92507, USA

Abstract: Statewide surveys in Texas for Huanglongbing (HLB) and its vector, the Asian citrus psyllid (ACP), *Diaphorina citri* began in 2006 following the confirmation of the disease in Florida. Suspect leaf and ACP samples from numerous sites in Texas were tested for *Ca.* Liberibacter asiaticus (*C*Las) by PCR. The first detection of HLB in a citrus tree in the Lower Rio Grande Valley occurred in early 2012, but the bacterium had to have been present several years earlier due to the long latent period. We present evidence that the bacterium was present 5 years earlier, albeit in a different part of Texas. Some of the ACP samples collected in 2007 were analyzed by USDA-ARS in a comparative study on samples from Florida and Texas, and one sample from a Star Ruby grapefruit tree in a residential site in Corpus Christi TX tested positive by PCR, triggering off a delimiting survey in the area; no positive trees or further positive psyllids were detected. This tree was then selected as one of the sentinel survey trees for the statewide survey, and has been sampled multiple times annually since then. No positive PCR results were obtained until March 2017 when a composite leaf sample gave a borderline Ct 36 result. A composite root sample collected by USDA produced a positive result, confirming *C*Las infection. No HLB symptoms were observed. Root and leaf samples were then taken four sides of the tree, and tested for *C*Las. All four roots samples were positive, but only leaves from the lower east side of the tree were positive (Ct 26.4). In March 2018, the sampling and testing was repeated with the same results, however 4 months later in July 2018, root and leaf samples were again tested, along with ACP feeding on young shoots – all root and leaf samples were positive, but the psyllids were negative. No HLB symptoms were observed in July 2018, but they were observed in November. The tree had suffered from dieback likely due to severe weather events earlier in 2018, but had multiple young shoots developing. If the exposure of the tree to infected psyllids 2007 resulted in infection by *C*Las, there was a 10-year incubation period before any detection in the upper portion of the tree occurred, with no symptom development until late 2018.

Non-Technical Summary: An Asian citrus psyllid sample collected of an apparently healthy grapefruit tree in Corpus Christi TX in 2007 tested positive for *Ca.* Liberibacter asiaticus in 2007, 5 years before the first infected tree was found in the state. This tree has been closely monitored since, and while still symptomless, first tested positive for *C*Las in the roots and one side of the tree in 2017, and then all sides in 2018, without symptom development.

IRCHLB-P10-122

Characterization of *Candidatus* Liberibacter asiaticus from Pakistan by different molecular methods.

Sagheer ATTA[1], Huawei LIU[2] and John. S. HARTUNG[2]

[1]Department of Plant Protection, Faculty of Agricultural Sciences, Ghazi University, Dera Ghazi Khan. [2]MPPL, ARS, BARC West, USDA, Maryland, USDA, Maryland, USA.

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is devastating pathogen of citrus associated with Huanglongbing (HLB), previously known as citrus greening disease. This disease is an important disease in Asia and has destroyed 100 million citrus trees worldwide during the last century. In recent years the presence of HLB was suspected in different citrus growing regions of Pakistan. Surveys conducted in citrus groves of Punjab, Pakistan, citrus samples exhibiting symptoms of HLB

were collected. The aim of this study was to investigate the presence and the genetic diversity of CLas in Pakistan. A PCR method using specific primers allowed the detection of CLas in citrus leaves by amplification of a 1160-bp fragment from the 16S rDNA. These samples were also tested by q-PCR. All the samples which were detected positive from standard PCR also showed positive FAM/ct value with q-PCR. We further used an anti-OmpA polyclonal antibody based direct tissue blot immunoassay to localize CLas in different citrus tissues. In citrus petioles, CLas was unevenly distributed in the phloem sieve tubes, and tended to colonize in phloem sieve tubes on the underside of petioles in preference to the upper side of petioles. These samples were then sequenced and phylogenetic analysis was drawn. These samples were phylogenetically related with other sequences from the rest of the world with varying degree of similarity. This is the first report of characterization of HLB from Pakistan using different molecular method.

Non-Technical Summary: Pakistan is considered among top ten citrus producing countries of the world. Citrus industry of Pakistan is facing many challenges these days; among them bacterial and viral diseases of of citrus are of utmost importance. Huanglongbing is present in citrus orchards from last one hundred years but hasn't been characterized well yet. This study can contribute to understand about the CLas presence in Pakistan and can define future researches on the disease.

IRCHLB-P10-123

Detection of citrus Huanglongbing and high throughput sequencing of citrus root fungi

Yongmei LIAO, Zhengzheng WANG, Yuantengji HUANG, Wen LU, Guixiang HUANG

College of Agriculture, Guangxi University, Nanning, China

Abstract: The citrus leaves symptom caused by Huanglongbing was complex, including homogeneous yellowing, mottled, element deficiency like etc. 45 yellowing leaves samples of *Citrus reticulate* cv. Shatangju were collected from Heng county in Guangxi Province of China, PCR detection results shown that only 16 samples were positive reactions, account for 35.56%. Among 3 samples with typical Huanglongbing symptoms collected from Nanning, only one sample has positive reaction by PCR detection. High throughput sequencings aim at the ITS1/ITS2 region of root total DNA of 4 samples (S1 healthy citrus tree, S2 Huanglongbing infected, S3 and S4 with leaves yellowing symptom but non Huanglongbing infected) were conducted. The analysis results shown that Ascomycota is the dominant phylum in all of root samples, the relative abundance of 4 samples (S1-S4) were 85.44%, 67.42%, 95.88% and 81.40% respectively, in which Nectriaceae is the dominant family in all samples, the relative abundance of 4 samples (S1-S4) were 53.94%, 58.07%, 75.32% and 60.28% respectively. In Huanglongbing infected sample (S2) the second highest relative abundance is Geastraceae (Basidiomycota) with 22.58%, while in the other samples is unclassified. It was reported that citrus root hairs are very short in the field, and it is generally considered that the citrus root hairs are few or even nonexistent and highly dependent on arbuscular mycorrhizal fungi instead of root hairs to absorb soil nutrients. However, the relative abundance of Glomeromycota which form arbuscular mycorrhizas with the roots of the majority of plant species is 0-0.28% only in 4 roots samples. It is concluded that most citrus tree with leaves yellowing were not infected by Huanglongbing, Nectriaceae is the dominant family in citrus roots, Geastraceae had a high relative abundance ratio in Huanglongbing infected citrus root, but Glomeromycota is almost lacking in citrus roots.

Non-Technical Summary: Leaves yellowing of citrus is very common in the field, but not all citrus trees with yellowing leaves were infected by Huanglongbing. Under the absence of detection conditions to determine whether the citrus trees were infected by Huanglongbing or not, it need to be prudent to cut down the yellowing leaves citrus trees. It is necessary to strengthen cultivation management and maintain root health to ensure healthy growth of citrus trees.

IRCHLB-P10-124

Effects and experiences of integrated control of Citrus Huanglongbing performed in the last decade in Guangxi, P. R. China

Xianjin BAI[1], Chongling DENG[2], Binghai LOU[2]

[1]Guangxi Academy of Agricultural Sciences, Nanning, Guangxi 530007, P. R. China, [2]Guangxi Key Laboratory of Citrus Biology, Guangxi Academy of Specialty Crops, Guilin, Guangxi 541004, P. R. China

Abstract: Citrus Huanglongbing is one of the most destructive diseases in citrus industry worldwide. Effective control of Citrus Huanglongbing is one of the most important tasks in citrus producing areas infected by Huanglongbing. In Guangxi, P. R. China, integrated control of Huanglongbing had been performed throughout the whole province for ten years, and numerous of successful experiences had been achieved. The core steps of integrated control of Huanglongbing performed in Guangxi consisted of controlling psyllids in large scale, removing Huanglongbing-infected trees in early stage and planting healthy nursery trees. By carrying out integrated control of Huanglongbing during the last decade, the cultivation area of citrus reached to 370,400 hm^2 in 2016 from 173,000 hm^2 in 2005, and the products reached to 5,782,200 t in 2016 from 1,871,000 t in 2005 in Guangxi. Currently, Guangxi is the biggest province of cultivation of citrus in China.

Non-Technical summary: Although Citrus Huanglongbing is one of the most destructive diseases in citrus industry, successful control of this disease can still be achieved by strictly carrying out integrated control techniques composed of controlling psyllids in large scale, removing Huanglongbing-infected trees in early stage and planting healthy nursery trees.

IRCHLB-P10-125

Ensuring security and integrity of valuable breeding, research, and germplasm collections

MaryLou POLEK[1], Tim GOTTWALD[2], John HARTUNG[3], Georgios VIDALAKIS[4], Wenbo MA[4], Tracy KAHN[4], Carolyn SLUPSKY[5], Peggy MAUK[4], Elizabeth GRAFTON-CARDWELL[4], Mikeal ROOSE[4]

[1]USDA ARS, Riverside, CA; [2]USDA ARS, Fort Pierce, FL; [3]USDA ARS, Beltsville, MD; [4]University of California, Riverside; [5]University of California, Davis Davis

Abstract: University of California Riverside (UCR) Citrus Research Center in Riverside has ~200 acres of citrus research blocks as well as a diverse collection of citrus varieties and related species, which are maintained by UCR and USDA ARS. To ensure the integrity of the field collections, several detection methods were utilized to survey for huanglongbing (HLB). These included the regulatory standard method of qPCR and several early detection technologies (EDTs) including canines, antibody detection of secreted and structural proteins, and metabolomics analysis. Canines, trained in Florida, visited the station in 2017, scanned selected field perimeters, several tree-by-tree surveys of blocks of viroid-infected trees, and other designated areas. Overall, the canines alerted on 3% of the perimeter trees scanned.

Canines also scanned more than 100 trees infected with various viroids or *Spiroplasma citri* and alerted on one known viroid-infected tree. Extensive qPCR analyses were subsequently conducted on the canine-alert trees, but no samples tested positive. Serological assays resulted in suspect positive samples but lacked agreement on which tree was suspect. Fifteen trees were deemed suspect by 3 methods; canines, secreted proteins, and structural proteins. Several trees were caged to prevent further feeding by psyllids and re-tested; these have also remained negative. The canine-alerted trees were re-tested in late 2018. Canines returned early in 2019 for a more extensive survey of the Citrus Research Center in Riverside and perimeter surveys of the University of California Research and Extension Center in Exeter, California located in the San Joaquin Valley. Results of the 2018 and 2019 surveys will be presented.

Non-Technical Summary: Valuable variety and research collections of citrus on the University of California (UC) Citrus Research Center (CRC) located in Riverside, California and the UC Lindcove Research and Extension Center located in Exeter, California are being monitored for the presence of *Candidatus* Liberibacter asiaticus using multiple detection methods. These methods include the standard qPCR and technologies (Early Detection Technologies (EDT)) that may detect the bacteria before qPCR. This effort will provide data to determine practical, accurate, and efficient detection technologies for growers, researchers, and regulators.

Citations:

Li, W.; Hartung, J.S.; Levy, L. 2006. Quantitative real-time PCR for detection and identification of *Candidatus* Liberibacter species associated with citrus huanglongbing. J Microbiological Methds. 66: 104-115.

Chin, E., D.O. Mishchuk, J. Bruce, M. Cilia, G. Coaker, C. Davis, H. Jin, W. Ma, G. Sellar, C. LeVesque, K. Godfrey, C.M. Slupsky. 2014. An interdisciplinary approach to combat HLB. Citrograph: 5(1):28-34.

IRCHLB-P10-126

Establishment of a citrus (*Citrus* spp.) / dodder (*Cuscuta campestris*) system to study the in planta biology of "*Candidatus* Liberibacter asiaticus"

Zheng ZHENG[1], Tao LI[1], Minli BAO[1], Xiaoling DENG[1]

[1]Citrus Huanglongbing Research Laboratory, South China Agricultural University, Guangzhou, 510642, China

Abstract: "*Candidatus* Liberibacter asiaticus" (CLas) is an α-proteobacterium associated with Huanglongbing (HLB, yellow shoot disease) of citrus (*Citrus* spp.; Rutaceae). Little is known about the bacterial biology, which is important for HLB management. CLas inhabits the phloem system of citrus. Dodder (*Cuscuta campestri*s; Convolvulaceae) is a parasitic plant and acquires nutrients from its host through haustorial connection in phloem tissue. For this reason, CLas can move between citrus and dodder, providing a good model to study the bacterium under different in planta conditions. In this research, the growth of CLas between citrus and dodder hosts were examined and compared using both PCR and next generation sequencing approaches. Citrus shoots showing typical HLB symptoms were maintained hydroponically under a screen- house condition between May and July, 2017 in Guangzhou, China. Clean dodder tendrils maintained in periwinkle (*Catharanthus roseus*) were used to wrap around the HLB citrus shoots for CLas acquisition. 1). For 55 citrus-dodder sets, 48 dodders (48/55, 87.3%) were successfully established parasitism. Higher titers of CLas (30/48, 62.5%) were detected in dodder tissue samples than in citrus leaf midrib samples. The titer increase ranged from 2- to 419-folds with an average of 53-fold; 2). A citrus-dodder set was selected for total

DNA extractions. The DNA samples were sequenced by Illumina Hiseq (2x150-bp) platform. As expected, the read ratio of CLas / dodder was significantly higher than the read ratio of CLas / citrus (2.99 % vs. 0.99%); and 3). A pararetrovirus detected in citrus was found to move into the dodder. However, citrus maintained higher titer of pararetrovirus (242 virus reads) than that in dodder (53 virus reads). Altogether, data from this study suggested that the in planta condition of dodder phloem tissue was more favorable to the growth of CLas. This feature has a potential to be used for CLas enrichment for whole genome sequencing purpose.

Non-Technical summary: Dodder is a parasitic plant capable of connecting the phloem tissue of citrus host. The citrus-dodder combination provided a unique opportunity to study the phloem limited HLB pathogen between different hosts. Data from this study suggested that the in planta condition of dodder phloem tissue was more favorable to the growth of CLas.

IRCHLB-P10-128

Evidence of the Occurrence of Huanglongbing (Citrus Greening) in Citrus Orchards and Nurseries in Suriname.

Ferdinand KLAS

University of Suriname, Faculty of Mathematics and Natural Sciences, Dept. of Biology. Paramaribo - Suriname.

Abstract: Suriname is surrounded by countries and areas, such as Brazil and the Caribbean where Huanglongbing (HLB) widely occurs. Therefore citrus nurseries (Tamansarie, Houttuin), orchards (Katwijk) and residential areas of the coastal region (fertile clay soils) were inspected for HLB symptoms and sampled (leaves and fruits) for HLB analysis . Trees showed typical yellow shoots and blotchy mottled leaves. In the residential areas HLB-suspicious trees were monitored for three consecutive years and randomly treated with Winpotion humic acid liquid fertilizer). Of the majority of the untreated trees twigs and branches died back and trees totally declined during this period. In the Coesewijne area in the interior (sandy unfertile soils) of Suriname citrus plantations planted with Pera orange, originating from the district of Belém do Pará (Brazil), were established. High percentages of totally green-colored fruits from this area showed blotchy mottling, lopsidedness, stained columellae and sponginess. As Brazil is known for *Candidatus* Liberibacter asiaticus *(*CLas) and *Candidatus* Liberibacter americanus (CLam), there is a great deal of evidence that we are dealing here with HLB. However, qPCR and PCR analyses of samples from both, the coastal region and the interior, were inconclusive, so far. Therefore, further biotechnology studies are needed to support and corroborate the evidence of the occurrence of Huanglongbing in Suriname.

Non-technical Summary: In different citrus-growing areas of Suriname, citrus trees with suspicious symptoms have been encountered. As Suriname is surrounded by several countries like Brazil with a HLB history, and as there is a busy traffic of persons and planting material between Suriname and its surrounding countries it was worthwhile conducting studies to verify and corroborate the evidence of the occurrence of HLB in Suriname. The outcomes of these studies are to protect the currently lingering citrus industry of Suriname from completely disappearing.

Citations:

Coleta-Filho, H.D. ,Targon, M.L.P.N., Takita, M.A., De Negri, J.D., Pompeu, J.J., and Machado, M.A. 2004 . First report of the causal agent of huanglongbing (‘ *Candidatus* Liberibacter asiaticus’ in Brazil. Plant Dis. 88: 1382

Halbert, S.E., 2005. The discovery of huanglongbing in Florida. Page H-3 in : Proc. 2nd International Citrus Canker and

Huanglongbing Research Worshop. Florida Citrus Mutual, Orlando, FL.

Lin, K.H., 1956. Observations on yellow shoot of citrus . Etiological studies of yellow shoot of citrus. Acta Phytopathologica Sinica, 2, 1-12.

IRCHLB-P10-129

High throughput sequencings and analysis of fungi population in citrus rhizosphere soil

Guixiang HUANG, Yuantengji HUANG, Zhengzheng WANG, Congcong GE, Wen LU, Yongmei LIAO*

College of Agriculture, Guangxi University, Nanning, China

Abstract: Soil samples from citrus rhizosphere were collected from two orchards, orchard No.1 is natural grass, while orchard No.2 is herbicide weeding. Two soil samples (S1 and S2) were collected from orchard No.1 in citrus rhizosphere, and four soil samples (S3 to S6) were collected from orchard No.2. Among six samples, only S3 was infected by Huanglongbing with yellow shoots, S4 and S5 had yellowing leaves, but non Huanglongbing infected. S1, S2 and S6 had normal green leaves, also non Huanglongbing infected. High throughput sequencings aim at the ITS1/ITS2 region of soil total DNA were conducted, the results shown that Ascomycota was the dominant phylum in all six samples, in which S3 infected by Huanglongbing had the highest relative abundance (82.52%), the relative abundance in other five samples were among 48.05%-78.18%. Sort by the ratio of relative abundance, in two samples from orchard No.1, the dominant phyla were Ascomycota, Basidiomycota and Zygomycota consecutively, the dominant families were Trichocomaceae, Mortierellaceae, Hypocreaceae and Nectriaceae; while from orchard No.2, the dominant phyla were Ascomycota, Zygomycota and Basidiomycota consecutively, the dominant families were Nectriaceae, Hypocreaceae, Chaetomiaceae and Mortierellaceae. Among healthy with normal green leaves citrus tree rhizosphere soil samples (S1, S2, S6), the dominant families were Nectriaceae, Trichocomaceae, Mortierellaceae and Hypocreaceae; among samples (S4, S5) with yellowing leaves but non Huanglongbing infected, the dominant families were Hypocreaceae, Nectriaceae, Chaetomiaceae and Mortierellaceae; in Huanglongbing infected sample (S3), the dominant families were Nectriaceae, Chaetomiaceae, Trichocomaceae and Hypocreaceae. It was illustrated that Chaetomiaceae appeared at high frequency in the citrus trees rhizosphere soil with yellowing leaves, whether the citrus trees were infected by Huanglongbing or not.

Non-Technical Summary: The causes of citrus leaves yellowing are complex. In addition to the Huanglongbing infected, the root rotten and its function of absorbing soil nutrients destroyed could cause citrus leaves yellowing also. Root health or not would affect the structure of fungi population in rhizosphere soil directly, conserving soil and maintaining root health is one of the important measures to avoid citrus leaves yellowing.

IRCHLB-P10-130

HLB and other stress factors – understanding the challenge of citriculture in the Tropics.

Juan DELGADO[1], Andrés CASTILLO[1], Bryan PALMA[1], Dennis ZAVALA[1], Bolívar TORRES[2], Esteban BLANCO[3], Thiago ANTUNES[3], Julio MEJÍA[4], Arturo FORTÍN [4], German ALFARO[5].

[1] LIFE-RID Amvac/Agricenter, Costa Rica. [2] TradeWindsCitrus Ltd, Jamaica. [3] TicoFrut, Costa Rica/Nicaragua. [4] Colon Fruit Company, Honduras. [5] Greenplants, Costa Rica.

Abstract: Citriculture is a never ending challenge for those who have and embrace the mission of assuring high quality citrus for a very

dynamic and demanding market. Climate change, global warming, new pests and diseases and market restrictions are changing the game and growers have to keep an open mind and a resilient attitude to remain playing. This scenario is specially complicated for citrus growers in the tropics. The lack of seasonality, the restricted or delayed access to technologies, the high rainfall, temperatures and radiation and the global competitiveness of the tropical fruit market are pieces of a puzzle that the stakeholders of the citrus business in the tropics have to deal with. Also, an extended rainy season reduces the window for xenobiotic application for crop protection and plant nutrition, so products must have fast and efficient uptake mechanisms. Our arena is ¨in the field¨ and is there where abiotic and biotic factors sum up to reduce productivity. In the end, the more stress factors you can reduce, the higher the yields you will achieve. In the case of citrus, HLB has become the main biotic limitation but the successful management of the disease implies overcoming also the related biotic and abiotic threats and their interactions. Continuous flushing due to constantly active metabolism sets a very difficult scenario for ACP control but allow trees to constantly replace lost foliage. Droughts and floods harm the roots while tropical storms and hurricanes affect the canopy and increase fruit drop. Hydroperiodic blooms coincide with heavy rainfalls that limit PFD control and long rainy seasons provide year round conditions for Greasy spot infection and dissemination. Having the tools (technologies, products, information and knowledge) and understanding how to use them will allow citrus growers to succeed in this scenario.

Non-Technical summary: Citriculture is a never ending challenge for those who have and embrace the mission of assuring high quality citrus for a very dynamic and demanding market. The lack of seasonality, the restricted or delayed access to technologies, the high rainfall, temperatures and radiation and the reduction of subsidies due to free trade agreements are some of the pieces of a puzzle that the stakeholders of the citrus business in the tropics have to deal with. HLB, PFD, Greasy spot, ENSO phenomenon and many more biotic and abiotic factors are all part of the challenge of citrus industry in the tropics.

IRCHLB-P10-132

Impact of interactions among endemic citrus pathogens in California and CLas

Karla ARAUJO[1], Kris GODFREY[1] and Georgios VIDALAKIS[2]

[1]University of California, Davis, USA, [2]University of California, Riverside, USA

Abstract: California citrus trees may harbor the endemic pathogens citrus tristeza virus (CTV) and *Spiroplasma citri* (SC), the citrus stubborn disease pathogen. These two pathogens are associated with the phloem like *Candidatus* Liberibacter asiaticus (CLas), the huanglongbing (HLB) pathogen. Because of this, CTV and SC may impact CLas establishment and/or growth. We challenged Washington Navel seedlings with combinations of these three pathogens by graft-inoculation. Control plants included non-grafted plants and plants grafted with healthy navel scion. Plant samples were taken monthly. Control plants remained healthy. For single pathogen treatments, graft inoculations were successful in 60% of the plants inoculated with SC, 100% of the plants inoculated with CTV, and 90% of the plants inoculated with CLas. For dual pathogen treatments, graft inoculations were successful in 20% of the plants inoculated with SC, then CLas; 70% of the plants inoculated with CTV, then CLas; and 10% of the plants inoculated with CLas, then SC. For the triple pathogen treatment, all of the pathogens could be detected in 20% of the plants. These results demonstrate that CTV, SC, and CLas can coexist in a plant. The inoculation of more than one pathogen lengthened the time to first detection for a given pathogen, suggesting that the pathogens are interacting in some manner.

Understanding these interactions could provide information useful for predicting spread and severity of HLB in California.

Non-Technical Summary: The presence of endemic pathogens in California citrus trees may impact the ability of the HLB pathogen to establish and grow within a tree. Our study has shown that two California endemic pathogens can co-exist with the HLB pathogen. However, detection of all the pathogens is delayed when multiple pathogens are present, suggesting interactions among the pathogens that may impact the severity and spread of HLB in California citrus.

