USDA, NIFA, SCRI Citrus Disease Research and Extension Program
2014 (Year 1) Approved Project Profiles

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Summary Tables of approved HLB projects.

Title	Investigator	Institution
CHARACTERIZATION OF LIBERIBACTER POPULATIONS AND DEVELOPMENT OF FIELD DETECTION SYSTEM FOR CITRUS HUANGLONGBING	Ramadugu, C.	The Regents of University of California Riverside, CALIFORNIA
NON-TRANSGENIC, NEAR TERM RNA INTERFERENCE-BASED APPLICATION STRATEGIES FOR MANAGING DIAPHORINA CITRI AND CITRUS GREENING HUANGLONGBING	Falk, B.	UNIVERSITY OF CALIFORNIA, DAVIS DAVIS, CALIFORNIA
STEAM-GENERATED SUPPLEMENTARY HEAT THERMOTHERAPY AS AN IMMEDIATE TREATMENT FOR PROLONGING PRODUCTIVITY OF HLB-INFECTED CITRUS TREES	Ehsani, R. J.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA
ZINKICIDE A NANOTHERAPEUTIC FOR HLB	Johnson, EV, G.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA
A NOVEL ANTIMICROBIAL APPROACH TO COMBAT HUANGLONGBING DISEASE	Lorca, G. L.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA
DETERMINING THE ROLES OF CANDIDATE GENES IN CITRUS-HLB INTERACTIONS AND CREATING HLB-RESISTANT CITRUS CULTIVARS	Gmitter, FR, G.	UNIVERSITY OF FLORIDA GAINESVILLE, FLORIDA
DEVELOPING AN INFRASTRUCTURE AND PRODUCT TEST PIPELINE TO DELIVER NOVEL THERAPIES FOR CITRUS GREENING DISEASE	Brown, S. J.	KANSAS STATE UNIV MANHATTAN, KANSAS

ACCESSION NO: 1005572 **SUBFILE:** CRIS
PROJ NO: CALW-2014-10148 **AGENCY:** NIFA CALW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-22992 **PROPOSAL NO:** 2014-10148
START: 01 FEB 2015 **TERM:** 31 JAN 2020
GRANT AMT: $1,683,429 **GRANT YR:** 2015
AWARD TOTAL: $1,683,429

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CHARACTERIZATION OF LIBERIBACTER POPULATIONS AND DEVELOPMENT OF FIELD DETECTION SYSTEM FOR CITRUS HUANGLONGBING

NON-TECHNICAL SUMMARY: Citrus is a very important fruit crop in the US. Huanglongbing (HLB) is a serious disease that has destroyed about half of Florida citrus and is now threatening the citrus industries in Texas and California. The pathogen is a bacterium known as Liberibacter and the disease is transmitted by an insect vector, Asian citrus psyllid. There is no known cure for HLB. Early detection and proper management done in a timely fashion are essential for disease management. Three species of the HLB pathogen are known. We have recently discovered a fourth species from Colombia. Proper identification of all forms of the pathogen is crucial for disease management. We propose to characterize pathogen variants and generate information that will be used for development of a testing system capable of detecting all species. Since the pathogen can be detected in the insect vectors several years before it is detectable in plants, we will focus mainly on testing the insect vectors of HLB. There is a need to trap the psyllids efficiently so that reliable testing can be conducted. The SmartTraps that we propose to develop will chiefly trap the insect vector of HLB and will preserve the psyllids such that they can be used efficiently for pathogen detection tests. We will use the genetic information developed for pathogen variants and design assays to detect all forms of the pathogen. In addition we will develop detection technologies using a very simple SmartDART unit that can be operated by growers and used for testing. Expensive equipment or scientific training will not be needed to utilize this system. The most important aspect of our project is extension. We will train a total of 30 growers in two states, California and Texas, in using these new technologies. SmartDART units, SmartTraps, and reagent kits will be provided to the participants. We believe that if the growers are capable of detecting HLB the surveillance system will be stronger and disease management becomes easier. Our goal is to engage a large number

of growers and extension personnel in early detection of HLB so that the disease can be better managed in areas where it is already present and prevent and suppress the disease in other areas.