IRCHLB-P10-133

Naturalized Rutaceae as hosts of *Diaphorina citri* and ‘*Candidatus* Liberibacter asiaticus’

Patrícia FELISBERTO[1], Eduardo GIRARDI[2], Leandro PEÑA[3,4], Guilherme FELISBERTO[1], Andrew BEATTIE[5], **Silvio LOPES[3]**

[1]São Paulo State University (UNESP), Jaboticabal, [2]Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA), [3]Fund for Citrus Protection (FUNDECITRUS), Araraquara, Brasil, [4]Universidad Politécnica de Valencia (UPV), Spain, [5]Western Sydney University, Penrith, Australia

Abstract: Various species of Rutaceae have become naturalized and can be found growing inside forests near citrus orchards in Brazil. In greenhouse experiments we assessed their suitability for *Diaphorina citri* (ACP) reproduction and survival, and for infection and multiplication of ‘*Candidatus* Liberibacter asiaticus’ (CLas), which are, respectively, the vector and the bacterium associated with huanglongbing (HLB). ACP reproduction was assessed by counting egg, hatched nymphs, and newly emerged adults after caging two ACP couples on newly developed shoots, and ACP survival by counting the individuals that remained alive after caging five adults for 30 days on newly differentiated leaves (NDL) or fully-expanded soft leaves (FESL). CLas infection and multiplication was assessed through qPCR analysis of leaf of eight-months insect-inoculated plants. ACP reproduced profusely and survived for 30 days on NDL and FESL of *Citrus sinensis* ‘Valencia’, ‘*C. limonia*’, *Murraya paniculata* (syn. *Murraya exotica* L.), and *Bergera koenigii*. On *Poncirus trifoliata* ‘Pomeroy’, *Microcitrus wintersii*, *Swinglea glutinosa*, and *Clausena lansium* ACP also survived and reproduced, but less than on the previous species. The insect survived as nymphs, but none developed to adults, on *Aegle marmelos*, *Atalantia buxifolia*, *Microcitrus* sp., and *Helietta apiculata*. On *Glycosmis pentaphylla*, *Balfourodendron riedelianum*, *Casimiroa edulis*, *Esenbeckia febrifuga*, *E. leiocarpa*, *Metrodorea stipularis*, *Zanthoxylum rhoifolium*, and *Dictyoloma vandellianum* ACP adults did not reproduce but survived up to 17 days, with the exception of *G. pentaphylla, B. riedelianum*, *D. vandellianum*, and *Z. rhoifolium* on NDL and *B. riedelianum* on FESL, which survived a few days longer. CLas infected only ‘Valencia’ (5.2 log cells/g tissue), ‘*C. limonia*’ (4.0 log), and *P. trifoliata* (3.9 log). The unsuitability to ACP plus the immunity or high levels of resistance to CLas infection observed for the naturalized Rutaceae tested indicated they are not contributing to HLB epidemics in Brazil.

Non-Technical summary: In Brazil, in several instances, citrus relatives may be found growing in forests near citrus orchards affected by HLB. Since they pertain to the same botanical family of *Citrus*, growers were concerned that these trees could be serving as source of inoculum and/or reproduction/survival sites of ACP, contributing, in one way or another, to HLB epidemics. This assumption can be discarded. On plants exposed to environments highly favorable to CLas and ACP, the pathogen did not infect and the insect did not reproduce well or survived for long time, on any of the plants tested.

IRCHLB-P10-134

Investigation and detection of '*Candidatus Liberibacter asiaticus*' in Hunan province, China

Feng ZHANG[1], Dan TANG[1], Runxian YAO[1], Dazhi LI[1], Ronghua LI[2], Hongmei LONG[2], Zhenhua TAN[2], Suming DAI[1], Na LI[1], Xianfeng MA[1], Ziniu DENG[1]

[1]National Center for Citrus Improvement and Horticulture and Landscape College, Hunan Agricultural University, Changsha, Hunan 410128, P.R. China. [2]Department of Agriculture, Yizhang County, Hunan, P.R.China

Abstract: Citrus huanglongbing (HLB) causes highly destructive damages to China's citrus industry in recent decades. It was first reported in Guangdong in 1919[1] and its pathogen was identified as '*Candidatus* Liberibacter asiaticus' (CLas). The pathogen primarily spreads via infective nursery plants and then by insect vector Asian citrus psyllid (ACP)[2]. During the last century, the affective region was mainly restricted in the southern area of China, like the provinces Guangdong, Guangxi and Fujian etc. However, up to date, 11 provinces reported HLB infection and it has destroyed multiple groves[3]. HLB-infective symptom trees and ACP were observed in the southern regions of Hunan, where adjoins HLB infective provinces Guangdong and Guangxi. To further evaluate the status of HLB in Hunan, we collected blotchy mottling leaves and shoots in citrus orchards from the major production regions of Hunan including 27 counties and cities. All suspect trees were budded on *Poncirus trifoliata* and maintained in the isolated greenhouse. Each sample was examined for *C. L*as by normal PCR and qRT-PCR and observation of the typical symptoms of leaf. Out of 586 tested samples, 43.68% resulted in positive for *C. Las*, which included 16 cultivars. And it was also found that cross-infection with other viruses and/or viroids was a quite frequent phenomenon in the investigated citrus groves. Further verification indicated that most of HLB positive trees were infected also by one or more viral pathogens, such as CTV, CTLV, CEVD, and CYVCV, which caused more severe symptoms of HLB infected trees. Among the tested trees, 57% of them were infected by HLB and CTV, and 30 HLB-positive trees were infected with the most severe viral diseases CTV, CTLV, CEVD and CYVCV, the most commonly present viral diseases in Hunan. Only a few HLB-infected trees did not have the above mentioned viral diseases. The cross-infection status indicated that the study of the influence of *C. Las* infection is more complicate, the field symptoms may be caused by HLB and other viral diseases combination. What's more, among the tested plants of popular pomelo cultivar, guanximiyou pomelo from Fujian province, most were examined as HLB positive even in the HLB- and psyllid-free counties of Hunan, indicating that it might be caused by the transportation of HLB-infected buds or small plants, especially they were the source of HLB and it may cause the rapid spread of HLB in the local regions. Therefore, these comprehensive situations result the difficulty of field management.

Non-technical summary: The infection and distribution of HLB was investigated in Hunan Province, China. The detection results showed that most of HLB positive trees are planted near the epidemic areas, Guangxi and Guangdong provinces. These regions are the most adaptive areas for sweet orange cultivation in Hunan Province. Combined with previous large scale virus and viroid survey, further detection indicated that HLB-infected trees were cross-infected with a variety of severe diseases, such as CTV, CTLV, CEV and CYVCV. Hence, the field management and the study of only HLB-infection is more complicated.

Citations:

Reinking O A. 1919. Diseases of economic plants in South China. *Philippine Agricalture*, 1919, 109-135.

Alabi O J, Kunta M, Dale J, et al. Survey and detection of 'Candidatus Liberibacter asiaticus' in a citrus nursery facility in South Texas.[J]. Plant Health Progress, 2014, 15(4):184-188.

IRCHLB-P10-135

Investigation of old pummelo trees (*Citrus maxima* (Burm.) Merr. cv. 'Shatian') tolerant to Huanglongbing in Rongxian County

Huihong LIAO[1], Yaoliang ZHANG[2], Fuping LIU[1], Xi WANG[1], Bonian DONG[2], Dongmo HE[2], Gaoyun LIANG[3], Hongming HUANG[1], Nina WANG[1], Yunfeng LIANG[2], Tiejun DENG[1], Dongkui CHEN[1] *

[1]Horticulture Research Institute of Guangxi Academy of Agricultural Sciences, Nanning, Guangxi, P.R.China, [2]Agricultural Science Research Institute of Rongxian County, Rongxian, Guangxi, P.R.China, [3]Agricultural Bureau of Rongxian County, Rongxian, Guangxi, P.R.China

Abstract: Shatian village, which is located in Songshan Town of Rongxian County of Guangxi Province of China, is the origin of *Citrus maxima* (Burm.) Merr. cv. 'Shatian'. There has been a long history planting 'Shatian' pummelo in Rongxian county for more than 2000 years. In order to find a tolerant citrus tree, we surveyed old trees of 'Shatian yu' in Rongxian County. More than 100 old pummelo trees were found in this county. There are five towns with old pummelo trees older than 100 years including Ziliang, Langshui, Shili, Liuwang, and Songshan. More than 40 old pummelo trees older than 100 years were found in Ziliang town. Six old pummelo trees older than 100 years are growing in Zhong ping village of Ziliang town. We collected information on old pummelo trees regarding their yield, quality, and resistance to huanglongbing. Most of the old pummelo trees keep their good yield and quality, with no huanglongbing symptoms. Of which, one old pummelo tree older than 100 years bore 600 fruits in 2018. This tree had a high yield and the fruit had a high sugar:acid ration. In addition, no HLB symptoms were found on the tree. The few old pummelo trees with huanglongbing symptoms had lower yield and quality.

Non-Technical Summary: By 2017, the 'Shatian' pummelo industry in Rongxian County had grown more than 14 thousand hectares, with an annual output of more than 220,000 tons and an output value of more than 1.8 billion yuan. The development of 'Shatian' pummelo industry in Rongxian County is related to the long-term exploitation and utilization of 100-year-old germplasm resources of 'Shatian' pummelo in Rongxian County, some of which yield well and do not have symptoms of huanglongbing (HLB). In the future, we should strengthen the protection of 100-year-old pummelo trees, deepen the exploitation and utilization of the disease-resistant characteristics of 100-year-old pummelo trees to HLB disease.

IRCHLB-P10-136

Long-term evaluation of *Candidatus* Liberibacter asiaticus detection in citrus root tissue as a tool for improved CLas detection

Jong-Won PARK[a], Eliezer S. LOUZADA[a], W. Evan BRASWELL[b], Philip A. STANSLY[c], T. Gregory McCOLLUM[d], John V. DA GRAÇA[a] and Madhurababu KUNTA[a]

[a]Texas A&M University-Kingsville Citrus Center, 312 N. International Blvd, Weslaco, TX 78599 [b]USDA APHIS PPQ CPHST, Mission Laboratory, Edinburg, TX 78539

[c]University of Florida-IFAS, Southwest Florida Research and Education Center, Immokalee, FL 334142

[d]USDA-ARS Horticultural Research Laboratory, Fort Pierce, FL 34945

Abstract: Previously, we reported a diagnostic real-time PCR protocol for *Candidatus* Liberibacter asiaticus (CLas) detection optimized for citrus fibrous (feeder) root tissue, which showed a potential for earlier detection of Huanglongbing (HLB) at a pre-symptomatic stage. In the current study, we summarized the data obtained from surveying CLas incidence on a monthly basis for two consecutive years (2016 and 2017) using both leaf and fibrous root tissue collected from field grown, 4-5 year-old trees in Texas (110 grapefruit trees) and Florida (99 sweet orange trees). For this study, we used three different sets of real-time PCR primers for CLas detection, HLBaspr (for leaf) and TXCChlb (for root) targeting CLas 16s rDNA and RNR (for both leaf and root) targeting CLas *nrdB* gene. The 2-year survey data showed that (1) Root assays have a higher CLas detection rate than leaf assays in both Texas and Florida, confirming that the root assay is more sensitive than the leaf assay in terms of the number of CLas positive trees detected; (2) CLas detection in roots preceded detection in leaves by ~ 6 months to 1 year (no. of CLas positives: TX leaf vs. root = 10 vs. 27 in June 2016 and 28 vs. 77 in June 2017; FL leaf vs. root = 7 vs. 58 in June 2016 and 71 vs. 73 in June 2017), indicating that earlier CLas detection can be achieved by the root assay; (3) A drastic increase in the number of positive trees was observed in Spring in both Texas and Florida, suggesting the seasonal effect on HLB progression; and (4) HLB progression in Texas was slower than in Florida, implicating the possible involvement of environmental factors leading to different HLB progressions in the two states. The current study showed that the CLas detection rate can be greatly improved by incorporating the root assay into the routine diagnosis procedure.

Non-technical summary: Huanglongbing (HLB) is a destructive bacterial disease in citrus. Since there are no known resistant commercial citrus cultivars available, earlier detection of HLB-associated bacteria, "*Candidatus* Liberibacter asiaticus" (CLas), is critical for efficient disease control. The current HLB diagnostic procedure relies on a visual inspection for foliar HLB followed by molecular biological confirmation and is not suitable for pre-symptomatic CLas detection. In this study we evaluated a root assay as a tool for improved CLas detection, using leaf and fibrous root samples collected over two consecutive years from 4-5-year old trees from Texas and Florida. The data indicated that the root HLB assay can greatly improve CLas detection rate and confirmed that earlier CLas detection can be achieved by root HLB assay.

IRCHLB-P10-137

Natural viral infections do not have obvious effects on Huanglongbing progression

Tyler DANG[1], Nichole A. GINNAN[1], Sohrab BODAGHI[1], Paul M. RUEGGER[1], Beth B. PEACOCK[1], Greg MCCOLLUM[2], Gary ENGLAND[3], Caroline ROPER[1], Philippe ROLSHAUSEN[1], James BORNEMAN[1] and Georgios VIDALAKIS[1]

[1]University of California, Riverside, USA, [2]U.S. Department of Agriculture, ARS, Fort Pierce, USA, [3]University of Florida, Hastings, USA

Abstract: A survey for naturally infecting citrus viruses and viroids was performed annually from 2016 to 2018, on four Huanglongbing (HLB) affected citrus orchards in different Florida counties. The planting year/scion/rootstock of the surveyed orchards were 1990/Hamlin/Swingle, 1990/Parson Brown/Sour Orange, 2005/Valencia/Swingle and 2013/Ruby Red/US-897. At the time of the survey, 10 trees per orchard were rated for HLB symptoms on a scale 1-5, with rating 1-2 representing a non-declining tree with full canopy and mild HLB symptoms and rating 3-5 representing a declining tree with foliage discoloration and dieback (3) and extensive canopy thinning and severe HLB symptoms (4-5). For each tree, eight stems were collected from around the canopy and nucleic acids was extracted for quantitative polymerase chain reaction (qPCR) testing for *Candidatus* Liberibacter asiaticus (CLas) as well as three common citrus viruses and eight citrus viroids. The HLB progression in CLas positive trees, was captured by the disease ratings with an annual reduction in the ratio of non-declining (1-

2)/declining (3-5) number of trees i.e. 2016: 0.91 (19/21), 2017: 0.48 (13/27) and 2018: 0.18 (6/34). qPCR testing did not identify citrus psorosis or citrus leaf blotch virus in any of the 40 trees tested. Citrus tristeza virus (CTV) was detected in 32 trees. Citrus dwarfing viroid (CDVd) was detected in 4 trees along with CTV. One tree tested positive for CTV, CDVd, hop stunt viroid and citrus exocortis viroid. All 6 non-declining trees in 2018 tested positive for CTV or CDVd. Similarly, 26 of the 34 declining trees in 2018 tested positive for CTV or viroids while the remaining 8 declining 2018 trees were negative for CTV and viroids. The observed lack of association of natural viral infections and HLB progression was also confirmed by a weak statistical correlation between viral infections and declining and non-declining trees.

Non-technical summary: It is not uncommon for citrus to harbor multiple pathogens which can act synergistically and exacerbate disease symptoms. From 2016 to 2018, we surveyed for citrus viruses and viroids in HLB affected trees in Florida. Our results suggested that citrus viruses or viroids do not have any obvious effects on HLB disease progression.

IRCHLB-P10-138

Response of Government and Industry to the Presence of Asian Citrus Psyllid and to the Potential Presence of Huanglongbing in Arizona

Glenn C. WRIGHT[1],
Elene STEFANAKOS[2], John CARAVETTA[3]

[1]University of Arizona - Yuma Agriculture Center, Yuma, AZ USA, [2]Yuma County Citrus Pest Control District, Somerton, AZ USA, [3]Arizona Department of Agriculture - Plant Services Division, Phoenix, AZ USA

Abstract: Arizona remains the only U.S. state with a commercial citrus industry that does not have a confirmed finding of Huanglongbing (HLB). However, the Asian Citrus Psyllid (ACP) was found in the state in 2010 and can now be found in areas where citrus is grown. Consequently, Arizona has initiated an aggressive tree and ACP sampling program to validate its freedom from HLB, which comprised about 6400 samples in 2018, and will be similar in 2019. Also, there is a network of almost 900 ACP sticky traps that are intended to ascertain the extent of ACP infestation in commercial citrus. Currently, ACP finds on traps in commercial citrus are very low. In 2016, citrus growers in Yuma County formed a Citrus Health Management Area (CHMA) to plan and verify the application of one annual coordinated ACP spray that reduces the ACP population in the late winter. Growers must provide local packinghouses with evidence that this spray has been applied in order to have their fruit packed. There has been no effort to control ACP in dooryard citrus, other than through releases of *Tamarixia radiata* in residential areas. Because of the lack of HLB in the state, Arizona authorities assisted the USDA with validation of root sampling as a method for determining the presence of HLB in citrus trees. That method is now an approved method that can serve for regulatory purposes. Regulatory authorities and growers remain concerned about the potential arrival of HLB from California and from Mexico. When HLB is found in Arizona, the intent is to eradicate the disease before it becomes widespread.

Non-Technical Summary: HLB has not yet been found in Arizona, yet ACP is widespread. An aggressive sampling and trapping program is in place in order to find any outbreaks of HLB, and to determine ACP population levels. Citrus growers have a mandatory coordinated spray program that reduces ACP populations. Tamarixia wasps are being released in residential areas. Growers and regulatory authorities are vigilant because of the presence of HLB in California and in Mexico, and when it arrives, the goal is disease eradication.

IRCHLB-P10-139

The Science for Citrus Health website as a source of information on existing efforts and new technologies to fight huanglongbing.

Sara GARCIA-FIGUERA[1], Brianna MCGUIRE[1], Barbara ALONSO[2], Monique RIVERA[4], Lukasz STELINSKI[3], Peggy LEMAUX[2], Elizabeth GRAFTON-CARDWELL[4]

[1]Department of Plant Pathology, University of California, Davis, CA; [2]Department of Plant and Microbial Biology, University of California, Berkeley, CA; [3]Department of Entomology and Nematology, Citrus Research and Education Center, University of Florida, Lake Alfred, FL; [4]Department of Entomology, University of California, Riverside, CA

Abstract: The Science for Citrus Health website (http://ucanr.edu/sites/scienceforcitrushealth/) is a collaborative effort between scientists from different universities whose goal is to describe the existing efforts and new technologies being developed to protect citrus from damage from the devastating disease huanglongbing (HLB). Because information on cutting-edge research tends to be scattered and difficult to access, this website is intended as a source of updated information for other scientists, citrus stakeholders, media and the general public. Technologies are organized in six categories (Early Detection Techniques, Established Orchards, Existing Tools, Psyllid, Replants and General Discussion). Each technology is thoroughly explained in a Research Snapshot, which is a factsheet containing all the information required to understand its characteristics and potential use. We encourage scientists to contact us if they have a technology that could be featured on the website; and citrus stakeholders and media to explore the website and learn more about the different strategies that are being developed to fight HLB.

Non-Technical summary: The Science for Citrus Health website is an initiative to describe tools that are in use or being developed by researchers to protect citrus from huanglongbing (HLB), written in a language that is accessible to all.

IRCHLB-11: Economics and Management Oral Presentations

IRCHLB-O6a-01

Main research findings leading to improvements in HLB control in Brazil

Silvio LOPES[1], Renato BASSANEZI[1], Marcelo MIRANDA[1], Nelson WULFF[1], Leandro PEÑA[2], Juliano AYRES[1]

[1]Fund for Citrus Protection (FUNDECITRUS), Araraquara, Brazil, [2]Universidad Politécnica de Valencia (UPV), Valencia, Spain

Abstract: Huanglongbing (HLB) was first detected in Brazil in 2004 in São Paulo (SP) state. Today it occurs also in the states of Paraná and Minas Gerais (MG). HLB is associated with '*Candidatus* Liberibacter asiaticus' (CLas), currently the most prevalent species, and *Candidatus* Liberibacter americanus (CLam), both transmitted by the Asian citrus psyllid (ACP), first reported in Brazil in 1942. In the main citrus belt (SP+MG), symptomatic tree incidence has been lower in regions of higher temperatures and lower rainfall. The incidence averaged 18.2% in the 2018 survey with as much as 54% in the center, and 0-2% in southern/northern regions. Highest incidence also happens in smaller farms (≤10,000 trees). HLB control relies on actions to prevent plant infections. The success is highly dependent on how extensive inoculum sources (infected citrus) and ACP populations are reduced, and how effective the new shoots (NS) are protected from ACP access. *Tamarixia radiata* releases are limited to abandoned groves and backyards and application of chemicals has been the only method used against ACP in commercial groves. Dose and spray volumes were reduced but the frequency of applications increased, especially at perimeters of blocks and farms during flushing periods. Biweekly ACP occurrences on 26,000 traps (ACP Alert System) also determine regional spray timings. This control strategy results from a better understanding of the HLB pathosystem. Investigations showed that after inoculation (i) CLas colonizes the tree faster than the time required for symptom expression, cannot be eliminated by pruning or heat treatments, and is acquired at highest rates by nymphs, (ii) regular and trifoliate citrus are the only hosts important to ACP and CLas, (iii) ACP moves long distances and reaches highest incidences in the first 100 meters from farm edges, (iv) NS phenology impacts ACP feeding and reproduction, tree vulnerability to infections, and spray effectiveness, and (v) the environment strongly impacts CLas, ACP and NS growth, explaining the historical seasonal and regional variation in HLB incidence in SP and MG.

Non-technical summary: In the main citrus growing region of Brazil, timing application of insecticides and elimination of symptomatic citrus trees, inside and outside commercial farms and in a regional way, have been used to control HLB. A better understanding of how much the host, the pathogen and the vector are impacted by the climate has allowed recent improvements, particularly in the control of the insect vector. The use of kaolin to make trees repellant and of insecticide-treated orange jasmine to attract and kill ACP have been tested in farm borders with promising results. Conventional and/or biotechnological approaches to develop citrus varieties resistant to CLas or repellant to ACP also been have tried, with the outcome more difficult to predict.

IRCHLB-O6a-02

Strategic Uncertainty Impacts Area-wide Pest Management Decisions of Florida Citrus Growers

Ariel SINGERMAN[1] and Pilar USECHE[2]

[1]University of Florida, Lake Alfred, FL, USA.;
[2]University of Florida, Gainesville, FL, USA.

Abstract: We conducted a choice experiment based on the theory of global games to analyze the impact of strategic uncertainty on participation decisions of Florida citrus growers in area-wide pest management programs to control the vector of citrus greening. We found that the farmers' average certainty equivalent in a strategically uncertain setting, under a high (low) coordination requirement for obtaining a Pareto superior outcome, was lower (higher) compared to that of a lottery. In addition, we also found evidence that farmers' strong beliefs about neighbors not coordinating negatively impacted their choices. A measure of strategic uncertainty was also found to influence the likelihood of growers to actually participate in area-wide pest management. Thus, our results help explain why participation in area-wide pest management to control the vector of citrus greening across Florida has not been as widespread as expected; the strategic uncertainty involved in relying on neighbors has made many of them choose self-reliance in spraying despite the lower payoff.

Non-technical summary: We summarize the results of an experiment we conducted to better understand growers' choices regarding insecticide sprays coordination. We found that coordinating insecticide sprays encompasses more than just the application cost; it also includes the uncertainty growers face regarding their neighbors' behavior. Coordination among a higher percentage and larger number of growers increases the perceived risk. In addition, when growers believe their neighbors do not coordinate, they do not coordinate themselves. Therefore, to avoid the risk of relying on others, many growers prefer self-reliance in spraying.

IRCHLB-O6a-03

Upstream Effect of Agricultural Diseases: The Case of US Orange Juice Market

Sheng LI[1], Feng WU[2], Zhengfei GUAN[3]

[1] Gulf Coast Research and Education Center, University of Florida. [3] Gulf Coast Research and Education Center, University of Florida. [3] Corresponding author, Gulf Coast Research and Education Center, University of Florida.

Abstract: Huanglongbing (HLB) is the serious citrus plant diseases has been destroyed the Florida citrus grove for a decade. The effect on the orange grove has been spread into the processing industry and shrunk the U.S. orange juice market. A multi-stage partial equilibrium model is build to access the impact of HLB on the U.S citrus industry, with a particular focus on the distribution and allocation patterns among domestic orange farmer, juice producer, and foreign exports. Product differentiation and imperfect competition are considered in the model. The simulation results indicated 1) the effects on equilibrium prices and quantities in the supply chain responding to shocks in the supply of upstream; 2) the welfare changes among the stakeholders in the market. The implications of there results are discussed as the potential changes in tariff and market power (oligopsony).