OBJECTIVES: Overall goal isdevelopment of methodologies for early detection of all disease-associated forms ofLiberibacter (in psyllids), monitoring of the pathogen using field based diagnostic tools and development of integrated management strategies.HLB has resulted in significant losses to citrus industries worldwide. Reliable identification of all disease-associated Liberibacter pathogens may be crucial for taking necessary steps for disease management. Development of information required for designing generic testing capabilities for all Liberibacters may be an integral part of disease management. Secondly, if we make these technologies available to the interested parties including citrus growers and extension personnel, it will facilitate large-scale testing in future and will make the surveillance system stronger.We have four major goals:a) to develop sequence information on diverse Liberibacters; b) to develop methodologies for field detection of all Liberibacters associated with huanglongbing (HLB) in psyllids and plants; c) to develop improved methodologies collection and storage of psyllid vectors, and, d) to make the technologyavailable to citrus growers and extension workers.The four objectives are outlined below:1. Since Liberibacter variants are known to exist and we propose to develop methods to detect all HLB-associated Liberibacters, we will develop genomic sequence information for Liberibacter variants currently known to exist in California, Texas, and other regions (objective 1). Our focus will be on detection of the pathogen in psyllids and plants in regions where HLB is not yet widespread (California and Texas).2. We will thenuse this information to design inexpensive, sensitive, field deployable kits using Loop-mediated amplification (LAMP) technology, and a field adaptable SmartDART™ detection device (objective 2).The methodology will be adapted to detect all disease-associated Liberibacter pathogens.As LAMP method is user-friendly, it can be utilized byinterested non-scientific personnel.3. Since the project is focused on detection of the pathogen in psyllid vectors, it is essential to have efficient vector trapping and storage. Towards this goal, we will improvepsyllid captureusing a 'SmartTrap' that traps only psyllids and stores them at ambient temperatures (objective 3).4. We believe that if these technologies are made available to a wider audience, it will strengthen the surveillance systems and help in disease mitigation. Enabling citrus growers and nurserymen to use the technologies will result in grower empowerment and facilitate management decisions. We will provide selected growers in California and Texas with tools and reagents to trap psyllids and test for Liberibacters. Participating labs will collect data and samples from growers for confirmatory analysis. With grower participation we will develop a system that helps in containment and management of HLB (objective 4).

APPROACH: Objective one: Develop genomic sequence information of Liberibacter variants found in California, Texas and other citrus-growing regions1.1 Develop universal Liberibacter detection system. Using alignments of available genomic sequences of Rhizobiaceae members, several targets will be selected and several universal Liberibacter detection systems will be designed and used to screen existing collection of psyllid DNAs to find out sequence variants.1.2. Selection of samples: We will analyze the Liberibacter strain from California (samples from CRF in Davis), include samples from Texas, citrus relative samples showing HLB symptoms from FL, Central and South American countries; primarily using single psyllid extractions.1.3. 16S metagenome analysis. We will conduct this using services of Mr. DNA Company. Analysis of the 16S metagenome data will be helpful in understanding the nature of