Non-Technical Summary: HLB has been destroyed the Florida citrus grove and shrunk the U.S. orange juice market. This study showed how supply chains of orange juice respond to shocks in the supply of a primary input (fresh oranges). The associated changes in prices and quantities in the juice market, farm-level orange market, and raw juice import market are estimated. We also simulated the losses/benefits of the market players in the supply chain. The results will help to understand the impact of HLB in the U.S citrus industry and provide a reference for the stakeholders and policymakers for decision.

IRCHLB-O6a-04

Reducing the Human Spread of Huanglongbing via the Internet

Dan WILLEY

Fruitmentor, Inc.

Abstract: Human spread of huanglongbing is a significant threat to citrus in California. YouTube videos that demonstrated the grafting of citrus with backyard budwood were observed to be popular. Comments on these videos showed that many Californians were unaware of the pathogen-free budwood from the Citrus Clonal Protection Program (CCPP) and were grafting with backyard budwood. YouTube videos were published demonstrating grafting of citrus with pathogen-free budwood from the CCPP. Different videos targeted the various keywords commonly searched on Google and YouTube by people interested in the topic of citrus grafting. Upon publication of the videos many people began to sign up for CCPP budwood accounts and order pathogen-free budwood from the CCPP. The number of users has grown from a few hundred to more than two thousand today. The videos were translated from English into other languages and published on new YouTube channels for Spanish, Chinese, Vietnamese, and Korean. The videos on these channels have surpassed 15 million views and have attracted more than 130 thousand subscribers.

Non-Technical Summary: YouTube videos promoting the use of pathogen-free budwood from the Citrus Clonal Protection Program have led to an increase in the number of hobbyists grafting with pathogen-free budwood.

IRCHLB-O6a-05

Risk based HLB survey extension: optimizing manpower/resource availability

W. LIAO[1,2], W. LUO[1,3], D. POSNY[1,3], K.

WESTERMAN[2], S. HAJERIA[2], J. BARNIER[2] and T. GOTTWALD[1]

[1] U.S. Department of Agriculture, Agricultural Research Service, Fort Pierce, FL, USA
[2] Central California Citrus Tristeza Eradication Agency, Tulare, CA, USA
[3] Center for Integrated Pest Management, North Carolina State University, Raleigh, NC, USA

Abstract: Conducting surveys can greatly help growers and regulatory agencies to detect diseases and pests earlier in the epidemic phase, take appropriate mitigation action, and reduce economic and production losses. In fact, California's ACP/HLB risk-based survey design protocol has led to the detection of more than 900 HLB+ citrus trees in residential areas. However, most survey methodologies are not directly linked or driven by real-time manpower availability and fiscal resources that can impact effective survey programs. These constraints are dynamically interconnected with the epidemic development, and may change frequently throughout the survey period (e.g., delimitation responses or budget adjustments). To address this issue, we built a platform (web application) that integrates resource availability and California's ACP/HLB risk-based survey in a real-time setting. Users and decision makers will be able to optimize and balance the sampling selection procedure at the desired spatial level (e.g., state, county, risk survey district) to allocate the available resources within the current budget, appropriately. Given user-defined sampling criteria (e.g., target particular host varieties), single realization of the risk-based survey design as well as an overall, representative sampling range (from 1,000) simulation runs are provided. These informative outputs provide opportunities for refinement of the sampling strategy to fit the existing or proposed budget. Additionally, sampling protocols can be defined at the citrus block level (perimeter, hierarchical, cluster, or full survey) to assist in comprehensive, commercial survey design. A case study is presented to demonstrate the utility of the web application to compare different sampling choices and survey design scenarios given various resource and fiscal constraints.

Non-Technical summary: Manpower availability and fiscal constraints can impact the effectiveness of survey protocols. We developed a web application that integrates the ACP/HLB risk-based survey design and resource availability dynamically to assist decision makers in defining reasonable survey strategies corresponding to their real-time manpower and resource budgets.

IRCHLB-O6a-06

Structural impacts of HLB on Florida citrus production and implications for Texas, Arizona, and California.

Earl L. TAYLOR[1] and Tim R. GOTTWALD[1]

[1]US Department of Agriculture – ARS, Fort Pierce, FL, USA

Abstract: Florida citrus production has experience a number of structural changes throughout its history. These structural changes have been brought about by weather, disease, and changes in production and technology. Arguably, the most pronounced structural change has occurred since 2005 when Huanglongbing (HLB) was first discovered in Florida. Citrus production in Florida from 1980 thru 2017 was examined to identify and parse the structural changes and the associated impacts. In order to identify and quantify these structural changes, an event study methodology was utilized. The model developed examines the impact of significant weather events, disease, and the reallocation of resources associated with shifts in land use or urbanization. The goal of this study is to identify the structural changes associated with the HLB epidemic in Florida, and then quantify the impact upon citrus

production. HLB disease models indicate an exponential increase in disease incidence thru time and an associated exponential reduction in production. Florida production history indicates this relationship exists and given that Florida appears to be in the terminal end of the disease progress curve, the model provides information about the impacts of structural changes, in this case, HLB disease progress, on citrus production. Utilizing the results, we can examine citrus production in Texas, Arizona and California to examine potential impacts on citrus production as each of these states are in different stages of the disease progress curve.

Non-Technical summary: Florida citrus production from 1980 thru 2017 was examined to identify and quantify factors that have led to significant structural changes within the citrus industry. The factors examined include weather, disease, and reallocation of resources. Since Florida has been dealing with HLB and the associated impacts on production, we can utilize the resulting models parameters to provide insight into the associated impacts facing citrus production in Texas, Arizona and California.

IRCHLB-11:
Economics and Management Poster Presentations

IRCHLB-P11-140

The Profitability of New Citrus Plantings in Florida in the Era of HLB

Ariel SINGERMAN[1], Marina BURANI-AROUCA[1], and Stephen H. FUTCH[1]

[1]University of Florida, Lake Alfred, FL, USA.

Abstract: We use investment analysis to evaluate the profitability of an investment in an orange grove in Florida in the era of HLB. Planting new groves is key for ensuring a supply of fruit for processors and packinghouses to stay in business. However, a key question is whether it makes economic sense to plant a new grove in the current environment. We estimate the establishment and production costs for a new grove under endemic HLB conditions for three different tree planting densities under different market conditions and examine their profitability. Our results show that establishing a new grove with a tree density similar to that of the state's average is not profitable under current market conditions. However, higher tree densities are profitable despite the higher level of investment required. Therefore, planting higher density groves could help offset some of the impact of HLB by generating a simultaneous increase in yield per acre and decrease in cost of production per box (due to costs being allocated to a higher number of boxes); ultimately resulting in an increase in profitability per acre.

Non-technical summary: We analyzed the investment of planting a new grove in Florida under the current endemic HLB environment across the state. We found that establishing a new grove with a tree density similar to that of the state's average is not profitable under current market conditions; such density only attains a modest return under potential higher prices. Despite the higher level of investment of high-density plantings, such investments lower the average cost per box and result in increased profitability.

IRCHLB-12:
Molecular Plant Manipulations for the Future Oral Presentations

IRCHLB-O6b-01

Engineered oranges ectopically emitting β-caryophyllene to combat HLB

Berta ALQUÉZAR[1,2]; Haroldo Xavier LINHARES VOLPE[1]; Rodrigo FACCHINI MAGNANI[1,3]; Marcelo PEDREIRA DE

MIRANDA[1]; Márcia ALMEIDA[1], Mateus ALMEIDA SANTOS[1]; Nelson ARNO WULFF[1]; Michel DE VRIES[4]; Rob SCHUURINK[4]; Harro BOUWMEESTER[4]; A. Juliano AYRES[1]; **Leandro PEÑA[1,2]***

[1]Fund for Citrus Protection (FUNDECITRUS), Av. Dr. Adhemar Pereira de Barros 201, Melhado, 14.708-040, Araraquara, São Paulo, Brazil.

[2]Instituto de Biología Molecular y Celular de Plantas (IBMCP). Consejo Superior de Investigaciones Científicas (CSIC)-Universidad Politécnica de Valencia (UPV), Valencia, Spain.

[3]Chemistry Department, Federal University of Sao Carlos, Rod. Washington Luiz, Km 235, Postal Code 13.565-905, São Carlos-SP, Brazil.

[4]Swammerdam Institute for Life Sciences. University of Amsterdam. Science Park 904. 1098 XH Amsterdam, Netherlands.

Abstract: Huanglongbing (HLB) is currently the most serious citrus disease. Despite intensive research during last decades there is no known cure for it and, although some citrus relatives have been reported as putatively resistant to the causal agent/s, there are no conclusive scientific evidences yet. Nowadays HLB management is mainly based on the control of transmitting psyllids populations by intensive insecticide applications, which harm the environment and increase citrus production costs. This extra investment joined to the loss of benefits derived from the reduction of production and fruit quality caused by HLB infection is seriously compromising the possibility of maintaining an economically viable citriculture in most Asia, most Central America and South America and Florida (USA). Moreover, the disease is quickly spreading worldwide, remaining nowadays HLB-free just two important citrus growing regions, namely Australia/New Zealand and the Mediterranean basin. In the long-term the only solution to ensure an economically viable and environmentally friendly citriculture is through the development of cultivars resistant to the psyllid vectors and/or to HLB-causing bacteria, namely *Candidatus* Liberibacter asiaticus (CLas)[1]. No gene for CLas resistance has yet been identified. In addition, conventional breeding in citrus is hampered by its complex genetics and reproductive characteristics (apomixis, sterility, incompatibility, heterozygosity…). Thus, the best chance to obtain HLB-resistant elite cultivars is through biotechnological approaches. Recently, it has been reported that pure β-caryophyllene is able to repel *Diaphorina citri*, the main CLas vector[2]. Moreover, modified *Arabidopsis* plants emitting β-caryophyllene altered *D. citri* behavior, even if presented in a background of citrus volatiles[2], envisaging the potential of this strategy to control HLB spread. Valencia orange has been engineered to constitutively emit β-caryophyllene. Characterization of this new putatively *D. citri*-repellent cultivar in relation to its volatile content/emission and *D. citri* behavior will be presented.

Non-Technical Summary: HLB is nowadays the most devastating citrus disease and it is seriously threatening citrus production around the world. There is no effective cure to combat this disease and its management is based on expensive and environmental harmful strategies. We have developed a Valencia orange cultivar with the ability to emit β- caryophyllene, a volatile compound repellent to *D. citri*, at high concentration. The potential use of this new cultivar for psyllid (and thus HLB-spread) control will be discussed.

Citations:

Bové, J.M. 2014. Phytoparasitica 42(5):579

Alquézar et al. 2017. Scientific Reports 7(1):5639

IRCHLB-O6b-02

Transgenic Carrizo citrange mitigated HLB

development based on the bacterial "quorum sensing" system.

Eduardo Fermino CARLOS[1], Juliana DEGANELLO[1], Angélica Luana KEHL DA SILVA[1], Ananda COVRE DA SILVA[1], Gisele SILVA DE AQUINO[1], Luiz GONZAGA ESTEVES VIEIRA[2]

[1]IAPAR, Londrina, PR, Brazil), [2]UNOESTE, Presidente Prudente, SP, Brazil.

Abstract: The citrus industry based in the central part of São Paulo State in Brazil has been dramatically affected by Huanglongbing (HLB) since its first detection in that area in 2004. The disease has damaged the entire industry nowadays, including losses in other States, such as Paraná and Minas Gerais. There is a clear demand for more stable solutions than eradication management and intensive psyllid control for sustainable production of citrus in endemic areas. However, traditional plant breeding faces the lack of resistant genotype sources while genetic engineering is an alternative to provide candidate tolerant plants. *Candidatus* Liberibacter sp., the HLB associated agent,has the LuxR gene, a key component of the bacterial "quorum sensing" system. DONG et al (2000) identified the AiiA gene, which codes for a lactonase enzyme that inactivates the acyl homoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora* pathogen. The AiiA gene (Genebank AF196486) was used in this work and several juvenile transgenic Carrizo citrange (*Citrus sinensis* L. Osb.×*Poncirus trifoliata* L. Raf.) events were confirmed by PCR. The transgenic plants were grown up under greenhouse conditions, multiplied by grafting on rangpur lime rootstock and inoculated with buds from a HLB positive Valencia sweet orange (*Citrus sinensis* L. Osb.) tree by side grafting. Both sprouts, from the Valencia inoculum and from the transgenic Carrizo canopy were periodically evaluated by visual assessment and by qPCR. Results indicated the transgene did not prevented infection, but indeed reduced the load of the *Ca.* L. asiaticus in the transgenic Carrizo canopies. Four events had less than 1% of bacterial load compared to non- transgenic control plants. Those plants must still prove to be tolerant to HLB under field conditions but this transgenic strategy seems efficient to mitigate HLB development. (Patent pending: BR1020150260784/PCT201620107).

Non-technical summary: The development of HLB symptoms and the consequent economical losses may be minimized in a commercial citrus block if the host trees presented an unfavorable tissue environment to the bacterial associated agent. The tree may be infected but does not get sick! This strategy may be an additional valuable tool against HLB, and other scion and rootstock cultivars are being transformed as well.

Citations:

Yi-Hu Dong, Jin-Ling Xu, Xian-Zhen Li, and Lian-Hui Zhang. AiiA, an enzyme that inactivates the acylhomoserine lactone quorum-sensing signal and attenuates the virulence of *Erwinia carotovora*. *PNAS*, vol.97, n.7, p.3526–3531, 2000.

IRCHLB-O6b-03

Transgenic Single-chain Variable Fragment Antibodies Directed at CLas Significantly Reduce CLas Titer in ACP- Inoculated Carrizo

Joseph KRYSTEL[1], Qingchun SHI[1], John HARTUNG[2], Huawei LIU[2], David HALL[1] and Ed STOVER[1]

[1]USDA-ARS, United States Horticultural Research Laboratory, Ft. Pierce, USA, [2] USDA-ARS, Molecular Plant Pathology Laboratory, BARC, Beltsville, USA

Abstract: *Candidatus* Liberibacter asiaticus (CLas) is the associated agent of Huanglongbing (HLB), the most serious disease afflicting citrus worldwide. In previous work, we selected a population of single-chain variable fragment (scFv) antibodies that interact with two CLas peptides. The targets are homologs of Invasin (InvA) and Tolerance to Colicin (TolC). Both are predicted to be necessary for the pathogenicity of CLas. Transgenic Carrizo citrange were produced by *Agrobacterium* mediated transformation with gene cassettes driving expression of the scFv sequences with a modified 2x35s constitutive promoter. Confirmed transgenic plants were infected with CLas through infestation by CLas+ Asian Citrus Psyllids (ACP). Plants were maintained under greenhouse conditions for twelve months and monitored every three months by qPCR analysis. Transgenic plants showed a significant reduction in CLas titer (increase in Ct value) and had a higher proportion of plants with no measurable bacterial DNA amplification. Reduction in average titer over twelve months, comparing only those plants showing amplification, was as high as 99.0% for TolC targeting scFv expressers and 99.2% for InvA. Additional independent scFv transgenics have been subjected to ACP infestation with data collection ongoing. Plants showing promising results from greenhouse studies have been clonally replicated and grafted with 'Ray Ruby' grapefruit scions for field testing.

Non-technical summary: *Candidatus* Liberibacter asiaticus (CLas) is the associated agent of Huanglongbing (HLB), the most serious disease of citrus worldwide. In previous work, we selected a population of antigen-binding fragments that interact with two CLas peptides. Disruption of these peptides was predicted to interfere with the infection process. Multiple lines of transgenic citrus expressing these fragments were generated and inoculated with CLas. The transgenic plants showed significantly lower titers (99% reduction) of bacteria in greenhouse trials and are now being tested in the field.

IRCHLB-O6b-04

Construction of transgenic citrus rootstocks and scions for effective CLas clearance and robust HLB protection

Shujian ZHANG[1,2], Guixia HAO[1], Loan HUYNH[2], Qingchun SHI[1], Spencer MARSHALL[1], Ellen COCHRANE[1], Jefferson SHAW[1], **Supratim BASU[2]**, Ed STOVER[1] and Goutam GUPTA[2]

[1]U.S. Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL, USA
[2]New Mexico Consortium, Los Alamos, NM. U.S.A

Abstract: There appear to be no naturally occurring HLB-resistant citrus cultivars, therefore, transgenic strategies offer our best opportunity to create HLB resistant citrus for use in commercial production. Our aim is to construct transgenic citrus rootstocks and scions expressing *Candidatus* liberibacter asiaticus (CLas)-killer proteins that eliminate the associated agent and provide robust protection against HLB. We have already reported successful greenhouse efficacy studies on our 1st generation citrus transgenic expressing a modified tobacco Thionin (Hao et al, 2016). Preliminary data from field trials showed that the 1st generation citrus transgenics exhibited less blotchy mottle, greater overall health and bigger trees. Subsequently to improve upon the CLas-killing activity, we generated 2nd and 3rd generations of transgenic citrus. The 2nd generation transgenic citrus was designed to express a chimera of citrus Thionin (modified to increase activity and lower toxicity) and a citrus LPS-Binding Peptide (LBP) to enhance affinity to the CLas membrane. Preliminary studies in

the lab showed that the 2nd generation citrus transgenics are more efficient in clearing CLas than the 1st generation transgenics expressing only the modified tobacco Thionin. The 3rd generation citrus transgenics were designed to express chimeras of modified citrus Thionin and citrus Bacterial Permeability Increasing/Lipid-binding protein (BPI/LBP) or citrus Subtilisin. BPI/LBP was chosen to recognize the CLas membrane and Subtilisin was selected to cleave the CLas outer-membrane protein. The efficacy of CLas clearance by the 3rd generation transgenic plants are under evaluation. We expect that these studies will demonstrate the efficacy of citrus-derived anti-CLas proteins in CLas clearance and HLB protection.

Non-Technical Summary: Our project focuses on constructing transgenic citrus rootstocks and scions that facilitate effective clearance of CLas (the HLB-associated agent) and provide robust HLB protection. Successful completion of this project will provide critical planting material for HLB management and help sustain a productive and economically viable citrus industry.

Citations:

Hao, Guixia, Ed Stover, and Goutam Gupta. "Overexpression of a modified plant thionin enhances disease resistance to citrus canker and huanglongbing (HLB)." *Frontiers in plant science* 7 (2016): 1078.

IRCHLB-O6b-05

Editing citrus *DMR6* via a CRISPR/Cas9 system to improve Huanglongbing tolerance

Shujian ZHANG, Qingchun SHI, Spencer MARSHALL, Yongping DUAN, David HALL, Ed STOVER

U.S. Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL, USA

Abstract: Huanglongbing (HLB) is a devastating disease in citrus worldwide. Genetic resistance from commercial varieties is lacking but tolerance has been observed. Downy Mildew Resistance 6 (DMR6) is a salicylic acid (SA) 5-hydroxylase that acts as a suppressor of plant immunity, and its expression is upregulated during pathogen infection[1]. Mutation of *Arabidopsis DMR6* and its orthologs in potato and tomato resulted in elevated salicylic acid levels and conferred broad-spectrum disease resistance[2,3]. In citrus, we found *DMR6*-like genes were downregulated in the more HLB-tolerant 'Jackson' grapefruit compared with those in the HLB-susceptible 'Marsh' grapefruit. Here, we conducted an expression survey of citrus *DMR6* orthologs (*CsDMR6-1* and *CsDMR6-2*) in seven citrus genotypes. The expression levels were significantly higher in infected trees than in uninfected ones regardless of genotype, suggesting a universal role of *DMR6* in the defense against the HLB associated bacterium, *Candidatus* Liberibacter asiaticus. A CRISPR/Cas9 system with the *Yao* promoter driving Cas9, was used to create stable transgenics to knock- out these *DMR6* orthologs in Carrizo. Targeted DNA mutations were achieved at variable rates in different lines and the highest was 64% in a *CsDMR6-1*-targeting line. We also conducted RNA interference-mediated gene silencing in Carrizo, through transgenic insertion of a hairpin, resulting in an average silencing efficiency of 67.1% for *CsDMR6-1* and of 96.9% for *CsDMR6-2. DMR6* silenced plants exhibited moderate to strong activation of defense-related genes compared to non- transgenic controls. Ongoing experiments include targeted next-generation sequencing of CRISPR transgenic lines and evaluations of disease resistance of *DMR6* silenced and mutated plants against HLB.

Non-Technical Summary: Negative defense regulators are genes that suppress plant defense when they are expressed, and plant pathogens such as the one causing HLB can use these genes to conquer plant cells. We identified such negative regulators in citrus, called DMR6, and used bio technology to reduce their expression and hence activate citrus defense. These defenses boosted citrus lines will be tested for enhanced HLB tolerance.

Citations:

Zeilmaker, Tieme, et al. *The Plant Journal* 81.2 (2015): 210-222.
de Toledo Thomazella, Daniela Paula, et al. *bioRxiv* (2016): 064824.
Sun, Kaile, et al. *Transgenic research* 25.5 (2016): 731-742.

IRCHLB-O6b-06

Insight into the mechanism of the citrus Huanglongbing pathosystem and its implications in disease control

Zhiqian PANG, Hang SU, **Nian WANG**

University of Florida, Lake Alfred, FL, USA

Abstract: Citrus is one of the most important fruit crops worldwide. However, citrus production in the USA is facing an unprecedented challenge due to Huanglongbing (HLB, also known as citrus greening). *Candidatus* Liberibacter asiaticus (CLas) is the most common associated agent of HLB. As an intracellular pathogen multiplying in the cytosol of living phloem cells of host plants, CLas's survival is dependent on live host cells. CLas is known to secrete putative virulence proteins (effectors) in a Sec-dependent manner. Here we show that one of these secreted proteins, SDE15, targets a well-known negative regulator of plant programmed cell death (PCD) to promote infection. SDE15 could be detected in the phloem sap of CLas-infected plants. Transgenic expression of SDE15 in citrus promotes CLas multiplication and HLB symptom development. SDE15 suppresses not only PCD induced by *Xanthomonas citri* subsp. *citri* (*Xcc*) in citrus, but also PCD induced by AvrBsT (a PCD-eliciting *Xanthomonas* effector) in tobacco, suggesting that SDE15 is a broad-spectrum bacterial suppressor of plant PCD. Yeast two-hybrid, in vitro protein pull-down and in vivo bimolecular fluorescence complementation assays showed that SDE15 interacts with ACD2 (ACCELERATED CELL DEATH 2),
a repressor of plant PCD and that it enhances the red chlorophyll catabolite reductase (RCCR) activity of ACD2 to remove porphyrin-related molecules, accumulation of which causes PCD. SDE15 promotes the chlorophyll break-down in planta and contributes to the development of yellowing symptom associated with HLB. Characterization of SDE15 unravels an elusive aspect of the mechanism of a major plant disease. In addition, I will present our current progress in application of CRISPR in citrus improvement against HLB and other approaches in HLB control.

Non-Technical summary: Here we described one important effector of CLas which might be involved in suppressing plant defense and promoting pathogen growth. We have been developing genome editing tools to modify citrus to generate disease resistant citrus. Understanding the virulence mechanism of CLas helps us develop suitable HLB control strategies.