bacteria present in the sample. The methodology helped us to confirm a new species of Liberibacter in Colombia.1.4. 384 primer array. We have designed and used this array in our Riverside labs. Will use selected samples to do the array and study populations.1.5. Agilent Sureselect hybridization. We have already designed this panel. We will use this approach for select samples only.1.6. PACBIO and Illumina NextGen sequencing and Bioinformatic analysis. Isolates carefully selected by several of the above methods will be used for full genome sequencing. This aspect will be conducted at the core facilities of UCR and UC Irvine/Univ. of Florida.1.7. Selection of sequences conserved in Liberibacters. We will refine our universal Liberibacter detection system as needed when new sequences become available.Objective two: Develop a LAMP-based field testing system capable of detecting all HLB-associated Liberibacter populations from both psyllids and plants2.1. Develop LAMP capable of detecting all relevant Liberibacters: Identify suitable regions conserved in Liberibacters, absent in other Rhizobiaceae, design LAMP primers (Jenkins et al. 2011) and screen against multiple plant/psyllid extractions collected from various hosts and geographical locations (extractions available in the collection of the Riverside labs). We will design assimilating probes for promising primer sets and multiplex with internal controls.2.2. Standardize and optimize LAMP assays using a qPCR machine: Development of LAMP primer sets and assimilating probes for all HLB-associated Liberibacters will be done in year 1.2.3. Prepare and evaluate a lyophilized, ready-to-use, diagnostic kit for HLB: We will develop formulations which can be freeze-dried, to achieve stability at room temperature over long durations of time (> 6 months). Coordinated by Kubota, and Jenkins at the Univ. of Hawaii, Manoa.2.4. Develop Assimilating Probe-based detection systems for Liberibacter and psyllids: To enable use of CLas- and ACP-specific internal control probes in duplex assays for validation.2.5. Develop an assay for detection of Liberibacters in plants: Psyllid assay is fairly easy to handle without cross-contaminations. LAMP works well with purified plant DNAs (Keremane et al., 2014). Will develop a crude extraction method for plant samples. Instead of the COX (multicopy), we will use single copy gene as internal control (Ramadugu et al. 2013).2.6. Customization of Smart-DARTTM software tools/ interface: Existing Smart-DARTTM software has automated classification algorithms to reliably identify and report outcomes of tests. As new tests are developed with internal controls, we will customize the user interface for citrus industry stakeholders to simplify and standardize interpretation of results (i.e. sample is infected or not, or test results are inconclusive/invalid based on the presence of inhibitors or inappropriate use of sample or sample preparation).Objective three: Improve methods for psyllid collection, shipping and preservation3.1. Purchase two new 3-D printers to make variations to the shape and design of SmartTraps. Modify previous designs by adding texture, more appropriate lighting (different colors and LED lights. Placement, color and deployment of light are key elements of research in the second year.3.2 Compare SmartTraps on citrus with those on separate poles. Improve lighting parameters. Provide additional light at dawn and dusk only.3.3. Collect psyllids and the low level of non-psyllids from traps. Psyllids will be sent to Riverside lab for analysis. Document non-psyllid samples and preserve in DPI.Objective 3 will be conducted in FL by Smith and Halbert with help from Jenkins for improving the LED aspect.Objective four: Promote the use of LAMP assay and train growers, nurserymen, pest control agents, extension workers and other stakeholders for widespread testing of Liberibacters as a component of an overall

integrated pest management (IPM) program.4.1 Train citrus growers (selected through advisory Board; 10 in year 1, 30 in years 3, 4 and 5; total no. is 30) in using SmartTrap, SmartDART TM and LAMP, sampling procedures, discuss anticipated project outcomes and expectations. Participants will collect psyllids from SmartTraps at biweekly intervals, conduct SmartDARTTM testing, initially for CLas and then for all Liberibacters (as soon a method is available; see objective 1). Each participant will periodically test pooled (1-10) psyllids for CLas using SmartDART TM and the remaining cohorts of trapped psyllids and crude extracts will be provided to Alabi in Texas and Keremane in Riverside for test validation. All data will be maintained in databases along with details of extension activities and test results. HLB finds in new locations will be subjected to standard reporting requirements with the local, state and federal regulatory agencies while maintaining grower confidentiality. As modifications and/or updates become available, we will provide participants with the improved units. Similarly, updated kits and protocols will be provided when the universal Liberibacter detection kit becomes available. All reagent kits will be prepared at Riverside and shipped to individual participants in coordination with project collaborators. We will work on room temperature stable reagent kits (objective 2), and the improved kits will be supplied to all participants when they become available. Detection systems for plant samples using Smart-DART™ will also be provided when they become available (objective 2).

ACCESSION NO: 1005650 **SUBFILE:** CRIS
PROJ NO: CA-D-PPA-2283-CG **AGENCY:** NIFA CALB
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23011 **PROPOSAL NO:** 2014-10128
START: 01 MAR 2015 **TERM:** 28 FEB 2018
GRANT AMT: $4,579,067 **GRANT YR:** 2015
AWARD TOTAL: $4,579,067

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NON-TRANSGENIC, NEAR TERM RNA INTERFERENCE-BASED APPLICATION STRATEGIES FOR MANAGING DIAPHORINA CITRI AND CITRUS GREENING HUANGLONGBING