IRCHLB-12:
Molecular Plant Manipulations for the Future Poster Presentations

IRCHLB-P12-141

β-caryophyllene repels *Diaphorina citri*, vector of *Candidatus* Liberibacters

Berta ALQUÉZAR[1,2]; Haroldo Xavier LINHARES VOLPE[1]; Rodrigo FACCHINI MAGNANI[1,3]; Marcelo PEDREIRA DE MIRANDA[1]; Márcia ALMEIDA[1], Mateus ALMEIDA SANTOS[1]; Nelson ARNO WULFF[1]; Michel DE VRIES[4]; Rob SCHUURINK[4]; Harro BOUWMEESTER[4];

A. Juliano AYRES[1]; **Leandro PEÑA[1,2*]**

[1]Fund for Citrus Protection (FUNDECITRUS), Av. Dr. Adhemar Pereira de Barros 201, Melhado, 14.708-040, Araraquara, São Paulo, Brazil. [2]Instituto de Biología Molecular y Celular de Plantas (IBMCP). Consejo Superior de Investigaciones Científicas (CSIC)- Universidad Politécnica de Valencia (UPV), Valencia, Spain. [3]Chemistry Department, Federal University of Sao Carlos, Rod. Washington Luiz, Km 235, Postal Code 13.565-905, São Carlos-SP, Brazil. [4]Swammerdam Institute for Life Sciences. University of Amsterdam. Science Park 904. 1098 XH Amsterdam, Netherlands.

Abstract: Citrus production is severely threatened by Huanglongbing (HLB), for which as yet a cure is not available. Spread of HLB associated bacteria is intimately connected with dispersal and feeding of the psyllid *Diaphorina citri*, oligophagous on rutaceous host plants. Effective control of this insect is an important component in successful HLB management programs. Organic volatile compounds (VOCs) released from the non- host guava have been shown to be repellent to the psyllid and to inhibit its response to citrus odour. By comparative analyses of citrus and guava VOC emission profiles we identified one volatile compound, namely (*E*)-β- caryophyllene, always present in guava VOC profiles and barely detected in those of non- stressed citrus. In olfatometric assays, pure commercial (*E*)-β-caryophyllene exerted, at certain doses, a repellent effect on *D. citri*. However, the volatility, instability and production costs of β-caryophyllene may be problematic for its direct use in agriculture. Overcoming these inconveniences will be feasible by generating a transgenic plant constitutively emitting the VOC of interest at the desired level. To validate this strategy we generated *Arabidopsis* (*E*)-β-caryophyllene synthase over-expression and knock-out lines and tested the response of *D. citri* to the VOCs they emit. Olfatometry results indicate that *D. citri* behavior can be modified by altering VOC emission. β-caryophyllene induced- emission had the ability of turning *Arabidopsis* neutral non-host plant to *D. citri* into a repellent one, and the effect that this compound exerts on the psyllid is maintained even if presented in a background of citrus volatiles.

Non-Technical Summary: Non-host plant rejection mediated by (*E*)-β-caryophyllene is demonstrated here by using chemical dispensers and genetically engineered *Arabidopsis* plants with modified emission of VOCs. This study shows that transgenic plants with an inherent ability to release (*E*)-β- caryophyllene can potentially be used in new protection strategies of citrus trees against HLB.

IRCHLB-13: Bacteriocides and Plant Defenses Oral Presentations

IRCHLB-O7a-01

Characterization of a novel virus-like RNA that accumulates to near rRNA levels in phloem and systemically infects all commercial varieties of citrus with limited or no symptoms for use as expression vector in anti-Huanglongbing management programs

Anne E. SIMON[1], Jingyuan LIU[1], Feng GAO[1], Sohrab BODAGHI[2], and Georgios VIDALAKIS[2]

[1]University of Maryland, College Park, MD, USA, [2]University of California, Riverside, CA USA

Abstract: We report the discovery of a new type of citrus infectious agent. Unlike traditional plant viruses, this agent is substantially smaller and capable of replication and phloem-limited systemic movement throughout a host without

encoding any movement proteins, coat proteins, or silencing suppressors and without any requirement for a helper virus. This movement protein-independent infectious RNA (MPI2RNA) is 100% graft transmissible into citron, sweet orange, sour orange, grapefruit, lime, lemon, mandarins, tangelo, and kumquat but is not transmissible by mechanical wounding or by aphids. Symptoms, if present, are confined to a few yellow veins on a few leaves scattered through the tree. In *Nicotiana benthamiana*, this MPI2RNA moves bi-directionally into emerging inflorescence and roots after agro- infiltration, reaching levels visible on ethidium-bromide stained gels despite only being present in companion cells, phloem parenchyma cells and sieve elements. Only two proteins are encoded, with the RNA-dependent RNA polymerase generated by an astonishingly competent ribosomal frameshifting event. Almost half of the extensive 3'UTR is unimportant, whereas the remainder includes replication and translation elements, and several required long-distance RNA:RNA interactions. This MPI2RNA should be a highly efficient vector for delivery of small RNAs, peptides and possibly proteins into the phloem of mature citrus.

Non-technical summary: Effective treatment for HLB-diseased trees and protection of uninfected trees will likely require virus vectors that can deposit anti-bacterial and anti- phyllid substances directly into the phloem (veins) of trees where the bacteria live and the insects feed. We have discovered a novel virus-like agent that can accumulate to high levels in the phloem of virtually all citrus, generates few if any symptoms, and should be capable of producing copious amounts of anti- HLB substances where they are needed.

IRCHLB-O7a-02

Antisense Oligonucleotides Targeting HLB in its Vector and Host

Andrés SANDOVAL-MOJICA and Kirsten PELZ-STELINSKI
University of Florida, Lake Alfred, USA

Abstract: The Asian citrus psyllid (ACP), *Diaphorina citri* (Hemiptera: Liviidae), is the vector of the bacterium *Candidatus* Liberibacter asiaticus (CLas), which is associated with citrus greening disease (HLB). Using novel gene-based bactericidal agents to target CLas, in both vector and host, may represent a viable strategy for controlling the transmission of HLB. Development of oligonucleotide-based bactericides to manage HLB offers a significant advantage over traditional insecticide use or antibiotic therapy. These engineered molecules can be delivered specifically to target bacteria based on gene sequence, avoiding the problems of effecting non-target bacteria. We assessed the bactericidal capabilities of FANA antisense oligonucleotides (FANA ASOs) and peptide phosphorodiamidate morpholino oligomers (PPMOs) by (1) confirming their capacity to penetrate insect cells, (2) silence bacterial essential genes in the ACP and (3) evaluating their effect in bacterial titer in both ACP and *Citrus* plants. Fluorescence microscopy demonstrated that a labeled oligonucleotides were transfected into insect cells 24 hours post treatment, without any delivery agent. Fluorescently labeled FANA ASOs were detected in the digestive tracts of ACP that fed on treated citrus leaves. CLas-specific olionucleotides delivered to excised citrus leaves inhibited expression of a CLas essential gene up to 4-fold and reduced the relative CLas titer in CLas-infected ACP significantly compared with ACP not exposed to oligonucleotides. Finally, root infusions of CLas-specific FANA ASO and PPMOs into one-year-old *Citrus* trees reduced bacterial titers in infected plants and reduced the new CLas infections among healthy plants. Specific targeting of CLas and *D. citri* endosymbionts using gene-based bacterial therapy strategies may be an effective alternative to traditional insecticides for integrated pest management.

Non-Technical summary: We investigated the capacity of modified nucleic acids to eliminate the HLB pathogen and psyllid vectors. Synthetic DNA-based bactericidal agents can be used to specifically knockdown CLas or bacteria necessary for psyllid survival. These molecules are more effective than broad-spectrum antibiotics because of their specificity and offer greater flexibility because they can target multiple bacterial genes, reducing the likelihood of resistance. Treating infected psyllids and plants with these engineered molecules decreased the density of the bacterium and reduced infection of healthy plants. These synthetic molecules represent a novel tool for HLB management.

IRCHLB-O7a-03

Use of dsRNA spray treatments to activate native citrus defenses and suppress CLas metabolic activity.

Tina STRAUSS[1], Shujian ZHANG[1], Giovana PERAZZO[1], Dean W. GABRIEL[1, 2]

[1]Integrated Plant Genetics, Inc., Gainesville, FL, 32653, USA.

[2]Plant Pathology Dept., University of Florida, Gainesville, FL 32611, USA

Abstract. Programmed cell death (PCD) or apoptosis is a plant defense response that can be triggered upon pathogen infection. Some PCD triggers, such as Reactive Oxygen Species (ROS), can activate or prime plant defenses over long distances. There are also some genes in plants which dampen plant defense responses. By suppressing expression of genes that suppress either ROS generation or apoptosis, PCD should become hypersensitive to pathogen infection. Huanglongbing (HLB), associated with *Ca.* Liberibacter asiaticus (CLas), threatens citrus production worldwide. In plants, CLas grows strictly inside living citrus phloem cells. Citrus cells detect CLas, but fail to trigger adequate native defenses or PCD. CLas secretes both a functional peroxiredoxin[1] and a peroxidase[2] directly into the citrus cell cytoplasm that significantly dampen both the ROS response and signaling pathways. We have developed an RNA interference (RNAi) strategy to help prime and hypersensitize the natural citrus defense reactions to CLas. Initial proof of concept was established using transgenic Carrizo rootstocks. Average silencing efficiencies of the transformed rootstocks ranged from 77.30% to 82.35%. The silencing signals moved from transgenic rootstocks to nontransgenic (NT) mature citrus scions and enhanced the citrus defense reaction sufficiently to cure CLas from infected NT branches in selected lines. Informed by these results, dsRNAs were commercially synthesized and used in spray applications in field trials on commercially grown, heavily CLas- infected and HLB symptomatic citrus. Since DNA is very long lived in citrus, living vs. dead bacterial cells cannot be differentiated by DNA-based PCR methodologies, making anti-Las treatment effects difficult to assess. Since bacterial mRNAs have an average half life of only a few minutes, qRT-PCR of mRNA expression was used to assess treatment effects. Two citrus gene targets and a streptomycin-based nanoemulsion proved to significantly suppress CLas mRNA activity both in treated branches and long distance in untreated branches of the same tree.

Non-Technical Summary. Programmed cell death (PCD) in plants is a cellular response to physical, chemical or biological insults, including pathogen attack. PCD is highly regulated to ensure it is not triggered lightly. Plant pathogens often interfere with this regulation if it helps their survival and multiplication. Citrus cells detect Las bacteria after initial infection, but fail to trigger an adequate defense. Based on several years of evaluating transgenic plants that are more hypersensitive to CLas than nontransgenics, we designed and applied two very different (and safe) dsRNA based spray treatments in field trials of commercially grown Hamlins in Florida that are symptomatic for HLB. Initial results are encouraging, based on suppression of Las gene expression both in treated and untreated branches.

Citations:

Jain M, Munoz-Bodnar A, Zhang S, Gabriel DW. 2018. Mol. Plant-Microbe Interact. 31:1312

Jain M, Fleites LA, Gabriel DW. 2015. Mol. Plant-Microbe Interact. 28:1330

IRCHLB-O7a-04

Candidatus Liberibacter asiaticus peroxiredoxin (LasBCP) suppresses oxylipin-mediated defense responses in citrus host, providing a potential target for chemical control of HLB

Mukesh JAIN, Alejandra MUNOZ-BODNAR, Dean W. GABRIEL

Department of Plant Pathology, University of Florida, Gainesville, FL 32611, USA

Abstract: Two peroxiredoxin genes *Lasprx5* (CLIBASIA_RS00940) and *Lasbcp* (CLIBASIA_RS00445) are conserved among all (even phage-less) strains of *Candidatus* Liberibacter asiaticus[1-2]. The LasBCP peroxiredoxin was functionally characterized as a secreted pathogenicity effector, with substrate specificity for organic peroxides. Transient expression of LasBCP in citrus leaves (a) provided significant protection against peroxidation of free and membrane- bound lipids, thereby preserving molecular integrity of chlorophyll apparatus and (b) reduced accumulation of lipid peroxidation products following exposure to organic peroxides. The lipid peroxidation products (oxylipins) extracted from GUS-expressing citrus leaves reduced viability of *Liberibacter crescens*, the only Liberibacter species cultured to date. However, a similar oxylipin extract obtained from the LasBCP-expressing leaves was less inhibitory to *L. crescens* growth and viability in culture. Quantitative RT-PCR analyses showed transcriptional downregulation of oxylipin-regulated jasmonic acid (*JAR1*, *COI1* and *JAZ1*) and salicylic acid (*PAL*, *ICS* and *PR1*) signaling pathways[3] in citrus leaves expressing LasBCP. These data clearly demonstrated a protective role of secreted LasBCP in favor of Las survival and colonization by (a) alleviating ROS-induced lipid peroxidation in citrus host, (b) preventing accumulation of antimicrobial oxylipins, and (c) suppressing both localized and systemic immune responses *in planta*. A high throughput assay was developed to screen two Prestwick small molecule libraries (1600 chemical and phytochemical compounds) for inhibition of purified LasBCP enzymatic activity. Out of 28 initially identified chemicals that inhibited peroxiredoxin activity in the primary and dose-dependent secondary screens, three lead chemicals were selected which are currently being examined for HLB control.

Non-technical summary: Host-generated apoplastic oxidative defenses in response to pathogen invasion results in a diverse array of oxygenated unsaturated fatty acid derivatives called as oxylipins. Oxylipins are directly antimicrobial as well as potent defense signal molecules eliciting both hypersensitive cell death response (HR) and systemic acquired resistance (SAR). Las secreted peroxiredoxin (LasBCP) suppresses lipid peroxidation and accumulation of oxylipins in the citrus host, thereby suppressing innate plant immunity. Inhibition of LasBCP activity may provide an attractive molecular target for chemical control of HLB during early stages of infection or preventing HLB infection in new citrus plantations.

Citations:

Jain M, Munoz-Bodnar A, Zhang S, Gabriel DW. 2018. Mol. Plant-Microbe Interact. 31:1312

Jain M, Fleites LA, Gabriel DW. 2015. Mol. Plant-Microbe Interact. 28:1330

Farmer EE, Müller MJ. 2013. Annu. Rev. Plant Biol. 64:429

IRCHLB-O7a-05

Molecular modeling and high throughput antimicrobial screening approaches to identify novel CLas inhibitors

Kranthi MANDADI[1, 2], Sonia IRIGOYEN[1], Shankar PANT[1], Sandun FERNANDO[3], Veronica ANCONA[4], Gitta COAKER[5], Shree P. THAPA[5], Elizabeth E. GRAFTON-CARDWELL[6], William O. DAWSON[7], Choaa EL-MOHTAR[7], Stephen FUTCH[7], Mike IREY[8]

[1]Texas A&M AgriLife Research and Extension Center, 2415 E. Hwy. 83, Weslaco, Texas
[2]Department of Plant Pathology and Microbiology, Texas A&M University, 2132 TAMU, College Station, Texas
[3]Department of Biological and Agricultural Engineering, Texas A&M University, 2132 TAMU, College Station, Texas
[4]Department of Agriculture, Agribusiness, and Environmental Sciences, Texas A&M University-Kingsville, Citrus Center, 312 N. International Blvd., Weslaco Texas 78599
[5]Department of Plant Pathology, University of California, Davis, California
[6]University of California, Riverside, California
[7]University of Florida, Citrus Research and Education Center, IFAS, Lake Alfred, Florida
[8]Southern Gardens Citrus, Clewiston, Florida

Abstract: Citrus greening or Huanglongbing (HLB) disease is devastating the citrus industry in Florida and starting to show its effects in Texas and California. Multiple antibiotics/bactericides are currently being evaluated to mitigate HLB losses, however they only show limited success. There is an urgent need to evaluate new antimicrobials and deploy technologies that facilitate rapid screening of antimicrobials. Here, we employed an *in-silico* target-based pharmacophore screening strategy, and an *in- vitro* *Candidatus* Liberibacter asiaticus (CLas) citrus hairy root culture bioassay to rapidly screen and identify novel CLas antimicrobials. The efforts so far identified two potent small- molecules targeting a CLas virulence protein, and multiple broad-spectrum antimicrobial peptides (AMPs) that suppressed CLas accumulation in hairy root cultures. Greenhouse and field trials are underway to determine the antimicrobial activities *in planta* using foliar sprays and Citrus tristeza virus viral vector (CTVvv)-based deliveries.

Non-Technical Summary: Rapid screening and deployment of new antimicrobials that can inhibit *Candidatus* Liberibacter asiaticus (CLas) should help mitigate the tremendous losses inflicted by Citrus greening disease (syn. Huanglongbing, HLB). Utilizing state- of-the-art computational modeling tools, and an innovative, high throughput antimicrobial screening approach, we identified multiple novel CLas inhibitors. These inhibitors, after further evaluation, could be deployed in the field and will offer sustainable, non-antibiotic based antimicrobial strategies for HLB management.

IRCHLB-O7a-06

HLB treatment: engineering of novel citrus innate immunity

Jeanette C. Velásquez GUZMÁN[1], Supratim BASU[1], Loan HUYNH[1], Joseph KRYSTEL[2], Roel RABARA[1], Godfrey MILES[2], Shujian ZHANG[1], Guixia HAO[2], Qingchun SHI[2], Jefferson SHAW[2], David HALL[2], Ed STOVER[2], and **Goutam GUPTA**[1*]

[1]Biolab, New Mexico Consortium (NMC) 100 Entrada Drive, Los Alamos, NM 87544, USA;

[2]U. S. Horticultural Research Laboratory 2001 South Rock Road, Fort Pierce, FL 34945, USA

Abstract:

In our previous work (see the preceding abstract), we have demonstrated that several citrus antimicrobial/anti-infective proteins are suppressed during *Candidatus* Liberibacter asiaticus (CLas) infection. In this work, we (Gupta and Stover Laboratories) have focused on overcoming this response. The first option one might consider is to supplement one of the endogenous defense proteins that is suppressed during CLas infection. However, this may not be a viable solution for HLB therapy because during host defense, multiple (and not one) proteins are induced at a low level but act in concert to clear the bacterium and/or block infection. Thus, if a single defense peptide/protein is chosen for HLB therapy, the high dose needed for its action may be toxic to the host or non-target organisms. Therefore, we chose an altogether new approach in that we selected ones from the class of the most suitable citrus defense peptides and modified their sequence/structure to increase activity and lower toxicity. Toward this end, we selected candidates from the class of citrus amphipathic helical peptides that show some (albeit low) bactericidal activity against gram- negative bacteria such as CLas. We designed novel helix-turn-helix peptides based upon several citrus amphipathic helical peptides. We performed *in vitro* assays to screen one or a few helix-turn-helix peptides with high CLas-killing activity and no toxicity to human and plant cells. Also, we demonstrated that unlike the endogenous single helix, the designed helix-turn-helix peptides are less susceptible to bacterial resistance. Finally, we showed by gene expression studies that the designed helix-turn-helix peptides may activate innate immune pathways that are suppressed by CLas. Therefore, we postulate that, in view of their anti-CLas efficacy, immune modulatory activity, and small/compact size, the helix-turn-helix peptides may be suitable for HLB treatment by topical delivery.

Non-Technical summary: Federal and industry investments allowed us to develop treatment of already CLas-infected orchards that can potentially sustain citrus productivity. The treatment is based upon topical delivery of the citrus-derived peptides that have anti- CLas efficacy, immune modulatory activity, and small/compact size. The citrus-based peptides are superior to the currently used antibiotics in terms of efficacy and consumer acceptability.

IRCHLB-13: Bacteriocides and Plant Defenses Poster Presentations

IRCHLB-P13-142

Toward in vitro production of the *Liberibacter asiaticus* phage SC1.

Janetta HARKOVIRTA[1], Jennie FAGEN[1], Kevin LINCE[2], Evan BRASWELL[2], Chase BEISEL[1,3]

[1]North Carolina State University, Raleigh NC, USA [2]U.S. Department of Agriculture, Edinburg TX, USA [3]Hemholtz Institute for RNA-based Infection Research,Wurzburg, Germany

Abstract: Phage therapy is a promising alternative to traditional antibiotics and has been used to control recalcitrant bacterial infections in human health as well as in plant agriculture. However, such therapies rely on the identification and isolation of sufficiently virulent phage for the targeted pathogen. Unfortunately, isolation and generation of phages has required culturing bacterial hosts, placing phage therapy out of reach for fastidious pathogens such as Liberibacter species. To overcome this restriction, we investigated the utility of two cell-free transcription/translation systems for production and assembly of *Candidatus* Liberibacter asiaticus (CLas) bacteriophage. Our study focuses on the phage

SC1 which was identified from genomic sequencing of CLas where it exists as an integrated prophage. We identified nine genes encoding the primary SC1 structural proteins through sequence homology, structural modeling, and genomic synteny with canonical Podoviridae. These proteins were then expressed, purified, and partially assembled in vitro providing the necessary components for SC1-based DNA delivery to its native host, CLas. When coupled with an amplified SC1 genome, these SC1 shells have the potential to facilitate wild- type CLas control via phage therapy. Alternatively, when coupled with a phagemid, SC1 may be used to deliver engineered DNA to CLas allowing for culture-independent manipulation of the pathogen. Ultimately, the ability to revive phage and phage components from sequencing data bypasses the need to culture the bacterial target and search for compatible phage which is a labor-intensive process and opens up new avenues of control for fastidious pathogens.

Non-technical summary: We are developing a natural predator of *Candidatus* Liberibacter asiaticus, the bacteriophage SC1. This bacteriophage may function as both a form ofbiocontrol and a potential engineering tool for the study of the pathogen.

Citations:

Rustad, Mark, et al. "Synthesis of Infectious Bacteriophages in an E. coli-based Cell-free Expression System." *JoVE (Journal of Visualized Experiments)* 126 (2017): e56144.

IRCHLB-14: Host Responses to Infection Oral Presentations

IRCHLB-O7b-01

Microbial Community Shifts Associated with Citrus Huanglongbing Severity

Nichole A. GINNAN[1], Tyler DANG[1], Sohrab BODAGHI[1], Paul M. RUEGGER[1], Beth B. PEACOCK[1], Greg MCCOLLUM[2], Gary ENGLAND[3], Georgios VIDALAKIS[1], Caroline ROPER[1], Philippe ROLSHAUSEN[4], and James BORNEMAN[1]

[1]Department of Microbiology and Plant Pathology, University of California, Riverside, USA, [2]U.S. Department of Agriculture, ARS, Fort Pierce, USA, [3]Hasting Agricultural Extension Center, University of Florida, Hastings, USA, [4]Department of Botany and Plant Sciences, University of California Riverside, USA

Abstract: Microbiomes are interactive cross-organismal microbial communities that influence host productivity, development, and tolerance to stresses, including disease. To better understand the microbial ecology underlying Huanglongbing (HLB) in citrus, we characterized the fungal and bacterial communities associated with different niche compartments (roots, stems, and leaves) of citrus trees in Florida. We structured our study to interrogate these communities to determine if microorganisms or groups of microorganisms play a role as HLB facilitators or as HLB antagonists. We sampled *Candidatus* Liberibacter asiaticus (CLas) infected citrus trees throughout Florida and discovered trees that display delayed disease development even while being surrounded by heavily symptomatic trees. These trees are termed survivor trees. We hypothesized that HLB symptoms could be directly related to the structure of the microbiome during disease development. To test this hypothesis, we sampled citrus leaves, stems, and roots in four Florida orchards over a 3-year period. In each orchard we sampled survivor trees and a companion set of citrus trees that displayed typical rapid HLB development. Using a Next-generation amplicon-based sequencing platform we have identified members of the citrus microbiome that negatively correlate with

both HLB disease rating and CLas titer. This indicates that members of the citrus microbiome could be directly antagonistic to CLas or curb HLB symptoms by another mechanism. Among these negative correlates are known antibiotic producing and plant growth promoting microbes. We have also identified microbes that positively correlate with disease development. These include some known pathogens, such as *Phytophthora* and *Fusarium* species residing in the roots. We have coupled this work with a robust culturing effort to create a repository of pure bacterial and fungal isolates that originate from citrus. These isolates are undergoing testing to better understand their biological role in the HLB disease phenotype.

Non-Technical summary: Our goal is to understand the community dynamics of the Huanglongbing pathosystem at the microbial level and identify microbial disease facilitators or suppressors to advance the development of HLB mitigation tools.

IRCHLB-O7b-02

Spatial Chemistry of Huanglongbing in Citrus

A. AKSENOV[1], A. BLACUTT[2], N. GINNAN[2], A. MELNIK[1], M. ERNST[1], J. BRADY[3], G. MCCOLLUM[3], P. DORRESTEIN[1], P. ROLSHAUSEN[2] and **C. ROPER[2]**

[1]Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California, San Diego, La Jolla, CA 92093; [2]Department of Microbiology and Plant Pathology, University of California, Riverside, CA 92521; [3]United States Department of Food and Agriculture, Agricultural Research Service, Fort Pierce, FL 34945.