NON-TECHNICAL SUMMARY: Effective techniques to reduce the rate of Huanglongbing (HLB) spread are key to slowing its incidence, especially for new citrus plantings. RNA-interference (RNAi) is a natural regulatory and anti-viral response in eukaryotes and can be

manipulated to target mRNA/gene expression, including to control insects. Our on-going collaboration has found that RNAi inducers, expressed in citrus trees using the Citrus tristeza virus (CTV) vector, reduce the survival of adult Diaphorina citri moving onto the trees, and greatly reduce their reproduction and acquisition of Candidatus Liberibacter asiaticus by psyllid progeny. Our goal is to further improve RNAi activity such that it can be used to help manage D. citri and HLB, allow reductions in pesticide use and lower grower costs for U.S. citrus. A second strategy for applying RNAi towards D. citri takes advantage of insect viruses that could be modified to deliver interfering RNAs directly to D. citri. This approach offers a non-modified plant alternative that could complement the use of CTV to express RNAi inducing sequences against psyllids and help manage HLB spread. Here we expand collaborative efforts in RNAi research with mathematical modeling, economic analysis, and extension/outreach to quickly move this technology to the growers' fields

OBJECTIVES: Objective 1). Optimize the Florida CTV to deliver efficacious D. citri-interfering RNAs in Florida non-transgenic citrus.Objective 2). Develop a CTV vector for applications in California citrus.Objective 3). Develop D. citri-infecting viruses for non-plant-based induction of RNAi effects in psyllids.Objective 4). Model/test RNAi systems under greenhouse and/or field conditions.Objective 5). Evaluate the economic impact of using RNAi technologies in citrus for controlling D. citri and HLB.Objective 6). Develop effective Extension Outreach programs for RNAi-based strategies.

APPROACH: Objective 1-The 35S binary plasmid constructs engineered to harbor the CTV vector, plus the different RNAi-inducer sequences, will be used to agro-inoculate Nicotiana benthaminia from which virions are extracted & used to inoculate small citrus trees. Inoculum from these trees are used to graft-inoculate test trees.Objective 2-The Ng lab has been developing the cloned infectious cDNA of a CTV isolate from California by using the approaches performed by the Dawson lab, culling from past experience constructing the infectious clone of Lettuce chlorosis virus, another member of the family Closteroviridae. The overarching guideline that we adhered to when choosing candidate CTV isolates for cDNA cloning is that they have to be mild or asymptomatic CTV isolates, and that they must be prevalent in California. We have identified several isolates that met those criteria: 1) CCTEA-5 and a few others that share a T30 genotype, 2) RB25, a Poncirus trifoliata-resistant breaking albeit mild isolate, and 3) CCTEA 96339, an isolate with a T36 genotype.Objective 3- This work is being led by the Falk lab. Because small RNAs are hallmarks ofantiviral responses in plants & insects, & with new sequencing platforms, small RNAs can be sequenced directly. We have & continue to sequence siRNAs from various populations of D. citri, and then use bioinformatics to identify D. citri-infecting viruses. So far RNAs have been extracted from 33 different D. citri populations collected from nine locations around the world, including U.S. populations from Florida, Texas, California & Hawaii. Four were used for small RNA deep sequencing & two for transcriptome sequencing on HiSeq and MiSeq platforms, respectively. Bioinformatics analyses has so far allowed us to identify three different types of virus sequences among both types of libraries (probably more virus sequences are present but not yet confirmed). D. citri from Taiwan, China, Hawaii & some from Florida were found to be infected with DcRV. We also found evidence for Densovirus sequences in all D. citri populations examined, but we are not yet sure if these data represent a replicating virus or possibly integrated Densovirus sequences. Densoviruses are widespread viruses with relatively simple ssDNA genomes. Furthermore, a

Densovirus has been used recently to induce RNAi effects in mosquitoes & we are further investigating this for D. citriObjective 4- The CRDF, which is coordinating the design and implementation of field tests, has enlisted the assistance of the Keesling lab to assist in the designs. The Keesling lab has already developed a mathematical model of CLas spread based on the lifecycle of D. citri populations. Their original model was designed as a proof of concept to predict the rapid asymptomatic spread of CLas through a grove. After a tree is infected, it is some time before symptoms of HLB appear. According to the model, the infection takes over a grove long before the first symptoms appear. The model takes into account factors such as psyllid movement patterns, psyllid aging & mortality, & citrus flushing patterns to make its predictions. This model is highly adaptable & is capable of incorporating factors such as pesticide spraying, invasion of psyllids from other groves, & the various effects that RNAi constructs may have on the psyllid population or CLas transmission. For this project, the model will be refined to be capable of accurately predicting the impact of different RNAi constructs & delivery means (CTV and/or D. citri virus) on the spread of HLB in conjunction with other strategies to insure that new citrus plantings can be protectedObjective 5-A market modeling approach will be used to estimate the changes in welfare for consumers & producers of oranges, lemon and tangerines in Florida and California. The market model contains linear equations for demand by consumers, total market supply, production by growers in each region, & trade. It is used to estimate the percentage changes in market supply, price, production and demand from an exogenous shift in the supply curve due to a sudden change in crop management, such as what occurs when an exotic pest becomes established, or a technological change. The percentage changes in prices, quantity demanded & production are used to calculate the changes in welfare to consumers & producers. For Florida, the model will be simulated based on current estimates of rates of spread & disease progression. For California, the model will be simulated based on expected values extrapolated from the Florida data.Objective 6-We will develop an extension outreach plan that enhances existing extension educator programming in citrus. There are four primary outreach approaches for this proposal.

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PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23030 **PROPOSAL NO:** 2014-10141
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GRANT AMT: $3,495,832 **GRANT YR:** 2015
AWARD TOTAL: $3,495,832

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STEAM-GENERATED SUPPLEMENTARY HEAT THERMOTHERAPY AS AN IMMEDIATE TREATMENT FOR PROLONGING PRODUCTIVITY OF HLB-INFECTED CITRUS TREES

NON-TECHNICAL SUMMARY: This four-year multi-state multi-disciplinary research and extension project aims to address the urgent need of citrus growers in Florida to an immediate treatment for HLB-affected citrus trees by utilizing steam for rapid thermotherapy. The overall goal of this project is to provide an immediate scalable technique using steam as well as synthesize scientific explanations on how the technique works in mitigating HLB and prolonging the production life of infected citrus trees. The central hypothesis of the project is that the HLB-affected trees can be treated for inoculum reduction using a rapid steam-based thermotherapy technique in a manner that is biologically and economically feasible with minimal environmental impact. This project covers both the practical and theoretical aspects of the thermotheraphy through an interdiscplinary approach involving researchers with expertise in plant pathology, plant physiology, engineering, nematology, economics, and plant biology. The project objectives include enhancement of the steaming system and operations used for the treatment, investigation of the effects of steam-based treatments on the survivability of CLas and the expression of HLB symptoms, determination of treatment efficacy and effectiveness, development of a comprehensive steaming system which includes treatment of the roots, studying the effects of heat treatment on yield and fruit quality, and conducting comprehensive economic analyses of the system. The enhanced steaming system will be used to treat citrus trees on a quarterly basis from different sites in collaboration with local citrus growers. An strong extension program is planned to disseminate the outcome of this research.

OBJECTIVES: Objective 1: Enhance the existing steam-generated supplementary heat thermotherapy system so that it will generate consistent heat and provide a uniform temperature to the canopy and roots.Objective 2: Determine the effect of steam treatment on CLas recolonization, overall tree health, and fruit yield and quality.Objective 3: Determine the efficacy of steam-based thermotherapy related to CLas viability.Objective 4: Evaluate the effectiveness of steam-based thermotherapy considering the pretreatment condition of roots as affected by pests and diseases and characterize the effects of the treatment on pests and diseases.Objective 5: To determine time-temperature combinations for the inactivation of CLas that do not result in tree defoliation.Objective 6: As a canopy post-treatment, horticultural mineral oil (HMO) applications to tree surfaces will improve the thermotherapy process and the development of new application technology.Objective -7: Conduct comprehensive economic analyses of the steam-generated supplementary heat thermotherapy system.

APPROACH: Objective 1:An existing heat treatment machine will be enhanced to provide rapid, precisely controlled, and uniform supplementary heat treatment into the canopy, roots, and surrounding soil of HLB-affected trees. Associated tasks include: (a) construction of an enhanced steaming system, (b) development of a mathematical model to predict both the heat transfer through the tree and the soil as the steam is applied and the temperature along the depth and direction of canopy and soil, (c) design and construction of a feedback control system to