Abstract: The utility of spatial mapping of diseases has been long appreciated. A famous historical example is of the London cholera outbreak where mapping of death cases by John Snow led to the understanding that cholera is a water-borne disease. We utilize several recently developed tools for Mass Spectrometry-based metabolomics to enable visualizations of complex chemistries within plant tissues at high resolution to explore how these chemistries change upon disease development in Huanglongbing (HLB). We demonstrate how molecular maps produced using metabolomic information on scales of an orchard or even an entire state down to a single branch or leaf may yield insight in the biology of the disease. This is accomplished by visualizing disease-linked perturbations in the complex metabolic processes of a living plant in a comprehensive manner using meta- metabolomic approaches. We further illustrate how juxtaposing such molecular maps for different compounds may aid in generating new hypotheses, such as the mechanism of formation of one of the HLB biomarker compounds, feruloylputrescine as well as suppression of flavonoid biosynthesis during disease. We aim to provide these maps as a public resource to enable other researchers to further explore the biology of HLB to potentially uncover hidden spatial patterns of metabolites associated with the disease. Moreover, these chemical maps present complex information in a visually appealing and easy to interpret format that can be helpful in dissecting complex chemical networks associated with plant health and the disease phenotype.

Non-technical summary: Visualizing distribution of compounds in plants at high resolution and in 3 dimensions is a new and exciting tool for studying the biology of a pathosystem. HLB causes major chemical shifts that we have visualized using 2D and 3D models of leaves and branches. These chemical maps aid in interpreting how and why these chemical transformations occur to better understand how trees become sick during HLB progression.

IRCHLB-O7b-03

Rootstock influences on tree health and growth in response to *Candidatus* Liberibacter asiaticus in grafted sweet orange trees

Kim D. BOWMAN[1], Rayane Barcelos BISI[2] and Ute ALBRECHT[2]

[1]U.S. Department of Agriculture/ARS, Horticultural Research Laboratory, Fort Pierce, FL, USA
[2]University of Florida/IFAS, Southwest Florida Research and Education Center, Immokalee, FL, USA

Abstract: A greenhouse study was conducted to examine the influence of rootstock on sweet orange tree health following infection of the trees with *Candidatus* Liberibacter asiaticus (CLas). Rootstocks compared in the study were ‘Cleopatra’, ‘Ridge’, sour orange, and eight USDA hybrid rootstocks of commercial importance. Valencia trees on each rootstock were inoculated with CLas, and maintained with non-infected controls in a greenhouse for 50 weeks. Leaves and roots were used to evaluate infection by PCR. Trees were measured for growth and leaf area, and scored for disease symptoms. Infection of trees with CLas resulted in symptoms of HLB on all rootstocks. The average percent reduction in the growth of the scion trunk due to infection ranged from 44% for trees on US-802, to 77 % for trees on Sour orange, but the rootstock effect was not significant. However, rootstock had a significant effect on the relative reduction in number of leaves resulting from infection with CLas at 50 weeks after inoculation. The two rootstocks with the least effects from CLas infection on leaf canopy were US-942 and US-812, with a 35 % and 41 % reduction in number of leaves, respectively. This was significantly better than all other rootstocks except US-802. Trees on ‘Cleopatra’, ‘Swingle’, and ‘Ridge’ rootstocks had the greatest percent reduction in the number of leaves, ranging from 81 – 78 %. A similar significant effect of rootstock was observed on total leaf area reduction due to infection, again with the rootstocks US-812, US-942, and US-802 showing the least damage. The observation that US-942 rootstock, which frequently has provided superior field performance under HLB-endemic conditions, was among the best in the effects on leaf area from CLas in the greenhouse, supports a classification of tolerant for US-942. Further, this indicates that greenhouse studies can be useful to evaluate relative cultivar response to CLas infection in grafted trees.

Non-technical summary: Eleven different rootstock varieties with commercial importance were compared for their influence on the CLas-tolerance of grafted one-year-old Valencia sweet orange trees. Rootstocks greatly differed in their influence on growth of non-infected trees. Following infection with CLas, Valencia trees on some rootstocks exhibited much greater relative decline from huanglongbing disease than others. Among the most important commercial rootstocks, US-942 and US-812 appeared to have the least canopy damage, while ‘Cleopatra’ and ‘Swingle’ suffered the most canopy damage. The findings of this study reinforce the importance of rootstock in the presence of HLB.

IRCHLB-O7b-04

Rootstock influences leaf and root metabolic response to *Candidatus* Liberibacter asiaticus in grafted sweet orange trees

Ute ALBRECHT[1], Indu TRIPATHI[1] and Kim D. BOWMAN[2]

[1]University of Florida/IFAS, Southwest Florida Research and Education Center, Immokalee, FL, USA; [2]U.S. Department of Agriculture/ARS, Horticultural Research Laboratory, Fort Pierce, FL, USA

Abstract: Eleven different rootstock varieties were investigated for their influence on the grafted tree response to *Candidatus* Liberibacter asiaticus (CLas) in a greenhouse environment. One-year-old Valencia trees were inoculated or mock-inoculated with CLas-infected or non-infected budwood from greenhouse-grown citrus plants. Rootstocks were Cleopatra mandarin, Ridge pineapple, sour orange, and 8 different hybrids of trifoliate orange: Carrizo citrange, Swingle citrumelo, US-802, US-1516, US-896, US-897, and US-942. Leaves and fibrous roots of mock-inoculated healthy and CLas-inoculated and confirmed CLas-positive plants were collected 6 months after inoculation at which time plants displayed foliar HLB symptoms. Metabolite profiles were assessed by untargeted gas chromatography time-of-flight mass spectrometry (GC TOF-MS). More than 500 unique metabolites were identified in leaves and roots of all plants. In the leaves, many metabolites were detected that responded significantly to CLas infection, but metabolites differed among plants on different rootstocks, demonstrating the metabolic influence of rootstock on the scion. In most Valencia/rootstock combinations, more leaf metabolites were down-regulated than up-regulated, indicating disruption of important metabolic pathways. Among the metabolites that responded most strongly were quinic acid and citrulline which were found in higher concentrations in leaves from infected than non-infected plants. The reverse was found for cerotinic acid and cyanoalanine. In the roots, more metabolites were up-regulated than down-regulated in response to infection. Most metabolites that responded most strongly to CLas were different than those responding in the leaves and included benzoic acid, salicylic acid, and fructose. Several metabolites showed an opposite response in leaves and roots such as quinic acid and raffinose. In addition to the rootstock-specific response in roots and leaves, rootstocks could be differentiated based on their metabolite profiles independent of infection. This demonstrates the influence of rootstock on the grafted tree response to HLB and underscores rootstock importance for disease management under HLB-endemic conditions.

Non-technical summary: In this study, eleven different rootstock varieties with commercial importance were investigated regarding their biochemical influence on grafted Valencia sweet orange trees. An analytical method which combines gas chromatography and mass spectrometry was used to identify metabolites (small molecules) present in leaves and roots of healthy and infected plants. We detected substantial metabolic disruptions in response to infection but, the response was different in plants grafted on different rootstock varieties. This demonstrates that the rootstock influences the biochemical profile of grafted citrus trees and underscores the importance of rootstock as a management tool for citrus production in the presence of HLB.

IRCHLB-O7b-05

Responses of citrus scion and rootstock combinations to artificial inoculation with *Candidatus* Liberibacter asiaticus

Everton CARVALHO[1], Laudecir RAIOL JÚNIOR[1], Walter SOARES FILHO[2], Silvio LOPES[3], Eduardo GIRARDI[2,3]

[1]São Paulo State University, Jaboticabal, Brazil, [2]Embrapa Cassava & Tropical Fruits, Cruz das Almas, Brazil, [3]Fund for Citrus Protection (Fundecitrus), Araraquara, Brazil

Abstract: In this study, scion and rootstock combinations were evaluated by artificial inoculation with *Candidatus* Liberibacter asiaticus (CLas) under controlled conditions. Ten rootstocks were evaluated, including Swingle citrumelo, Indio, San Diego and Riverside citrandarins (Sunki mandarin x trifoliate orange), and the hybrids Sunki x citrange Coleman-008, Sunki x Swingle

citrumelo-041, Sunki x (Rangpur lime x trifoliate orange) - 059 and 073 and Sunki x Flying Dragon trifoliate- 003 and 006, grafted or not with the scion varieties 'Pera' and 'Folha Murcha' sweet orange. For Las infection, 20 months old plants were inoculated 5 to 10 cm above the neck in the rootstocks, and 5 to 10 cm above the grafting line in the grafted trees, with two 2 to 3 cm long infected buds, on opposite sides of the canopy. The noninoculated plants (control) received healthy buds. Two leaves of each of the three shoots grown in each plant were collected and used for qPCR analysis at 3, 6 and 12 months after inoculation (MAI). The titration of Las in the roots was performed at 12 MAI, as well as the measurement of the dry matter of the root system. At 3 MAI, almost all combinations already had all the plants positive for Las. In the multiple comparison between all the treatments, the titration of Las in the canopy did not present significant difference at 6 and 12 MAI. However, HLB symptoms were more pronounced on plants grafted with Pera sweet orange. Although some rootstocks had lower CLas titers in the roots, symptoms on the canopy were severe for the same treatments. Healthy trees had dry matter of the root system 4-68% higher than the respective inoculated combination or rootstock. Therefore, all materials were susceptible to CLas multiplication irrespectively of the genetic background of the hybrid rootstocks either graft combination.

Non-Technical Summary: Finding new rootstocks that are tolerant to HLB could improve tree healthy of the graft combination. Several studies reported contraditory results on genetic tolerance or resistance of rootstocks in the field either in greenhouse. Our study indicated that ten different hybrid rootstocks, varying in the genetic background of the cross, were all susceptible to CLas multiplication within canopy and root tissues. A significant reduction in the dry mass of root system was observed in general. There was no evidence that the scion-rootstock combination of sweet orange varieties had major influence on the bacteria colonization.

Citations:

FOLIMONOVA, S. Y.; ROBERTSON, C. J.; GARNSEY, C. S.; GOWDA, S.; DAWSON, W.O. Examination of the responses of different genotypes of citrus to Huanglongbing (citrus greening) under different conditions. Phytopathology, v. 99, p. 1346-1354, 2009.

JOHNSON, E.G.; WU, J.; BRIGHT, D.B.; GRAHAM, J.H. Association of ‘Candidatus Liberibacter asiaticus’ root infection, but not phloem plugging with root loss on huanglongbing-affected trees prior to appearance of foliar symptoms. Plant Pathology, v. 63, p. 290- 298, 2014.

LOPES, S. A., FRARE, G. F. Graft transmission and cultivar reaction of citrus to Candidatus Liberibacter americanus. Plant Disease, v. 92, p. 21–24, 2008.

IRCHLB-O7b-06

Off-flavor compounds in huanglongbing-affected orange juice and their mitigation using natural citrus non-volatile compounds

Smita RAITHORE[1], Johannes KIEFL[2], Bruno Dala PAULA[3], Beatriz GLÓRIA[4], John MANTHEY[5], Anne PLOTTO[5], Jinhe BAI[5], Wei ZHAO[5], and **Elizabeth BALDWIN**[5]

[1] Symrise Inc., Teterboro, NJ, USA, [2] Symrise AG, Holzminden, Germany, [3] Laboratório de Nutrição Experimental, Faculdade de Nutrição, Universidade Federal de Alfenas, Alfenas, Minas Gerais, Brazil, [4] LBqA Laboratório de Bioquímica de Alimentos, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, [5] U.S. Horticultural Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Fort Pierce FL, USA

Abstract: Huanglongbing (HLB) has reduced yields in Florida by over 80%, while also reducing juice quality due to decreased sugars, sometimes increased acids, increased bitter limonoids and astringent/bitter flavonoids as well as an altered aroma profile. Compounds obtained from orange juice (OJ), affected or not by HLB, were tested for off-flavor revealing that flavonoids and hydroxycinnamic acids (HCA) imparted bitter and harsh flavor. Other compounds from OJ and orange peel molasses (PM), and as such considered “from the named fruit” (FTNF), were tested for their effects on HLB-affected OJ flavor for bitterness enhancement or suppression. These and other “target” compounds, reportedly found in citrus and with ability to affect flavor, were spiked into off-flavored HLB-affected reference juices. A trained sensory panel evaluated reference juices spiked with fractions or “target” compounds in comparison with unspiked juice for sweetness, bitterness, astringency and aftertaste. OJ spiked with fractions containing polymethoxyflavones (PMFs), HCAs and/or hesperidin induced bitterness. Of the “target” compounds, feruloyl putrescine, taxifolin and neodiosmin were most effective in suppressing bitterness, while feruloyl putrescine also suppressed astringency and aftertaste. Feruloyl putrescine and neodiosmin enhanced the perception of sweetness as well. Some OJ and PM fractions containing limonin, nomilin, HCAs, hesperidin, and/or PMFs enhanced HLB off- flavors such as sourness, bitterness, astringency, aftertaste, and/or suppressed sweetness. Other fractions, however, containing feruloyl putrescine enhanced sweetness and suppressed bitterness, sourness, astringency and/or aftertaste. Compounds that improved the flavor of HLB-affected juice, and are also FTNF, may be useful as a second generation add-back flavor management strategy for HLB-affected orange juice.

Non-Technical Summary: New compounds were identified in HLB-affected orange juice that impart off-flavor that are not bitter limonoids. Other compounds were identified in citrus fruit that mask bitterness and could be used to mitigate HLB-induced off-flavor.

Citations:

Dala Paula, B.D., Raithore, S.., Manthey, J.A. Baldwin, E.A., Bai, J., Gloria, B.M., Plotto, A. Active taste compounds in juice from oranges symptomatic for Huanglongbing (HLB) citrus greening dsease. LWT – Food Science and Technol. 91:518-525. 2018

Baldwin E.A., Plotto, A., Bai, J., Manthey, J. Zhao, W., Raithore, S. and Irey, M. Effect of abscission zone formation on orange (Citrus sinensis) fruit/juice quality for tres affected by Huanglongbing (HLB). J. Agric. Food Chem. In press

IRCHLB-14: Host Responses to Infection Poster Presentations

IRCHLB-P14-143

A deeper gene expression analysis of HLB pathogen and host to understand their complex relationship.

Sai ZADGAONKAR[1], Mikeal ROOSE[1]

[1]University of California, Riverside, USA

Abstract

Huanglongbing disease involves complex and intricate interactions among three biological species (psyllid, tree, and bacteria). However, most studies on molecular interactions are focused on understanding host molecular responses to the Liberibacter infection and are conducted separately for host and pathogen. We used dual RNA-Seq to capture and understand host and pathogen responses simultaneously at different levels of infection. To reveal the transcriptional dynamics between citrus and *Candidatus* Liberibacter asiaticus (CLas), we analyzed the same phloem-enriched tissue to quantify bacterial infection and gene expression for both species. Three Hamlin trees were

infested with CLas positive psyllids, and three Hamlin trees were infested with CLas negative psyllids. Post infestations, petiole samples were collected and tested for CLas using qPCR. Three leaf ages and four infection levels were used to classify stage-specific host/pathogen responses. Eighteen samples were individually sequenced in a high-throughput manner, and about 500 million reads were generated per sample. The reads were mapped to *Citrus clementina* genome and *Candidatus* Liberibacter asiaticus psy62 genome. The differential analysis revealed upregulation of most CLas genes and down-regulation of most citrus genes at higher infection levels. Many transcriptional and translational related bacterial genes were upregulated. Genes that play roles in bacterial virulence, are components of the general secretion pathway and are involved in cell to cell signaling were also differentially expressed among treatments. Citrus genes involved in response to stress, signal transduction, and metabolic processes were downregulated significantly in infected plants compared to healthy plants.

Non-technical summary:

This study provides knowledge about the physiological state of the disease and will help identify interacting proteins of citrus host and CLas pathogen. These proteins will be potential therapeutic targets for HLB disease.

IRCHLB-P14-144

A survey of transcriptionally active effector genes during CLas-citrus interactions

Qingchun SHI[1], Marco PITINO[2], Shujian ZHANG[1], Joseph KRYSTEL[1], Liliana M. CANO[2], Robert G. SHATTERS, Jr.[1], David G HALL[1], and Ed STOVER[1]

1. U.S. Horticultural Research Laboratory, USDA-ARS, Fort Pierce, FL University of Florida, UF/IFAS, Department of Plant Pathology, Indian River Research and Education Center, Fort Pierce, FL

Abstract: Phytopathogenic bacteria can secret a repertoire of effectors into host cells to succeed in parasitism. Temporal and sequential deployment of effectors define different stages of infection[1] and may affect the outcome of plant-pathogen interactions. Bioinformatics was used to filter the genome of *Candidatus* Liberibacter asiaticus (CLas, the associated agent of Huanglongbing/HLB) and identified 28 effector candidates. Using RT-qPCR, the transcriptional levels of these putative effectors were studied during bacterial interaction with HLB susceptible, tolerant and resistant citrus varieties (susceptible Duncan grapefruit and Washington navel orange, tolerant citron and Cleopatra mandarin, and resistant Pomeroy trifoliate and Carrizo citrange), at early (within 7 days) and late (over 1 year) infection stages, and from leaf and root tissues. Using a detached leaf inoculation, the expression of effector genes were observed as early as 6 hours after CLas-positive psyllid exposure. The number of effectors detected was higher in the citrus samples with higher bacterial titer. Relatively high expression of *CLIBASIA_03695*, *CLIBASIA_00460*, *CLIBASIA_00420*, *CLIBASIA_04580*, *CLIBASIA_05320*, *CLIBASIA_04425*, *CLIBASIA_00525* and *CLIBASIA_05315*

were observed in a host-specific or – nonspecific manners. Comparison between leaf and root tissues indicated consistently high expression of *CLIBASIA_03875*, *CLIBASIA_04800* and *CLIBASIA_05640*

between leaf and root tissues, and the pattern was similarly observed in citron, Duncan and Cleopatra. The identified transcriptionally active effector candidates may have important roles in bacterial pathogenicity. Further studies are underway including biochemical investigation to identify their host binding targets.

Non-Technical Summary: Bacterial pathogens including CLas can secret small proteins named effectors into plant cells. The effectors are able to help conquer the plants cells and achieve infection. Here the expression levels of CLas effectors were measured and certain effectors showed higher expression than others during citrus infection. These effectors may be important for CLas disease development and are worthy of further studies.

Citations:

Win, J. *et al. Cold Spring Harb Symp Quant Biol*, 2012. 77: p. 235-47.

IRCHLB-P14-145

'*Candidatus* Liberibacter asiaticus' effector SDE1 controlled by a complex promoter targets DEAD-box RNA helicase DDX3 to modulate chlorosis in *Nicotiana benthamiana*

Yanjiao LI[1], Xiangying WEI[2], Yongping DUAN[2], **Huasong ZOU**[1]

[1]College of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou, 350002, China，[2]U.S. Horticultural Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Fort Pierce, FL, USA

Abstract:'*Candidatus* Liberibacter asiaticus' (CLas) is the associated agent of Huanglongbing, the most destructive disease of citrus worldwide. Beside citrus, CLas also infects several experimental plants, such as the model plant, *Nicotiana benthamiana*. To carry out its pathogenesis, CLas secrets out effectors to modulate host responses. One of these critical effectors is the SDE1, which induces diverse host reactions in citrus and *N. benthamiana*, including chlorosis and starch accumulation. We revealed that the SDE1 gene possesses a promoter region containing 11 putative promoters centered in three distinct regions. Based on a GUS-monitored activity analysis, the SDE1 promoter was controlled by a complex regulation mode. Four SDE1-interacting proteins were identified from *N. benthamiana*, including DEAD-box ATP-dependent RNA helicase 3 (DDX3), 26S proteasome non-ATPase regulatory subunit 14, ARM repeat protein, and a hypothetical protein. Silencing the DDX3 gene resulted in mottled leaves, mimic the diagnostic symptom of HLB. Furthermore, using co-localization and bimolecular fluorescence complementation (BiFC) *in vivo*, we confirmed the interactions between SDE1 and NbDDX3 were localized at the cell membrane. Altogether, we demonstrated the SDE1 effector induced chlorosis in *N. benthamiana* by targeting NbDDX3, and the complex nature of the SDE1 promoters can be an important manipulation target for HLB control since it can be regulated by environmental stimuli, such as iron concentration and pH changes.

Non-technical summary: *Candidatus* Liberibacter asiaticus can be experimentally transferred to *N. benthamiana* by dodder, leading to a typical disease symptom includes blotchy mottles on leaves. Our purpose was to understand the transcriptional regulation of *SDE1* gene and to reveal the molecular mechanism underlying the modulation of chlorosis by SDE1.

Citations:

Pitino, M., Allen, V., and Duan, Y. P. 2018. LasΔ15315 effector induces extreme starch accumulation and chlorosis as '*Candidatus* Liberibacter asiaticus' infection in *Nicotiana benthamiana*. Front. Plant Sci. 9:113.

Pitino, M., Armstrong, C. M., Cano, L. M., and Duan, Y. P. 2016. Transient expression of '*Candidatus* Liberibacter asiaticus' effector induces cell death in *Nicotiana benthamiana*. Front. Plant Sci.7:982.

Prasad, S., Xu, J., Zhang, Y., and Wang, N. 2016. Sec-translocon dependent extracytoplasmic proteins of '*Candidatus* Liberibacter asiaticus'. Front. Microbiol.7:1989.

IRCHLB-P14-146

Chemical evaluation of the tolerance of newly developed citrus cultivars to Huanglongbing

Nabil KILLINY[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, USA

Abstract: Although there are no commercial citrus cultivars with strong tolerance to HLB, field observations and greenhouse-controlled studies showed that some citrus species are more tolerant to the HLB pathogen and its vector than others. Unfortunately, the mechanism behind the tolerance of these species has not been fully understood. Tolerance of these species to HLB disease could result from their resistance to the HLB pathogen or to its insect vector. We investigated the volatile and the non-volatile metabolite profile of many commercial citrus cultivars to determine why some cultivars are more tolerant to HLB than others. Interestingly, we found that tolerant cultivars were high in metabolites implicated in plant defense or metabolites that possess strong antimicrobial activities. We found the HLB- tolerant mandarin variety Sugar Belle was high in thymol, carvacrol, and eucalyptol. Interestingly, thymol and carvacrol have proven antimicrobial and anti-fungal activity. In addition, thymol was found to inhibit biofilm formation in some bacteria. On the other hand, eucalyptol has demonstrated repellency and toxicity to many insects. On the other hand, the profiles of the mandarin hybrid, 'Bingo' was different than 'Sugar Belle' including low amounts of most amino acids and sugar alcohols indicating that it may be less tolerant. We also investigated several newly developed/released citrus cultivars for their tolerance for the HLB pathogen and its vector under controlled conditions followed by our chemical analysis. Our aim is to build up a library of biomarkers that could be used as tolerance indicators. In addition, understanding of the mechanisms behind citrus tolerance to HLB could help citrus breeders to develop new commercially tolerant citrus cultivars.

Non-technical summary: With the active breeding programs in California and Florida, there is an urgent need to provide growers with the necessary information about the tolerance of the newly developed cultivars to HLB disease. We have developing a dynamic system to evaluate these newly released varieties for their tolerance to psyllid and *Candidatus* Liberibacter asiaticus by their metabolic profiles.