efficiently control the heat thermotherapy process, (d) development of a new mechanism for heat treating the root system, and (e) development of a post-treatment mineral oil application to improve the effect of the steam thermotherapy (objective 6).Objective 2:The steaming system from objective 1 will be used to conduct a comprehensive field trial in 'Valencia' sweet orange. The trial will be designed as a split-plot design with timing of treatment application being the main-plot. Main-plots will be steam applications done at quarterly intervals to determine if there is an optimal time of year for CLas reduction in the canopy. The main-plots will be laid out as either a completely randomized design (CRD) or a randomized complete block design (RCBD) depending on the aspects of the site selected for the experiment. The main plot will consist of a minimum of 50 trees, subdivided into 10 tree sub-plots. There will be five replications of the main plots. For the first year of study, the sub-plot treatments will include five levels of heat treatment: 1) no heat treatment (control); 2) 58°C for 15 s; 3) 58°C for 60 s; 4) 58°C for 90 s; 5) 58°C for 120 s; and 6) 60°C for 30 seconds. These heat and time combinations have been selected to create two extreme set-ups including one combination in which steam is applied but no defoliation occurs while the other combination will cause total defoliation based on prior experiments. Before the steam treatments in each main-plot, an HLB symptom assessment (scale 1= Vigorous, asymptomatic, 2= Slight decline, symptomatic, 3= Moderate decline, symptomatic, 4= Severe decline, symptomatic, 5= Nonviable, will not recover) and sampling for CLas will be done.Objective 3:The viability of CLas after heat treatments will be used to assess the effectiveness of the treatment to reduce or eliminate the bacteria in the tree. Two different approaches will be used to assess bacterial viability. One will be based on the propidium monoazide (PMA) real-time PCR assay adapted for CLas detection.The second approach to assessing CLas viability will be based on a comparison of specific mRNA populations in the bacteria either with total genomic DNA (CLas) or CLas 23 rRNA levels. The rationale is that mRNAs in bacteria (and other organisms) show much higher turnover rates compared to genomic DNA or 23S rRNA. Hence, fluctuations in mRNA population size can provide a sensitive means to assess the overall viability of bacteria soon after the heat treatment. The expectation is that the exposure to high temperatures will result in drastic changes in the pattern of RNA abundance within minutes to hours after treatment.Objective 4:Sub-objectives related Objective 4 are to i) characterize the effects of the treatment on soilborne pests and diseases and ii) determine whether additional IPM practices improve the thermotherapy outcome. In addition to the field experiment described in Objective 2, we will select groves based on the infestation status for three key pests of the citrus root system: the root weevil Diaprepes abbreviatus the phytoparasitic, nematode Tylenchulus semipenetrans, and the oomycete Phytophthora nicotianae. Tree responses to thermotherapy in groves infested and not infested by each pest will be compared. Additionally, in some infested groves, thermotherapy will be applied to trees that are treated or not treated with chemical and biological pesticides capable of reducing pest population densities.Objective 5:Sustained productivity of citrus trees after heat treatment requires that the metabolic functions of the tree be maintained and that CLas populations be reduced to minimize symptom development and tree decline; our work indicates both are possible. Two key underlying factors for the long-term success of thermal treatment will be established. First, mathematical functions that describe the amount of time that a leaf or twig can be exposed to steam at a given temperature without dying will be derived and verified for CLas inactivation in planta, and a rapid enzymatic method developed for assessing leaf viability. Understanding the rate of heat transfer between steam and trees is critical to establish a dependable system. In the proposed system, as steam is injected into the canopy, convective

streams of steam and air mixtures are produced; optimization of the system requires characterization of heat transfer to individual tree components. Secondly, to understand the thermal death kinetics of CLas in citrus branches, D-values and the z-value will be established. When microorganisms are heated at a constant temperature, the decrease of viable organisms follows a first order reaction, commonly defined by D-values (the time to inactivate 90% (1-log) of microorganisms). The temperature dependence of D-values are represented by z-values (the temperature change needed to change D-values by a factor of 10). D- and z- values allow for the determination of equivalent rates of microbial kill under varying time and temperature treatment combinations.Objective 6:In this objective, the effect of post-treatment with three HMO aerosols to improve effectiveness of thermotherapy on HLB-affected canopies will be evaluated. The HMO selection, application volume, and concentration (< 2%) will be based on preliminary laboratory studies (year 1). In regards to appropriate application technologies, an ultra-low volume (ULV) applicator will be evaluated to post-treat the enclosed citrus canopies. The ULV-based aerosols with < 50 um droplets are expected to have a thin, even coating of the material on canopies. Preliminary studies on effectiveness of an ULV applicator on coverage and deposition rates in varied parts of canopies will be conducted (year 1). The ULV application treatments, with an oil soluble fluorescent tracer dye, will be conducted to evaluate the deposition with fluorometry and coverage with oil sensitive paper based image analysis.Objective 7:Economic analysis into steam-generated thermotherapy will proceed in four steps: 1) estimate of capital costs; 2) estimate of annual operational costs; 3) assess the long-term impact of thermotherapy on yield and fruit quality; and 4) comparing the net present value (NPV) of thermotherapy with alternative HLB management strategies.