IRCHLB-P14-147

Constraint-based modeling of '*Candidatus* Liberibacter asiaticus' unravel interactions with its hosts

Cristal ZUNIGA[1], Bo LIANG[1,2], Beth PEACOCK[3], Georgios VIDALAKIS[3], James BORNEMAN[3], Karsten ZENGLER[1,5,6]

[1]Department of Pediatrics, University of California, San Diego, USA. [2]State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry, East China University of Science and Technology, Shanghai, P.R. China. [3]Department of Microbiology and Plant Pathology, University of California, Riverside, USA. [4]Facultad de Ingeniería Química, Universidad Autónoma de Yucatán, Mérida, Yucatán, Mexico. [5]Department of Bioengineering, University of California, San Diego, USA. [6]Center for Microbiome Innovation, University of California, San Diego, USA

Abstract: *Candidatus* Liberibacter asiaticus (*C*Las) is the bacterial pathogen associated with huanglongbing (HLB) of citrus. This lethal disease affects citrus crops worldwide, reducing crop productivity by up to 80%. Although this putative pathogen has eluded cultivation, recent omic tools have made possible the genome sequencing of multiple *C*Las strains collected from *in vivo* environments. Comparative analyses of these genome sequences have provided preliminary insights into the metabolic capabilities of *C*Las. A comprehensive functional characterization of *C*Las' metabolism is currently lacking, hindering targeted treatment against the pathogen. We reconstructed and manually curated genome- scale metabolic models for six *C*Las strains – A4, FL17, gxpsy, Ishi-1, psy62, and YCPsy. Furthermore, we generated a model of the most phylogenetically related cultured bacterium to C*Las*, *L. crescens* BT-1. Growth phenotypes for all seven microbes were evaluated for different media compositions. Additionally, *C*Las models were constrained using expression data obtained from C*Las* infected citrus plants as well as from the bacteria residing in the psyllid host *Diaphorina citri*. The metabolic networks were used to analyze the shared and unique metabolic capabilities for all strain-specific variants cultivable and uncultivable. Our results enabled us to identify metabolic similarities and differences between these bacteria, providing insights into the interactions between *C*Las and its different hosts.

Non-technical summary: We applied a systems biology modeling approach to understand the metabolism of six strains of C*Las* and its closest culturable relative, *L. crescens* BT-1. These models enable us to predict various phenotypic outcomes from genome information. They also allow us to predict the response of the organisms to diverse environmental conditions, giving detailed metabolic insight into the physiology and nutrient requirements for growth. Making such predictions in the context of key events associated with HLB pathology should provide new information leading to new treatments to combat HLB.

IRCHLB-P14-148

Coordination of multiple regulation pathways contributes to the tolerance of a wild citrus species (*Citrus ichangensis*) against Huanglongbing

Yan HU[1,2], Haodi WU[1], Cailing TENG[1], Xuelu LIU[1], Shiming FU[1], Changyong ZHOU[1*], **Xuefeng WANG**[1*1]

National Citrus Engineering Research Center, Citrus Research Institute, Southwest University, Chongqing, China, [2] Ganzhou Plant Protection Station of Fruit Industry, Jiangxi, China

Abstract: Huanglongbing (HLB), a highly destructive disease of citrus production worldwide, is associated with '*Candidatus* Liberibacter asiaticus' (CLas). No HLB resistant genotypes have been currently identified in commercial citrus species, inhibiting the development of resistance breeding. China is an important center of global citrus germplasm resources with abundant wild citrus. However, there has been little studies on susceptibility or resistance of these wild citrus and their response mechanism to CLas infection. In this study, we evaluated symptom development and transcriptional difference among two wild citrus species, *Citrus ichangensis*, *C. daoxianensis* and HLB susceptible sweet orange (*C. sinensis*). Biological characterization indicated that *C. ichangensis* did not show typical HLB symptoms and accumulated much lower level of bacteria, as compared to *C. daoxianensis* and *C. sinensis*. Global transcriptome analysis showed that *pp2* genes related with callose deposition, starch synthesis and photosynthesis process were not significantly changed in CLas- affected *C. ichangensis*. Notably, a group of genes in secondary metabolism, such as isoprenoid and flavonoid biosynthesis pathway, were significantly up-regulated in *C. ichangensis*. The coordination of carbohydrate metabolism, photosynthesis process, phloem protein, cell wall metabolism and secondary metabolism may play important roles in mediating the tolerance of *C.*

ichangensis against CLas infection. Evaluation of wild citrus resources to CLas infection is useful for citrus HLB resistance breeding in the future.

Non-technical summary: China is an important center of global citrus germplasm resources with abundant wild citrus, such as *C. ichangensis*, *C. daoxianensis* and *C. mangshanensis*. We evaluated symptom development and transcriptional difference among two wild citrus species, *C. ichangensis*, *C. daoxianensis* and highly susceptible sweet orange (*C. sinensis*). The understanding on the response mechanisms of the HLB-tolerant wild citrus species to CLas provided new clues to citrus breeder for development of HLB tolerant/resistant varieties.

IRCHLB-P14-149

Deciphering the Bacterial Microbiome of HLB-affected Citrus Roots Treated with Sulfonamide Antibiotics

Chuanyu YANG[1,3,4], Muqing ZHANG[1,2,3], Yongping DUAN[2], Veronica ANCONA[4], and Charles A. POWELL[1]

[1]IRREC-IFAS, University of Florida, 2199 S. Rock Rd, Fort Pierce, FL 34945, USA
[2]USHRL-USDA-ARS, 2011 S. Rock Rd, Fort Pierce, FL 34945, USA
[3] College Agricultural Science, Guangxi University, Nanning, 530005, China
[4]Citrus Center, Texas A&M University-Kingsville, 312 N. International Blvd, Weslaco, TX, 78599, USA

Abstract: Bacterial root endophytes are an important component of the root microbiome in many plant species. The root microbiome can modulate plant growth through phytohormone production or degradation, and maintain plant health through competition with pathogens or induction of plant disease resistance. Citrus Huanglongbing (HLB) is serious disease of citrus associated with *Candidatus* Liberibacter asiaticus (CLas), a fastidious Gram-negative phloem-limited bacterium. We have previously reported that treatment with sulfonamide antibiotics can not only suppress Las titer, but also promote lateral root growth of HLB-affected citrus. We hypothesize that sulfonamide antibiotics modify the endophyte community in HLB- affected citrus roots which may be beneficial for induction of lateral root growth and plant defense mechanisms. Therefore, in this study, the bacterial microbiome of HLB-affected grapefruit roots treated with sulfadimethoxine sodium (SDX, 10 ppm), indole butyric acid (IBA, 10 ppm) and tap water (CK), was analyzed by PhyloChip^{TM}G3. In total, 234 Operational Taxonomic Units (OTUs) were detected in roots from all treated HLB- affected citrus, of which 74 OTUs (31.62%) were shared by all treatments. Twenty four phyla were detected, of which 6 phyla had 10 or more OTUs: *Proteobacteria* (76, 32.48%), *Cyanobacteria* (29, 12.39%), *Planctomycetes* (25, 10.68%), *Bacteroidetes* (22, 9.40%), *Firmicutes* (14, 5.98%), and *Actinobacteria* (10, 4.27%). *Proteobacteria* was prominent in the bacterial population after all treatments. The specific OTUs enriched in SDX belonged to the *Sphingomonadaceae*, *Hyphomicrobiaceae*, and *Porphyromonadaceae* families, which are known to be implicated in increased resistance to plant pathogens. Other specific OTUs belonging to the families *Lachnospiraceae*, *Clostridiaceae*, *Comamonadaceae* and *Bradyrhizobiaceae*, may be related to lateral root growth. Deciphering the root microbiome in SDX treated HLB-affected citrus roots helps in the identification of bacterial groups that promote root health and disease resistance, which could be further studied as potential biocontrol strategies for citrus HLB management in the future.

Non-Technical summary: Sulfonamide is an antibiotic against citrus HLB pathogen, which can also promote lateral root growth in HLB-affected citrus. Utilizing a meta-genomic analysis, it was found that several bacterial families that can induce development of lateral root and plant disease resistance were enriched after Sulfonamide treatment, which can be used for HLB management in the future.

IRCHLB-P14-150

Developing tolerance/resistance in citrus cultivars to HLB

Olufemi J. ALABI[1], MaryLou POLEK[2], Liliana M. CANO[3], Zhanao DENG[4], Yongping DUAN[5], Frederick GMITTER Jr.[6], Feng LUO[7]

[1]Department of Plant Pathology & Microbiology, Texas A&M University, Weslaco, USA;
[2]USDA-ARS, National Clonal Germplasm Repository, Riverside, USA; [3]University of Florida Indian River Research and Education Center, Fort Pierce, USA; [4]University of Florida Gulf Coast Research and Education Center, Wimauma, USA; [5]USDA-ARS, Fort Pierce, USA;
[6]University of Florida Citrus Research and Education Center, Lake Alfred, USA; [7]School of Computing, Clemson University, Clemson, USA

Abstract: Huanglongbing (HLB), a destructive disease of citrus, has devastated Florida's $9 billion citrus industry and is now spreading throughout the citrus-production areas of Texas and in urban areas of southern California. The HLB epidemics have not abated despite significant resources committed to the control of the Asian citrus psyllid vector of the disease-associated bacterium. The long-term goal of this multi- institutional and multi-disciplinary USDA- NIFACDRE standard research and extension project entitled "Selection, molecular and genetic analysis of HLB tolerant/resistant variant citrus plants" is to develop new generation of citrus cultivars that will survive profitably in the presence of HLB. Specifically, we aim to 1) Select variant citrus plants or bud sports with HLB tolerance/resistance; 2) Identify citrus HLB susceptibility-related genes for potential citrus genome editing targets through transcriptome profiling; 3) Identify genetic variants that are associated with HLB susceptibility or tolerance/resistance in variant citrus plants; 4) Engineer HLB tolerance/resistance using genome editing technology; and
5) Disseminate project results to stakeholders and the public through outreach/education activities. The team is led by Dr. Feng Luo, Clemson University, and includes 6 co-project directors drawn from four institutions across the major US citrus producing states of Florida, California and Texas. An advisory board of citrus stakeholders from the 3 leading US citrus-producing states has been constituted and a project website (http://bigdata.clemson.edu/citrus/) has been created. The team is on track to achieve the project objectives which will culminate in the field evaluation of potentially HLB tolerant/resistant citrus cultivars in Florida, California and Texas as grower participatory trials.

Non-Technical summary: A project is being undertaken to develop new generation of citrus cultivars that are tolerant/resistant to HLB. Outcomes of the project will provide growers with resilient and GMO-free citrus cultivars to ensure profitability of citrus production in HLBendemic production areas.

IRCHLB-P14-151

Effect of CLas titers on tree health, root health and soilborne citrus pathogens

David LAUGHLIN, Catherine SIMPSON, Jorge SOLORZANO, Veronica ANCONA

Citrus Center, Texas A&M University-Kingsville, 312 N. International Blvd, Weslaco, TX, 78599, USA

Abstract: Huanglongbing (HLB), a devastating disease of citrus associated with *Candidatus* Liberibacter asiaticus (CLas), has caused major yield losses in citrus producing regions worldwide. In Florida, it has been shown that HLB causes extensive root loss resulting in tree decline, poor yields and unproductive orchards. Although CLas has been detected in Texas since early 2012, severe tree decline due to HLB has yet to be observed in Texas orchards. This study aims to investigate the factors leading to HLB related tree decline in Texas. To this aim, we are studying the relationship between tree health and root mass with CLas titers, and their possible association with *Phytophthora nicotianae* and *Tylenchus semipenetrans*, two other major soilborne pathogens of citrus in Texas orchards. Thirty Rio Red grapefruit trees on sour orange rootstock were randomly selected from three HLB positive orchards in South Texas. CLas titer in roots and leaves, and populations of *P. nicotianae* and *T. semipenetrans* in the rhizosphere were evaluated in summer and fall of 2018 and compared against a tree health index which was based on canopy density and fibrous root mass. Our results show that there were no negative correlations between CLas root titer and root mass, even in an orchard that has been HLB positive since 2012, indicating that thus far CLas infection has not resulted in root decline in the evaluated orchards. Also, CLas root titer declined from summer to fall following the fall root flush. In two orchards with no *P. nicotianae* control, there was a non-significant positive correlation between *P. nicotianae* soil propagules and CLas leaf titers. Interestingly, no correlation between *P. nicotianae* and CLas root titers or between *T. semipenetrans* and CLas titers was observed. These preliminary observations imply that, at this stage of the epidemic, CLas is not causing root decline in citrus grown in Texas. This may partially explain why Texas citrus orchards are not seeing the decline usually associated with HLB.

Non-Technical Summary: In Texas orchards, Citrus trees infected with CLas over several years have not exhibited fibrous root decline based on a correlative study between CLas root titers and root mass. However, there may be a positive interaction between CLas and *Phytophthora nicotianae* within in the rhizosphere.

IRCHLB-P14-152

Enhancing resistance to *Candidatus* Liberibacter asiaticus via heterologous expression of plant disease resistance (*R*) genes in citrus.

Marco PITINO[1], Stacy De BLASIO[3,4,5], Michelle L. HECK[3,4,5], Qingchung SHI[2], Ed STOVER[2], Liliana M. CANO[1] and Robert G. SHATTERS[2].

[1]Department of Plant Pathology, University of Florida, Institute of Food and Agricultural Sciences, Indian River Research and Education Center, Fort Pierce, Florida.

[2]United States Department of Agriculture ARS, Fort Pierce, Florida.

[3]United States Department of Agriculture, Biological Integrated Pest Management Research Unit, Robert W. Holley Center for Agriculture and Health, 538 Tower Road, Ithaca, New York.

[4]Boyce Thompson Institute for Plant Research, BTI, Ithaca, New York.

[5]Section of Plant Pathology and Plant- Microbe Biology, School of Integrated Plant Science, Cornell University, Ithaca, New York.

Abstract: Citrus Huanglongbing (HLB) is a citrus disease associated with three bacterial species, *Candidatus* Liberibacter asiaticus

(CLas), *Candidatus* Liberibacter Africanus (CLaf), and *Candidatus* Liberibacter Americanus (CLam). The pathogen is transmitted in a persistent propagative and circulative manner by its vector, the Asian citrus psyllid (*Diaphorina citri*). To date, no sustainable management practices have been established to overcome damage caused by HLB disease in the groves. Genetic improvement of citrus through genetic engineering (GE) remains one of the most promising methods for improvement of existing citrus cultivars, and understanding plant-pathogen interactions should provide excellent targets for creating HLB-resistant citrus through GE manipulation. Effector biology emerges as an important aspect of the investigation on plant-pathogen interactions, as effector proteins secreted by bacteria play many roles in the pathogenicity leading to the initial infection and host colonization. Binding targets for effectors *in planta* include host susceptibility and *R* genes. An understanding of fundamental effector biology, including their various subcellular localizations, and mechanisms of host cell modulation, is key to revealing how pathogens achieve virulence. A candidate set of 28 effectors from CLas is currently being subjected to functional characterization to reveal their potential biological roles during infection. This pipeline involves performing cell biology and protein-protein interaction assays *in planta*. Host protein targets and potential effective resistance genes will be revealed, this information will be used in developing citrus HLB resistance.

Non-Technical Summary: The HLB associated pathogen (CLas) produces proteins, known as effectors, which disrupt citrus defenses to permit pathogen establishment and proliferation. We identified 28 CLas proteins as potential effectors, and are identifying the citrus targets where they bind. Understanding the details of these pathogen-host interactions should be useful in developing HLB-resistant citrus through biotechnology.

IRCHLB-P14-153

eQTL mapping reveals a complex architecture of Callose Synthases transcript-level variation in hybrids of *Citrus sunki* and *Poncirus trifoliata* affected by Huanglongbing

Laís Moreira GRANATO[1]; Maiara CURTOLO[1,2]; Tatiany Aparecida Teixeira SORATTO[1]; Nathália Da Roz D'ALESSANDRE[1,3]; Mariângela CRISTOFANI-YALY[1]; **Marcos Antonio MACHADO**[1]

[1] Centro de Citricultura Sylvio Moreira, Instituto Agronômico de Campinas, Cordeirópolis/SP, Brazil; [2] Universidade Estadual de Campinas, Campinas/SP, Brazil; [3] Universidade Federal de São Carlos, Araras/SP, Brazil.

Abstract: The symptoms of Huanglongbing (HLB) have been related by the callose accumulation in the phloem sieve plates, which inhibits the phloem transport to consequently reducing the bacterial colonization via phloem, what possibly contribute to the starch accumulation in the leaves and the development of HLB symptoms. The key class of enzymes for callose synthesis is the Callose Synthases. The callose synthase genes (*calS*) expression are modulated for biotic and abiotic stresses. In this study, nine *Citrus calS* genes (*CscalS*) were identified and the expression patterns were analyzed in hybrids between *Citrus sunki* and *Poncirus trifoliata*, *Candidatus* Liberibacter asiaticus (CLas) inoculated and healthy plants. The objective of the present work was to localize possible tolerance loci in citrus combining the expression quantitative trait loci (eQTL) of different callose synthases and the SNP (single-nucleotide polymorphism) map obtained from DArTseq^{TM} markers genotyping of hybrids between *C. sunki* and *P. trifoliata*. This study presents the first map for citrus using SNP markers from DArTseq technology, which are a substantial resource for future citrus studies. The *C. sunki* map has 571 SNP divided into 9 groups, with a genomic cover of 2855 cM. The *P.*

trifoliata map was obtained with 568 SNP in 3334.1 cM, distributed into nine groups. The expression data from all *CscalS* ranged widely among the hybrids plants. However, this data made the detection of 19 eQTL in the *C. sunki* map and 28 eQTL in the *P. trifoliata* map possible. Some hot spots were located in the interval of the eQTL, thus a single region could regulate more than on *Cs*CalS. It is also proposed that more than one *Cs*CalS is involved in citrus defense against CLas, forming a complex in the phloem.

Non-technical summary: Callose Synthases are enzymes responsible by callose deposition in the Citrus tissues. The expression of all callose synthases genes are affected in the CLas infected hybrids and it is possible that just a single genome region regulates more than one calloses synthase.

IRCHLB-P14-154

Expression profiles of *Candidatus* Liberibacter asiaticus effectors and their citrus targets

Fatta B. GURUNG[1], Gitta COAKER[2], Wenbo MA[3], Veronica ANCONA[1]

1Texas A&M University-Kingsville Citrus Center, Weslaco, TX 78599. 2Department of Plant Pathology, University of California, Davis, CA, 95616. 3Department of Microbiology and Plant Pathology, University of California, Riverside, CA, 92521

Abstract: Huanglongbing (HLB) is a highly destructive disease of citrus worldwide associated with the Gram-negative bacterium *Candidatus* Liberibacter asiaticus (CLas). The pathogen has been detected in the three major citrus producing regions of the United States, Florida, California and Texas, threatening their citrus industries. As differences in HLB-related tree decline have been observed in different regions, it is important to assess if this is caused by variation of expression of CLas virulence factors. Sec-delivered Effectors (SDEs) are presumably important for CLas pathogenicity by targeting specific defense components in citrus such as the papain-like cysteine proteases (PLCPs). In this work, we evaluate the gene expression of four selected SDEs and five PLCPs in field trees in Texas by quantitative RT-PCR. Symptomatic and asymptomatic leaves at different maturity stages and roots were collected from CLas-infected Rio Red grapefruit in the Southern Research Farm in Weslaco, TX. Our results indicate that relative expression of SDEs was lower in asymptomatic leaves compared to symptomatic leaves, even when the samples had similar bacterial titers (ct=22±3). Furthermore, no SDE expression was detected in asymptomatic leaves with lower bacterial titers (ct≥31±2). These results imply that SDE expression is corelated with development of HLB symptoms. In contrast, the relative expression of PLCPs was significantly higher in the asymptomatic leaves (ct=31±2) compared to the symptomatic leaves (ct=22±3) irrespectively of the leaf maturity level. Additionally, transcripts of these SDEs and PLCPs were not detected in root samples, suggesting that their expression is tissue specific. Further analysis will reveal if seasonal changes play a role in the expression profiles of these important virulence determinants.

Non-Technical Summary: In Texas, severe tree decline due to HLB has not been observed. Therefore in this work, we are investigating the gene expression patterns of CLas virulence factors (SDEs) and their citrus targets (PLCPs) at different disease stages of CLas-infected citrus in TX. This work revealed that asymptomatic tissues do not express SDEs but have high PLCP expression, in contrast to symptomatic tissues which exhibit high SDE expression and lower PLCP expression compared to asymptomatic, demonstrating their role in HLB severity. This knowledge will facilitate the development of integrative management programs for HLB in a sustainable manner.

IRCHLB-P14-155

Flushing dynamics of 'Pera' sweet orange on different rootstocks

Everton CARVALHO[1], Juan Camilo CIFUENTES-ARENAS[2], Eduardo STUCHI[3,4], Silvio LOPES[2], Eduardo GIRARDI[2,4]

[1]São Paulo State University (UNESP), Jaboticabal, Brazil, [2]Fund for Citrus Protection (Fundecitrus), Araraquara, Brazil, [3]Bebedouro Citrus Experimental Station, Bebedouro, Brazil, [4]Embrapa Cassava & Tropical Fruits, Cruz das Almas, Brazil

Abstract: Understanding the dynamics of the flush. growth, and development of new shoots (NS), and the potential influence of the rootstock on those parameters, could help to improve the control of vector-borne citrus pathogens. On three-year-old trees of 'Pera' sweet orange grafted onto nine rootstocks, the NSs found within a 50 x 50 cm square frame placed at central outer surface of the canopy (ca. 1.5 m above ground level) were counted and classified[1]. The trees were assessed during the 2017/2018 season, at 21-day intervals. The work was carried out in an orchard located in the north region of the state of Sao Paulo, Brazil. The one-way split-plot analysis of variance for repeated measures, with the rootstocks as the main plot and the sampling time the subplot, were applied to the data. The area under NS occurrences (AUNS) also was calculated for each tree. Rootstock significantly affected NS number and NS flushing dynamics, with the average number found during the spring reaching 13.4 times that found during the winter. The average NS numbers allowed separating the rootstocks into three groups: one vigorous ('Sunki' mandarin, 'Rough' lemon, 'Sunki' mandarin x ('Rangpur' lime x trifoliate orange), 'Sunki' mandarin x 'Swingle' citrumelo) with 4.09 ± 0.11 NS plant^{-1}, one intermediate ('Cleopatra' mandarin x 'Swingle' trifoliate, 'Swingle' citrumelo, 'Cleopatra' mandarin x 'Barnes' trifoliate, 'Sunki' mandarin x 'Flying Dragon' trifoliate) with 2.95± 0.12 NS plant^{-1}, and one poor ('Flying Dragon' trifoliate) with 1.90 ± 0.15. Analysis of AUNS pooled the rootstocks into the same groups, with averages of 1864.0 ± 0.11, 1424.72 ± 0.14, and 941.06 ± 0.13 NS-days, respectively. Fewer NSs and flushing cycles were detected on 'Pera' grafted onto the dwarfing rootstocks ('Cleopatra' mandarin x 'Barnes' trifoliate and 'Flying Dragon' trifoliate), which was concentrated mostly in two or three periods over the year.

Non-Technical Summary:
Rootstocks significantly changed the flushing dynamics of the 'Pera' sweet orange variety used as scion, with lower NS quantity and cycle numbers detected on trees growing on dwarfing rootstocks. Since new shoots are the preferred feeding and reproduction sites for the various insect species that vector citrus pathogens, including *Diaphorina citri*, vector of the HLB-associated pathogen, lower flushing cycles and NS quantity may reduce the vulnerability of the trees to new infections.

Citations:
J. C. Cifuentes-Arenas, A. de Goes, M. P. de Miranda, G. A. C. Beattie, and S. A. Lopes, "Citrus flush shoot ontogeny modulates biotic potential of *Diaphorina citri*," *PLoS One*, vol. 13, no. 1, p. e0190563, 2018.

IRCHLB-P14-156

HLB pathogenesis: suppression of citrus innate immunity

Jeanette C. Velásquez GUZMÁN[1], Supratim BASU[1], Loan HUYNH[1], Joseph KRYSTEL[2], Roel RABARA[1], Godfrey MILES[2], Shujian ZHANG[1], Guixia HAO[2], Qingchun SHI[2], Jefferson SHAW[2], David HALL[2], Ed STOVER[2], and **Goutam GUPTA**[1]

[1]Biolab, New Mexico Consortium (NMC)
100 Entrada Drive, Los Alamos, NM 87544, USA; [2]U. S. Horticultural Research Laboratory
2001 South Rock Road, Fort Pierce, FL 34945,

USA

Abstract: Innate immunity is the first line of host defense against the invading microbes. However, the pathogens that are successful in establishing infection can overcome host innate immune defense. A long-standing collaboration between Gupta (NMC and LANL) and Stover (USDA-ARS) laboratories has focused *first on* understanding how *Candidatus* Liberibacter asiaticus (CLas) infection abrogate citrus innate immune defense during Huanglongbing (HLB) pathogenesis and *then on* developing treatment and prevention of HLB by engineering novel citrus innate immunity that is not defeated by CLas infection. Genome-based, microbiology, immunology, and structural biology tools have been applied to understand the mechanisms of suppression of citrus innate immune defense during HLB pathogenesis and to apply this knowledge to develop novel tools for HLB therapy. This collaboration was initiated after receiving funding from the Citrus Research Board (CRB), CA and was further developed after obtaining USDA-NIFA funding several years later. The CRB and NIFA funding was used to perform transcriptome and proteome studies to determine the following critical steps in HLB pathogenesis:

(i) how the citrus innate immune gene networks are totally disabled or compromised to offer host defense against CLas

(ii) how specific citrus microRNAs to block one or more nodes in the gene networks leading to the suppression of citrus defense proteins

(iii) how specific CLas effectors directly target and inhibit the citrus defense proteins.

Therefore, our collaborative studies enabled us to identify important steps of citrus innate immune defense, which are suppressed due to CLas infection and which can be targeted by engineering citrus amphipathic helical peptides for HLB treatment. See the following abstract.

Non-Technical summary: Federal and industry investments in the sequencing of Liberibacter and citrus genomes allowed genome-based, microbiology, immunology, and structural biology research to better understand the hallmarks of HLB pathogenesis, i.e., the suppression of the critical steps in citrus innate immune response during CLas infection to the detriment of the host and to the benefit of the bacteria.

IRCHLB-P14-157

HLB Pathogenesis: the role of two CLas effectors in the suppression of citrus innate immune defense

Supratim BASU[1], Loan HUYNH[1], Godfrey MILES[2], Shujian ZHANG[1], Guixia HAO[2], Qingchun SHI[2], Roel RABARA[1], Hau NGUYEN[1], Jeanette C. VELÁSQUEZ GUZMÁN[1], Ed STOVER[2], and Goutam GUPTA[1]

[1]Biolab, New Mexico Consortium 100 Entrada Drive, Los Alamos, NM 87544, USA
[2]USDA/ARS U. S. Horticultural Research Laboratory 2001 South Rock Road, Fort Pierce, FL 34945, USA

Abstract: A genome-based approach was used to investigate the role of two putative *Candidatus* Liberibacter asiaticus (CLas) effectors, P235 and Effector 3, in suppressing citrus innate immune defense. *First*, we expressed the His-tagged versions of these two effectors in *E. coli*. *Second*, the whole protein extracts from CLas-infected and uninfected citrus were incubated with the effectors bound on TALON columns. *Third*, the citrus proteins bound to the effectors were eluted and identified by mass spectrometric method, LC-MS/MS. *Finally*, the mode of interactions between the two CLas effectors and their prominent citrus protein interactors were analyzed by *in vitro* and *in planta* assays. Our studies show that both P235 and Effector 3 may directly target and inhibit more than one citrus protein in the innate immune pathway. For example, one of the targets of P235 is the citrus lipid transfer protein (LTP), which shows antimicrobial effect on gram-negative bacteria

such as CLas. One of the targets of Effector 3 is Kunitz protease inhibitor (KPI) that blocks programmed cell death (PCD), which is a defense mechanism by which the host prevents the bacteria from causing premature PCD. We performed molecular dynamics (MD) simulations in a model water: lipid bilayer interface to predict how P235 binds to the citrus LTP, prevents it from rupturing the bacterial membrane, and thus blocks its antimicrobial function. We also performed MD simulations in water alone to visualize how Effector 3 binds to the reactive loop of KPI, blocks its function, and leads to premature PCD to the benefit of CLas. Therefore, our studies reveal how two CLas effectors may adversely affect antimicrobial and anti-PCD functions critical to citrus innate immune defense. Countering the adverse effects of the two CLas effectors may provide the basis for developing an effective HLB therapy.

Non-Technical Summary: One hallmark of HLB pathogenesis is action of proteins produced by the HLB pathogen, called effectors, to disrupt citrus innate immune defense. In this study, we have used a multi-disciplinary approach to determine how two key HLB pathogen effectors interact with citrus proteins to prevent activity critical for blocking infection and suppressing pathogen growth. This knowledge will guide development of novel HLB therapies.

IRCHLB-P14-158

Identification of host response to HLB using mass spectrometry-based metabolomics

Yu WANG[1], Joon hyuk SUH[1], Wei-Lun HUNG[1] and Fred GMITTER[1]

[1]Citrus Research and Education Center, University of Florida, Lake Alfred, USA

Abstract: Since HLB was first identified in Florida in 2005, there have been a multitude of challenges in seeking solutions. *Candidatus* Liberibacter asiaticus (CLas), the presumed bacterial pathogen of HLB, is a focus to help us understand the interactions between pathogen and hosts (both psyllid and citrus plants). For example, CLas interacts with citrus plants triggering host defense mechanisms and thereby inducing the changes of metabolites and metabolism pathways. A better understanding of metabolite changes can unravel questions such as biomarkers for early diagnosis, biomarkers for potential tolerant/resistant cultivars, as well as metabolism pathways involved in the pathology. Therefore, we developed a metabolomics platform, focusing on untargeted and targeted approaches, both qualitatively and quantitatively characterizing the pool of small molecules/metabolites in the citrus plant. Metabolic fingerprinting, the high throughput qualitative screening of metabolite composition, has been utilized for comparison and discrimination analysis of CLas-free and CLas-infected plants. All the changed metabolites have been analyzed using pathway enrichment analysis to indicate biosynthesis areas for further investigation. For instance, metabolic profiling of a variety of long-chain fatty acids and their oxidation products was performed to elucidate altered host metabolic responses of disease. Fatty acid signals have been found to decrease obviously in response to HLB regardless of cultivar. Several lipid oxidation products strongly correlated with those fatty acids were also consistently reduced in the CLas-infected group. Using a series of statistical methods and metabolic pathway mapping, we also have identified significant markers contributing to the pathological symptoms, and their internal relationships within the metabolic network. All the studies suggest that the infection of CLas may cause altered metabolism of long-chain fatty acids, possibly leading to manipulation of the host's defense derived from fatty acids.

Non-Technical Summary: We have newly developed a platform studying how citrus plant metabolites change due to HLB. Some long-chain fatty acids have been identified as highly relevant to the development of HLB and manipulate the host defense. This information may eventually lead to the development of early diagnosis tools or tolerant/resistant cultivars.

Citations:

Hung W-L, Wang Y. A targeted mass spectrometry-based metabolomics approach toward the understanding of host responses to Huanglongbing disease. Journal of Agricultural and Food Chemistry 2018, 66(40), 10651-10661.

Suh JH, Niu YS, Wang Z, Gmitter FG, Wang Y. Metabolic analysis reveals altered long-chain
fatty acid metabolism in the host by Huanglongbing disease. Journal of Agricultural and Food Chemistry 2018, 66(5), 1296-1304.

IRCHLB-P14-159

Influence of huanglongbing on yield loss and fruit quality of ‘Tahiti’ acid lime

Juan Camilo CIFUENTES-ARENAS[1], Hermes Teixeira de OLIVEIRA[1], **Silvio LOPES[1]**

[1]Fundecitrus, Araraquara, Brazil

Abstract. Little is known on the impact of huanglongbing (HLB) on 'Tahiti' acid lime fruit production and quality. On a 10-year-old ‘Tahiti’ grafted onto ‘Flying Dragon’ rootstock trees we characterized changes in symptomatology, symptom severity, yield loss and fruit quality, during the 2016 to 2018 seasons. The work involved over 130 trees exhibiting different levels of symptom severity (0 to 100%). They were growing in a drip irrigated orchard located in north São Paulo State, Brazil. Mottling patterns on leaves were not as clear as those commonly found in sweet orange. Preliminary results indicate that in fruits, the bilateral asymmetry of the polar cut, and the orange-brown stain of the vascular columella were present, but they were less severe and frequent than those reported in sweet orange. The average symptom severity tripled from first to the third year, with an initial estimation of 5 to 6 years to reach 100% of the canopies. The rate of yield loss as a function of the increase in symptoms severity, measured by relative yield (fruit from symptomatic trees/fruit from asymptomatic trees within the same block), was lower than that reported for sweet oranges[1]. The weight of the fruit from asymptomatic and symptomatic portions of same diseased trees was reduced 5.0% and 3.2% respectively, when compared to those from HLB-negative trees. This represents less than the 4.8% and 32.1% found in sweet oranges[1]. Significant fruit drop was not observed. Unlike sweet oranges[2], in ‘Tahiti’ acid lime most of the fruit quality parameters remained stable in healthy and infected trees. In juice of symptomatic fruit, hesperidin and pectin levels increased 35.2% and 12.7% respectively, as compared to fruits from asymptomatic trees.

Non-technical summary: Most of the fruit quality parameters of ‘Tahiti’ acid lime were not influenced by HLB infection. Fruit size and weight were barely reduced, indicating that the main concern about the effect of HLB in ‘Tahiti’ acid lime was the number of fruits per plant, which declined as the severity of the symptoms increased but at lower rates than in sweet oranges. Fruit drop was not significant. This may be due to the harvest criteria of the crop, different from the sweet orange trees.

Citations:

R. B. Bassanezi, L. H. Montesino, M. C. G. Gasparoto, A. Bergamin Filho, and L. Amorim, “Yield loss caused by huanglongbing in different sweet orange cultivars in São Paulo, Brazil,” *Eur. J. Plant Pathol.*, vol. 130, no. 4, pp. 577–586, 2011.

E. Baldwin, A. Plotto, J. Manthey, G. McCollum, J. Bai, M. Irey, R. Cameron, and G. Luzio, “Effect of Liberibacter infection (Huanglongbing disease) of citrus on orange fruit physiology and fruit/fruit juice quality: chemical and physical analyses,” *J. Agric. Food Chem.*, vol. 58, no. 2, pp. 1247–62, 2010.

IRCHLB-P14-160

Isolation and characterization of a citrus RIN4 gene reveals its negative regulatory role in HLB response

Yun ZHONG, Chunzhen CHENG, Guangyan ZHONG, Bo WU, Bo JIANG, Huaxue YAN

Institute of Fruit Tree Research, Guangdong Academy of Agricultural Sciences, Guangzhou, Guangdong, China

Abstract: The citrus industry has been threatened by Huanglongbing (HLB) for over a century. Here, an HLB-induced Arabidopsis *RIN4* homologous gene was cloned from *Citrus clementina*, and its characteristics and function were analyzed to determine its role during citrus–*Candidatus* Liberibacter asiaticus (*C*Las) interactions. Quantitative real-time PCR showed that *RIN4* was expressed in roots, stems, leaves and flowers, with the greatest expression level in leaves. Its expression was suppressed by gibberellic acid, indole-3-acetic acid and salicylic acid treatments, but was induced by abscisic acid and salt treatments, as well as wounding. The transient expression of a RIN4-GFP showed that RIN4 was localized in the cell membrane. RIN4-overexpression and RNA-interference vectors were constructed, and transgenic *Citrus maxima* cv. Shatianyou plants were obtained following *Agrobacterium*-mediated transformation. Transgenic pomelo plants overexpressing *RIN4* had a greater sensitivity to *C*Las infection and HLB symptoms appeared earlier than in controls, while the detection of *C*Las infections and the appearance of HLB symptoms were delayed in *RIN4*-RNA interference pomelo trees. The upregulated expression of *RIN4* in HLB diseased citrus may aid *C*Las infection. Thus, RIN4 may play a negative regulatory role during citrus–*C*Las interactions.

Non-Technical Summary: *RIN4*acts as a negative regulator during citrus–*C*Las interactions. The silencing or knocking out of this gene may have great potential for improving citrus resistance to HLB.

Citations:

Mackey D, Belkhadir Y, Alonso JM, Ecker JR, Dangl JL (2003) Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell 112:379-389

Wang N, Trivedi P (2013) Citrus Huanglongbing: A newly relevant disease presents unprecedented challenges. Phytopathology 103:652–665

Zhong Y, Cheng CZ, Jiang B, Jiang NH, Zhang YY, Hu ML, Zhong GY (2016) Digital gene expression analysis of Ponkan mandarin (Citrus reticulata Blanco) in response to Asia citrus psyllid-vectored Huanglongbing infection. Int J Mol Sci 17:1063

IRCHLB-P14-161

Mining the culturable citrus microbiome as a reservoir for potential HLB mitigation tools

BLACUTT, A.A.[1], GINNAN, N.A.[1], VIRAVATHANA, P.[1], DANG, T., BOGDAHI, S.[1], PEACOCK, B.[1], RUEGGER, P.[1], MALONEY, K.[3], Greg MCCOLLUM[4], VIDALAKIS, G[1], BORNEMAN, J.[1], ROLSHAUSEN, P.[1], and ROPER, C.[1]

[1] University of California, Riverside
[2] University of California, San Diego
[3] Point Loma Nazarene University, San Diego, CA
[4] United States Department of Food and Agriculture, Agricultural Research Service

Abstract: Plants interact with a vast array of microbes that make up their microbiome. Plant health and resilience to disease are often a function of the activities of the resident microbiome. The focus of our research is to elucidate the composition and roles of microbial communities inhabiting different anatomical parts of citrus trees in the context of the

Huanglongbing (HLB) pathosystem. Using next generation amplicon-based sequencing methodologies we examined the bacterial and fungal inhabitants of Florida citrus roots, stems and leaves. We also captured the microbial diversity from these same tissue compartments using traditional culturing methods. We queried the resulting repository of cultured citrus microbial isolates for antimicrobial activity against *Liberibacter crescens*, a culturable surrogate for *Candidatus* Liberibacter asiaticus (CLas), the non-culturable bacterium associated with HLB. Using this bioassay-guided pipeline, we then further resolved the secretomes of the inhibitory isolates using flash column chromatography to fractionate the supernatants with the goal at arriving at purified natural products that retain inhibitory activity. This work serves as a foundation for unraveling the complexity of the chemistries associated with the collective citrus microbiome. These data build collective understanding of the functional roles members of the citrus microbiome fill, in an effort to determine whether these microbes or their natural products can be developed into tools to mitigate HLB.

Non-technical summary: Using next generation sequencing coupled with traditional culturing techniques, we are exploring the microbial diversity of the citrus microbiome. We are interrogating the culturable portion of the citrus microbiome for production of anti-CLas natural products. These data can be used to develop these microbes or their natural products as tools to combat HLB.

IRCHLB-P14-162

Movement and propagation of *Candidatus* Liberibacter asiaticus in its plant host and insect vector.

Diann ACHOR[1], Murad GHANIM[2], **Amit LEVY**[1,3]

[1] Citrus Research and Education Center, University of Florida, Lake Alfred, USA, [2] Department of Entomology, Volcani Center, Rishon Le-Zion, Israel [3] Department of Plant Pathology, University of Florida, Gainesville, USA

Abstract: Citrus greening is associated with the phloem-restricted bacteria *Candidatus* Liberibacter asiaticus (CLas), and is transmitted by the Asian citrus psyllid *Diaphorina citri*. CLas is an intracellular bacteria, but very little is known about its interactions with the plant host and insect vector at the cellular level. Here we used transmission electron microscopy and confocal microscopy to study and compare the accumulation and movement of CLas inside its host and vector. We show that CLas enters the *D. citri* gut cells by endocytosis and that it forms a large replicative vacuole inside the gut and the reproductive organs of the insect. The formation of these vacuoles inside the cells is accompanied by dramatic re-organization of the psyllid endoplasmic reticulum (ER) that associate with these intracellular bodies. Immunolocalization confirmed that CLas accumulates inside these vacuoles. CLas movement between the vacuoles and the cell surface is carried out inside vesicles. In the plants, CLas movement and propagation take place in the cytoplasm of the phloem sieve elements, without vacuole formation. Our results suggest that in its insect vector, CLas modifies the host cell membranes to move and to create an isolated and safe environment to support its own propagation. Understanding the molecular interactions that are involved in these membranal processes may open new opportunities for controlling CLas.

Non-technical summary: Blocking the bacteria propagation and/or movement inside the plant and the psyllid can prevent bacteria spread and transmission, but very little is currently known about the bacteria-plant and the bacteria-vector interactions at the cellular level. Here we studied CLas cellular interactions in the insect and plant, and identified a novel mechanism the bacteria employs to enable its propagation and movement inside the psyllid. Interfering with this mechanism may help block the bacterial spread.

IRCHLB-P14-163

New insight into the distribution of viable *Candidatus* Liberibacter asiaticus in citrus floral organs

Fang DING[1,2], Qinglian WANG[1,2], Shiling WANG[1], Guoliang XU[1], Jin JIA[1], Yongzhong LIU[2], Yongping DUAN[3], Ni HONG[1], Guoping WANG[1], Shu-ang PENG[2] and John S. HARTUNG[4]

[1]Hubei Key Laboratory of Plant Pathology, College of Plant Science and Technology, Huazhong Agricultural University, Wuhan, 430070 P. R. China. [2]Key Laboratory of Horticultural Plant Biology (Huazhong Agricultural University), Ministry of Education, Wuhan, 430070 P. R. China. [3]USDA ARS USHRL 2001 South Rock Road, Fort Pierce, FL34945 USA. [4]USDA ARS MPPL 10300 Baltimore Ave., Beltsville, MD 20705 USA.

Abstract: Huanglongbing (HLB) is the most serious disease threatening citrus industries worldwide. '*Candidatus* Liberibacter asiaticus' (CLas), the associated agent of HLB, is a phloem-limited gram-negative bacterium. The distribution of CLas in floral organs is still largely unknown. In the present study, we used PCR, Nested-PCR and direct tissue blot immuno assay (DTBIA) to identify whether viable CLas was distributed in floral parts or not. The flowers tested were taken from five citrus cultivars collected from three provinces in China. We used PCR-based methods to detect CLas DNA from flower petals, as well as in both male and female floral organs, including the anther filament, pollen grains, stigma, ovary and receptacle. CLas in the same floral organs were also detected with DTBIA using two antibodies against CLas. It showed that CLas bacteria were present in anthers, pollen grains, pollen tubes, stigmas, ovaries and receptacles of citrus flowers. This is the first report that viable CLas are present in pollen, pollen tubes and ovaries. Our results demonstrate the colonization of citrus pollen by CLas, especially in pollen tubes, which opened a new possible way for the dissemination of CLas. It should be taken into account for the integrated control to prevent HLB from wide spreading.

Non-Technical Summary: The distribution of CLas in planta has been studied extensively with DNA-based methods. Yet the distribution of CLas in citrus flowers especially whether it is viable, is largely unknown. We investigated the distribution pattern of CLas with both DNA and antibody-based techniques. Our results revealed the presence of CLas in citrus floral organs, especially in pollen and pollen tubes, indicating a high risk of a new possible dissemination pathway of CLas.

Citations:

Ding, F., Duan, Y., Paul, C., Brlansky, R.H. and Hartung, J.S. (2015) Localization and distribution of '*Candidatus* Liberibacter asiaticus' in citrus and periwinkle by direct tissue blot immuno assay with an Anti-OmpA polyclonal antibody. *PloS one* **10**, e0123939.

Hilf, M.E., Sims, K.R., Folimonov, S.Y. and Achor, D.S. (2013) Visualization of '*Candidatus* Liberibacter asiaticus' cells in the vascular bundle of citrus seed coats with fluorescence in situ hybridization and transmission electron microscopy. *Phytopathology* **103**, 545-554.

IRCHLB-P14-164

Off-flavor of fruits from citrus trees infected with *Candidatus* Liberibacter asiaticus is associated with a reduction in the levels of terpenoid products and the downregulation of proteins in glycolysis

Lixiao YAO[1,2], **Qibin YU[1]**, Ming HUANG[1], Weilun HUNG[1], Jude GROSSER[1], Shanchun CHEN[2], Yu WANG[1], Frederick G. GMITTER Jr.[1]

[1]University of Florida, IFAS-CREC, Lake Alfred, FL, United States
[2]Southwest University, Chongqing, P. R. China

Abstract: HLB not only reduces the quantity of citrus fruit but also affects fruit and juice quality. Affected orange fruit often do not look fully mature and exhibit off-flavors. The molecular mechanisms leading to the off-flavor is poorly understood. Proteomics and metabolomics, each with its own advantages, have different analytical objects. Proteomics aims to detail an overall and comprehensive understanding of protein characteristics directly relevant to physiological phenotypes and underlying processes. Metabolomics aims to analyze quantitatively all low molecular weight biomolecules that are the end products of reactions mediated by proteins during a specific physiological period. The integration of proteomic and metabolomic analyses in one study could create a more comprehensive overview of changes in protein expression and metabolite composition and aid the understanding of the metabolic and physiological changes of HLB-affected fruits. In this study, fruits were collected from symptomatic and healthy Valencia sweet orange (*Citrus sinensis*) trees grafted on Swingle (*C. paradisi* x *Poncirus trifoliata*) rootstock. The results showed that most of the differentially expressed proteins involved in glycolysis, the tricarboxylic acid (TCA) cycle, and amino acid biosynthesis were degraded, and terpenoid metabolism was significantly downregulated in the symptomatic fruit. Valencene, limonene, 3-carene, linalool, myrcene, and α-terpineol levels were significantly lower in fruit from (*Candidatus* Liberibacter asiaticus) *C*Las-infected trees than from healthy trees. Similar phenomena were observed for sucrose and glucose. The results from studies performed on citrus fruit pulp from *C*Las-infected trees revealed significant suppression and metabolic dysfunction in sugar and organic acid metabolism and homeostasis, and terpenoid metabolism. These cause major disruptions in the production of proteins, sugars, organic acids, and volatiles, resulting in lower levels of sugars and terpenoids in symptomatic fruit pulp, and thereby causes many of the negative attributes found in juice produced from such fruit.

Non-Technical summary: HLB not only reduces the quantity of citrus fruit but also affects the quality of fruit and juice. This study provides further understanding of the molecular mechanisms of the metabolic and physiological changes of HLB-affected fruits, such as identified proteins on off-flavor related biosynthetic pathways. Results from this study will be very valuable for researchers seeking solutions to HLB though breeding or genome editing, so the citrus industry can provide high quality juice for consumers.

IRCHLB-P14-165

Overexpression of the *Nicotiana tabacum* SALICYLIC ACID BINDING PROTEIN 2 (NtSABP2) enhances the defense response pathway in citrus

Juliana Moreira SOARES[1], Wenming QIU[1,2], Jude W. GROSSER[1] and Manjul DUTT[1]

[1]Citrus Research and Education Center, University of Florida, Lake Alfred, FL 33850
[2]Institute of Fruit and Tea, Hubei Academy of Agricultural Sciences, Wuhan, China 430064

Abstract: The SALICYLIC ACID BINDING PROTEIN 2 (SABP2) is essential for the establishment of systemic immune responses in *Nicotiana tabacum* (Nt). This protein catalyzes the conversion of methyl salicylic acid (MeSA) into salicylic acid (SA) which consequently induces plant defense. The aim of this study was to evaluate transgenic lines overexpressing SABP2 to enhance citrus defense pathways and mediate tolerance to Huanglongbing (HLB). A large population of sweet orange (cvs. 'Hamlin' and 'Valencia') were produced to express the NtSABP2 gene, either under the control of a constitutive CaMV 35S promoter (35S-SABP2)

or a phloem specific *Arabidopsis thaliana SUC2* promoter (AtSUC2-SABP2). The transgenic lines were screened for gene expression using qPCR and the protein expression verified by western blotting with NtSABP2 specific antibody. Eleven transgenic lines expressing high, medium and low levels of the transgene were selected and propagated for further experimentation. An increase in *PATHOGENESIS-RELATED*-2 (*PR-2*) transcripts was observed in all the selected 35S-SABP2 lines. The transgenic line with the highest SABP2 transgene expression also had the highest accumulation of *PR-2*. The relative expression of *NON-EXPRESSOR OF PR-1* (*NPR1*) and *PR-1* transcripts were also estimated, however, only wild type transcript levels were detected in both 35S-SABP2 and AtSUC2-SABP2 lines. All transgenic lines were planted in our USDA approved transgenic field site. Under field conditions, two of the 35S-SABP2 lines and one of the AtSUC2SABP2 lines had enhanced tolerance to HLB, remaining PCR negative even after several years in the field. Together, the results indicate that NtSABP2 expressed either constitutively or in the phloem tissues can confer tolerance to HLB. The increase in resistance might be mediated by the activation of plant defense pathways, however, the specific pathway and biochemical mechanism orchestrating this process remain to be investigated.

Non-Technical Summary:

The most desirable form of long term HLB management is genetic resistance. However, conventional breeding usually takes time and based on the HLB progression, citrus growers need a faster and efficient solution. The expression of transgenes involved in plant defense activation pathways have been demonstrating to be an effective approach to increase tolerance to HLB in citrus. In this work we have observed that the expression of the tobacco derived NtSABP2 led to increased tolerance towards HLB.

IRCHLB-P14-166

Poncirus Genes (*PtCDR2* and *PtCDR8*) Restore Disease Resistance in Arabidopsis *cdr1* Mutant

Xiaobao YING, Fred G. GMITTER, Jr., and Zhanao DENG

University of Florida, Gulf Coast Research and Education Center,
14625 CR 672, Wimauma, FL 33598
University of Florida, Citrus Research and Education Center, 700 Experiment Station Road, Lake Alfred, FL

Abstract: Huanglonbing (HLB) or citrus greening is the most destructive disease of citrus worldwide. Genetic resistance to this disease has not been identified in commercial citrus cultivars. Various transgenic approaches are being explored in a number of laboratories to introduce durable, effective genetic distance to HLB. Previous transcriptome profiling and gene expression studies revealed significant up-regulation of the constitutive disease resistance 1-like (*CDR1*-like) genes in HLB-tolerant *Poncirus-Citrus* hybrids after being challenged with *Candidatus* Liberibacter asiaticus (CLas), the associated agent of HLB. We identified several copies of *CDR1*-like genes, and in particularly, *Poncirus CDR2* and *CDR8* (*PtCDR2* and *PtCDR8*) were induced in HLB-tolerant *Poncirus trifoliata* and its hybrids under HLB pressures in the field condition. To confirm their function in disease resistance, *PtCDR2* and *PtCDR8* were cloned and introduced into the Arabidopsis *cdr1* mutant whose *CDR1* gene was disrupted. *Pseudomonas syringae* pv. tomato strain DC3000 (Pst DC3000) expressing the *avrRpt2* avirulence gene was used to infiltrate the wild type Arabidopsis, the *cdr1* mutant, and the PtCDR2 or PtCDR8-transgenic *cdr1* mutant. *PtCDR2* or *PtCDR8*-transgenic *cdr1* mutants showed similar hypersensitive responses (HR) with the Arabidopsis wild type after they were inoculated with Pst DC3000. On the contrary, the Arabidopsis *cdr1* mutant did not show an HR.

The *PR1* gene expressed at much higher levels in the wild type Arabidopsis plant and the *PtCDR2* or *PtCDR8*-transgenic *cdr1* mutant than in the *cdr1* mutant, with or without bacterial challenges. The results showed that *PtCDR2* and *PtCDR8* conferred the Arabidopsis *cdr1* mutant resistance to *Pseudomonas syringae*. Research is under way to produce transgenic citrus plants over-expressing *PtCDR2* or *PtCDR8* and to characterize their resistance to HLB.

Non-technical summary: Genetic resistance to Huanglongbing (HLB) has not been identified in commercial citrus cultivars. Various transgenic approaches are being explored to introduce genetic distance to HLB. Two genes *PtCDR2* and *PtCDR8* cloned from HLB-tolerant *Poncirus* can confer the Arabidopsis *cdr1* mutant resistance to the bacterial pathogen *Pseudomonas syringae*. Research is under way to produce transgenic citrus over-expressing *PtCDR2* or *PtCDR8.*

IRCHLB-P14-167

Progress of HLB severity and yield loss in different bearing sweet orange variety/rootstock combinations

Renato Beozzo BASSANEZI[1]

[1]Fundecitrus, Araraquara, Brazil

Abstract: The Huanglongbing (HLB) severity progress and its damage on crop yield in different bearing sweet orange variety/rootstock combinations considering the age of tree at the moment of symptoms onset was assessed for 4 to 6 years in Sao Paulo State, Brazil. All selected citrus blocks were frequently sprayed with insecticides for the Asian citrus psyllid control and the trees had a good nutritional status and yield. The aim of this study was to get information to support the decision-making of the citrus growers in keeping Huanglongbing (HLB)-affected trees in the orchard. The disease severity progress was well modelled by monomolecular model with the average annual rate of disease severity *Rm* equal to 0.16. There was no significant difference for *Rm* among the scion varieties, rootstocks or tree ages. The relationship between disease severity and the relative yield of diseased tree (proportion of diseased tree yield in relation to the yield of healthy tree) were fitted by the negative exponential model with the average *b* parameter equal to 1.922. There was no significant difference for estimated *b* parameter among different scion varieties or rootstocks. However, older trees had smaller *b* values than younger trees. Contrarily that was previously thought, the disease severity progress and the yield loss in adult sweet orange trees was relatively faster. In general, the disease severity reached 50% of the canopy area just after four years from HLB-symptoms onset, with 60% yield loss in comparison with healthy trees.

Non-Technical Summary: This research evaluated the disease severity and yield loss in different bearing sweet orange variety/rootstock combinations. There was no significant difference for the rate of disease symptoms progress among the scion varieties, rootstocks or tree ages. The relationship between disease severity and the proportion of diseased tree yield in relation to the yield of healthy tree were similar among different scion varieties or rootstocks. However, older HLB-symptomatic trees had slower decrease on yield than younger trees. In general, the disease symptoms reached 50% of the canopy area of sweet orange bearing trees just after four years from HLB-symptoms onset, with 60% yield loss in comparison with healthy trees.

IRCHLB-P14-168

Screening transgenic citrus trees for Huanglongbing (HLB) tolerant/resistance

Ahmad A. OMAR[1, 2] and Jude W. GROSSER[1]

[1]University of Florida, IFAS, Citrus Research and Education Center, 700 Experiment Station

Road, Lake Alfred, FL 33850; [2]Zagazig University, College of Agriculture, Biochemistry Department, Zagazig 44511, Egypt

Abstract: Citrus greening, also called Huanglongbing (HLB) or yellow dragon disease, is one of the most serious diseases of citrus. HLB, associated with *Candidatus Liberibacter* asiaticus, is a phloem-limited fastidious pathogen transmitted by the Asian citrus psyllid (*Diaphorina citri*) and appears to be an intracellular pathogen that maintains an intimate association with the psyllid or the plant throughout its life cycle. Genetic transformation approaches to achieve tolerant/resistance to HLB is one of the most important tools as a long-term solution. Over-expression of the citrus β-1,3-glucanase and lipid transfer protein (LTP) genes using constitutive and phloem-specific promoters was performed to achieve this goal. Both genes were amplified from Valencia leaf and embryogenic callus using PCR with adding a cMyc tag (to ease later western blot analysis). After several sub cloning ended in a transformation vectors designated as p35SBG3 (constitutive), pSuc2BG3 (phloem specific), p35SLTP (constitutive), pSuc2LTP (phloem specific). Using a modified *Agrobacterium*-mediated transformation protocol, more than 80 transgenic lines of sweet orange (Valencia, OLL8, OLL20, and Vernia) and 15 'Duncan' grapefruit were regenerated containing p35SBG3 or p35SLTP and 40 containing pSuc2BG3 or pSuc2LTP. PCR analysis of these transgenic plants is showing the specific band for the BG and LTP genes. Molecular analysis for these plants including Southern, Western blot analysis and RT-PCR are showing the integration and expression of the β-1,3-glucanase and LTP genes in the regenerated transgenic plants. All transgenic lines have been propagated using shoot-tip grafting technique producing 3-4 replicates of each clone to speed up their evaluation under HLB high-pressure condition. The propagated transgenic plants have been planted at two different APHIS approved field sites in Florida in October 2015. Transgenic plants in the field were screened using qRT-PCR for the presence of HLB pathogen yearly. More than 40% of the transgenic plants in the field are showing negative results for HLB as of spring 2018. Another screening will be performed early 2019 and the results will be presented at the meeting.

Non-Technical Summary:
Genetic transformation approaches to achieve tolerant/resistance to HLB were used to generate transgenic citrus plants containing β-1,3-glucanase or lipid transfer protein (LTP) genes under constitutive or phloem-specific promoters. More than 100 transgenic lines from sweet orange (Valencia, OLL8, OLL20, and Vernia) and 'Duncan' grapefruit were produced. Molecular analysis for these plants showed the integration and expression of the β-1,3-glucanase and LTP genes in the regenerated transgenic plants. The propagated transgenic plants were planted at an APHIS approved field sites in Florida in October 2015. Transgenic plants in the field were screened using qRT-PCR for the presence of HLB pathogen and more than 40% of the transgenic plants are showing negative results for HLB as of spring 2018.

IRCHLB-P14-169

The effect of HLB on volatile profile of orange oil

Jinhe BAI[1], Randall CAMERON[1], Huqing YANG[2], Anne PLOTTO[1], Mike IREY[3], Elizabeth BALDWIN[1]

[1] USDA/ARS, HRL, Ft. Pierce, FL, USA, [2] Zhejiang A&F University, Hangzhou, Zhejiang, China, [3] Southern Garden Citrus, Clewiston, FL, USA

Abstract: Orange peel collected from HLB-affected fruit produced 30% less peel oils in comparison with the healthy fruit. The HLB peel oils also exhibited dark green color instead of the bright yellow in healthy peel oil. The green color is an indicator of maturity due to HLB-associated

phloem malfunction and resulting retarded growth and development of the fruit. The HLB peel oil contained low concentrations of mostly sesquiterpene hydrocarbons and derivatives, such as, nootkatone, valencene, α-selinene, 7-epi-α-selinene and caryophyllene oxide, and some monoterpene hydrocarbons and derivatives with orange/citrus characteristics, such as, limonene, myrcene, α-pinene, neral, geranial, and α-terpineol. Medium-chain aldehydes, such as, octanal, nonanal and decanal are key quality indicators of citrus oil, and they were substantially lower in HLB peel oil in comparison with healthy peel oil. However, eight compounds, mostly oxidation/dehydrogenation products of monoterpenes were found in the HLB peel oil which were not present, or present in very low quantities, in the healthy fruit. Other compounds found in larger amounts in HLB-affected peel oil than in healthy peel oil were cis-3-hexenol, cis-pinocarveol, carvone, Z-carvone, α- and ß-farnesene and limonene oxide. Preliminary research on utilization of degraded HLB fruit for the creation of value-added co-products was conducted. In comparison with essential oil collected from HLB fruit peel, the oil from whole fruit showed higher abundance of aliphatic aldehydes, alcohols and esters which contribute fruity flavor, and monoterpene aldehydes, alcohols and esters which contribute citrus and fruit flavors.

Non-Technical Abstract: Orange peel from HLB fruit produced much less peel oil with dark green color instead of the bright yellow. The chemical components in the oil also substantially different from the healthy fruit oil. The results suggest that the peel oil users need to adjust their formulations based on the changed volatile profiles.

IRCHLB-P14-170

The mitogen activated protein kinase (MAPK) signaling pathway plays a crucial rule in early response to *Candidatus* Liberibacter asiaticus

Qibin YU[1], Dongliang DU[1], Ming HUANG[1], Yi ZHANG[1], Zhanao DENG[3], Frederick G. GMITTER Jr[1]

[1]University of Florida, IFAS-CREC, Lake Alfred, FL, United States
[2]University of Florida, IFAS-GCREC, Wimauma, FL, United States

Abstract: The citrus host response to *Candidatus* Liberibacter asiaticus (*C*Las) at the transcriptional level has been widely studied . However, previous transcriptomic studies of citrus-*C*Las interactions were more often conducted using different tissues (leaves, fruits or roots) from either field or greenhouse grown trees. Inconsistent experimental design or sampling methods, and inappropriate controls, has limited the comparison of results between studies. In addition, not analyzing the transcriptome over time following inoculation ignores the dynamic mechanisms underlying the biological processes, because disease symptoms develop gradually across a long latent period. In this study, RNA-seq was used to compare tolerant rough lemon and sensitive sweet orange at 7, 17, and 34 weeks post-inoculation in a greenhouse experiment. Both time zero and mock inoculation were used to normalize the data, and to maximize the number of significant differentially expressed genes. Therefore, it was possible to analyze networks of defense response between mock- and *C*Las-inoculation of rough lemon and sweet orange separately. Functional analysis revealed that the genes involved in mitogen activated protein kinase (MAPK) signaling pathway, such as MAPKKK19, MKK9, MPK3, ERF-1, JAZ1, were highly expressed among upregulated genes in rough lemon. The MAPKs can induce a strong activity of WRKY transcription factors which might turn on multiple defense responsible genes in early response to *C*Las. Although more defense and immunity genes were found in the later than early stages, response levels were generally stronger at earlier than later stages.

Reprograming of transcriptomic pathways was different not only between tolerant and susceptible host response to *C*Las in general, but also specifically at different stages of the infection process, as well.

Non-Technical summary: Rough lemon and sweet orange could recognize *C*Las via pathogen recognition receptors, and activate plant immune systems, even before qRT-PCR could detect the bacteria. Quantitative disease resistance, seen as a strong and balanced defense response, could contribute to the development of the durable tolerance of rough lemon. Findings of this study should be very valuable to researchers for understanding molecular mechanisms of tolerance to *C*Las and searching for solutions to HLB through citrus breeding and genome editing.

IRCHLB-P14-171

Transcriptome profiles of HLB-affected citrus roots in response to sulfonamide antibiotics

Chuanyu YANG[1,3,4], Muqing ZHANG[1,2,3], Yongping DUAN[2], Veronica ANCONA[4], and Charles A. POWELL[1]

[1]IRREC-IFAS, University of Florida, 2199 S. Rock Rd, Fort Pierce, FL 34945, USA
[2]USHRL-USDA-ARS, 2011 S. Rock Rd, Fort Pierce, FL 34945, USA
[3] College Agricultural Science, Guangxi University, Nanning, 530005, China
[4]Citrus Center, Texas A&M University-Kingsville, 312 N. International Blvd, Weslaco, TX, 78599, USA

Abstract: Plant root systems serve as a conduit for nutrient and water transport and promoting the length of small-diameter roots within a root system increases the amount of water and nutrients available to the plant. Citrus Huanglongbing (HLB) associated with *Candidatus* Liberibacter asiaticus (CLas), causes loss and degeneration of roots, resulting in reduced yield, tree decline and eventually tree mortality. Sulfonamide antibiotics have been proved to be effective against Las bacterium, but have also been shown to promote the development of lateral roots in willow and barely. Therefore, it would be important to assess if sulfonamide antibiotics will trigger lateral root growth in citrus infected with Las while suppressing the bacterial population. It would also be important to investigate the transcriptomic changes triggered by these antibiotics. In this study, HLB-affected roots were treated with sulfadimethoxine sodium (SDX, 10 ppm), indole butyric acid (IBA, 10 ppm) and water control (CK), to assess Las titers and lateral root growth, while RNA-seq analysis of the transcriptome was also performed. The results indicated that: 1) Las titers in HLB-affected citrus root treated with SDX was lower than those of IBA and CK treatments; 2) SDX and IBA treatments induced lateral root growth in HLB affected citrus compared to CK treatment; 3) Using RNA-sequencing method, 1,606 and 881 differentially expressed genes (DEGs) were present in HLB-affected citrus root treated with SDX and IBA, respectively. In SDX and IBA treatments, many genes which contribute to lateral root and root hairy growth were enriched such as genes from the phenylpropanoid biosynthesis pathway, starch and sucrose metabolism, plant hormone signal transduction and pentose and glucoronate interconversions. Moreover, several genes related to calcium-binding protein, and cyclic nucleotide-gated ion channel, which are involved in plant-pathogen recognition pathway, were up-regulated in response to SDX. The results from this study provide a new insight into the molecular mechanisms triggered by SDX in HLB-affected citrus which have the potential to be used in the development of management strategies of HLB in the future.

Non-Technical summary:
Sulfonamide antibiotics can not only suppress ‘*Candidatus* Liberibacter asiaticus’ (CLas) titers, but also induce lateral root in HLB-affected citrus. Utilizing a transcriptomic analysis, we

found that these antibiotics activate genes that promote lateral root growth and plant disease resistance which can be used in the future for HLB management.

IRCHLB-P14-172

Translocation of *Candidatus* Liberibacter asiaticus in split root citrus and progression of Huanglongbing disease in plants

Jeane Dayse V. S. PULICI[1,2,3]**,** Mayara M. MURATA[1], Kayla M. GERBERICH[1], Diane B. BRIGHT[1], Silvio A. LOPES[2], Evan G. JOHNSON[1]

[1]University of Florida, Citrus Research and Education Center, Lake Alfred, FL, USA; [2]Fund for Citrus Protection- FUNDECITRUS, Araraquara, SP, Brazil; [3]São Paulo State University (Unesp), School of Agricultural and Veterinarian Sciences, Jaboticabal, SP, Brazil.

Abstract: Huanglongbing (HLB), associated with '*Candidatus* Liberibacter asiaticus' (CLas) and transmitted by the insect *Diaphorina citri*, is considered the most destructive disease of citrus worldwide. After initial infection, CLas quickly colonizes the root system before visible canopy symptoms develop. Understanding CLas movement between leaf and root tissues and local and systemic effects of CLas on the tree is important to improve disease management. Split root rhizotrons and one-side graft inoculation below the trunk split in late summer was used to study the role of root infection. Unlike previously reported results from above the split inoculations, CLas infection remained isolated in the inoculated half of the root system through fall root flush and dormancy until after the spring flush (at least 8 months). The dynamics of root growth and dieback were altered on both CLas-infected and non-infected roots on inoculated trees compared to healthy mock-inoculated controls. Root dieback occurred on both sides, while root growth stimulation only occurred on the non-infected side. The canopy was negatively impacted directly above infected roots. The first detections of CLas in canopies (2 of 20) occurred 9 months after inoculation, with 6 canopies CLas-infected at the end of the experiment (11 months). The results showed that root system infection does move up and around the trunk within a year, likely linked to seasonal growth patterns with inconsistent movement all the way to the canopy. Split root inoculation provides the ability to study local and systemic effects of CLas on roots.

Non-Technical summary: Huanglongbing damages roots and canopies of affected plants. Understanding root infection, damage, and bacterial movement between leaf and root tissue is essential to develop and test treatments for HLB. We are investigating how the bacterium moves systemically within the plant and the role of root damage and infection on overall tree health.

IRCHLB-P14-173

Triploid lime is more tolerant to HLB than diploid lime because specific physiological and anatomical traits are associated with better detoxification processes

Raphaël MORILLON[1], Gary SIVAGER[1]; Leny CALVEZ[1], Benoit HEUGET[1], Saturnin BRUYERE[1], Rosiane BOISNE-NOC[1], Pierre BRAT[2], Olivier GROS[3] and Patrick OLLITRAULT[1]

[1]SEAPAG, UM AGAP, CIRAD, Station de Roujol, 97170 Petit Bourg, Guadeloupe, French West Indies; [2]UMR QualiSud, CIRAD, Station de Neufchâteau-Sainte Marie, 97130 Capesterre-Belle-Eau Guadeloupe, French West Indies.; [3]C$_3$MAG, UFR des Sciences Exactes et Naturelles, Université des Antilles, BP 592 - 97159 Pointe-à-Pitre, Guadeloupe, French West Indies.

Abstract: HLB is responsible for the synthesis of callose at sieve plates of the phloem sieve tubes leading to the obstruction of the pores that provide connection between adjacent sieve elements thus limiting the symplastic transport of sugars and starch synthesized in leaves to organs of the plants such as fruits or roots. Polyploidy is a frequent occurrence in the plant kingdom. Our previous results showed that polyploidization leads to an increase in the size of cells and organs in citrus. Also, the use of tetraploid citrus rootstocks was shown to confer increased tolerance to water deficit. Our objective was to decipher the potential traits of tolerance to HLB related to polyploidy. Diploid (2x, *Citrus aurantiifolia*) and triploid (3x, *Citrus latifolia*) limes grafted onto diploid citrumelo 4475 (*Citrus paradisi* × *Poncirus trifoliata*) rootstocks were investigated when naturally infected by HLB or infected by grafting. Plant physiology and the anatomy of leaf petiole were analyzed using a Scanning Electron Microscope to observe callose deposition at sieve plates of the phloem and leaf starch content and detoxification enzyme activities in 2x and 3x leaves were also investigated. The triploid variety was shown to present less symptoms of chlorosis due to HLB. Photosynthesis, stomatal conductance and transpiration decreased compared to control plants but values remained greater in 3x than in 2x. Analysis of the petiole sieve plate in control petiole samples shown that pores were about 30% larger in 3x than in 2x. SEM analysis of infected petiole samples shown important deposition of callose onto 2x and 3x pores of naturally infected trees while biochemical analysis revealed similar behavior in 3x than in 2x. SEM analysis of infected petiole samples that do not present leaf symptoms (samples of controlled infected trees by grafting) showed more deposition of callose onto 2x than 3x pores while biochemical analysis revealed much better behavior in 3x than in 2x. Taken together, our results suggest that polyploidy may present great interest to improved tolerance to HLB.

Non-Technical summary: Polyploidy promotes better tolerance to abiotic stress such as drought. Because of its specific anatomical traits such a bigger cells, we evaluated triploid lime compared to diploid when infected by HLB. Triploid lime is much more tolerant to HLB than diploid lime. In triploid, the phloem sap flux is probably less affected because the callose deposition that plugs the pores between cells is less than in diploid. Polyploidy may eventually be a tool to improve tolerance to HLB.

IRCHLB-P14-174

Using effectors of CLas as molecular probes to understand HLB pathogenesis

Kelley CLARK[1], Jessica FRANCO[2], Simon SCHWIZER[1], Zhiqian PANG[3], Eva HAWARA[1], Veronica ANOCONA[4], Nian WANG[3], Gitta COAKER[3], Wenbo MA[1]

[1]Department of Microbiology and Plant Pathology, University of California, Riverside, USA
[2] Department of Plant Pathology, University of California, Davis, USA
[3] Citrus Research and Education Center, University of Florida, Lake Alfred, USA
[4] Citrus Center, Department of Agriculture, Agribusiness and Environmental Sciences, Texas
A&M University, -Kingsville, Weslaco, TX 78599

Abstract: The Huanglongbing (HLB)-associated pathogen, *Candidatus* Liberibacter asiaticus (CLas), possesses the Sec secretion system that potentially delivers virulence proteins into the phloem of infected trees and promotes disease development. These Sec-delivered effectors (SDEs) can be used as molecular probes to uncover pathogenesis mechanisms of the host-pathogen arms race and set the foundation for the development of the urgently needed management strategies for HLB. Previous work from our lab predicted several SDEs from CLas that are highly expressed in infected trees. We have characterized one of these SDEs, named SDE1. We found that SDE1 interacts with several

proteases, known as papain-like cysteine proteases (PLCPs) and inhibits their protease activity. As such, SDE1 promotes bacterial infection. Further characterization of this interaction will help with the generation of tolerant or resistant citrus varieties via genome editing or transgenics. We will discuss recent progress on characterization of the SDE1-PLCP interaction, PLCP profiling across citrus varieties, and phenotypes associated with SDE1 expression *in planta*.

Non-Technical summary: HLB management is one of the major challenges faced by the citrus industry in lieu of this devastating disease. We focus on understanding the virulence strategies utilized by the CLas bacterium and the molecular interactions between CLas effectors and citrus host defense mechanisms. Our research findings work towards development of future sustainable management of HLB.