ACCESSION NO: 1005557 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10120 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
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GRANT AMT: $4,613,838 **GRANT YR:** 2015
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INVESTIGATOR: Johnson, EV, G.

PERFORMING INSTITUTION:
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ZINKICIDE A NANOTHERAPEUTIC FOR HLB

NON-TECHNICAL SUMMARY: Huanglongbing (HLB) is an invasive disease of citrus that is devastating the largest citrus industry in the US and threatening the other major citrus producing regions. This disease causes significant crop loss for citrus growers because of increased

premature fruit drop and reduced fruit quality (i.e. small, unpallatable flavor). The bacteria that causes the disease (Candidatus Liberibacter asiaticus) is transmitted by insects (Asian citrus psyllid). Control of bacterial plant pathogens is difficult because of the limited bactericides available and because they only act as a protective film on the outside of the plant. Insect transmission bypasses this protective barrier and insect control alone cannot prevent disease spread.This project aims to develop a specially formulated bactericidal particle that is small enough to enter the plant vascular tissue where the bacteria lives. It is made from plant nutrient and plant derived compounds to develop novel bactericidal activity not found in the raw ingredients. These particles are designed to breakdown into these nutrients after it has performed its bactericidal function. In conjunction with testing how effective this new bactericide is in controling HLB in citrus orchards, the safety and fate of the bactericide particles will be investigated to ensure safe use of the product. The effect of this new bactericide on citrus tree health, fruit production and quality will be determined. Crop improvement data from field trials will be used in economic analyses to determine if it will allow citrus growers impacted by HLB to return to profitable production. Once shown to be safe and effective for control of HLB, it will likely be useful for control of many other bacterial pathogens that significantly limit food production and threaten farmers livelihoods.

OBJECTIVES: The goal of this project is to develop an economical bactericide for control of HLB-affected trees in established citrus orchards, allowing growers to maintain production and profitability in the presence of endemic HLB. We have developed a prototype plant nutrient-based nanoparticle (novel vacancy-engineered (VE) Zinc oxide (ZnO) particle formulation) with unique bactericidal activity that can translocate into plant tissue with the goal of providing an economical HLB management option for infected trees that will allow efficient and profitable citrus production in the presence of HLB. We will pursue this goal with the following research and outreach objectives:Research objectivesObjective 1. Development, improvement, and characterization of Zinkicide nanoparticles.Objective 2. Test the efficacy of new Zinkicide formulations and optimize field application for HLB control while minimizing non-target effects on beneficial organisms.Objective 3. Determine residue lifespan of Zinkicide in planta and toxicology on non-target organisms to ensure safety and expedite product registration for grower use to combat HLB.Objective 4. Evaluate the economic feasibility of using Zinkicide to manage HLB compared to existing methods of citriculture in the presence of HLB.Outreach objectivesObjective 1. Develop interactive media tools including a website and related tools to educate citrus growers about the efficacy, viability, and best use practices of Zinkicide as an HLB management optionObjective 2. Provide training for safe handling and field use of nanoparticlesObjective 3. Provide in-service training on Zinkicide for extension agents from major citrus producing regions across the U.S.

APPROACH: Objective 1. Production of the Zinkicide nanoparticles is done in a unique single pot reaction under conditions that don't require a second purification stage. This process will be optimized for large scale synthesis. To be able to identify nanoparticle half-life and location in plants methods will be developed to detect and quantify the Zinkicide particles instead of just their chemical components using multiple microscopy techniques based on the particles unique emission spectra.Objective 2. Greenhouse and field trials on grapefruit and sweet orange will be performed to determine efficacy and the most effective method and timing of application. Currently used field application methods (i.e. foliar spray and soil drench) will be the methods

tested to maintain economic sustainability of the treatment. Efficacy will be determined based on fruit production and quality. Samples will also be taken from these trees to determine the systemic movement and residue of the particles using the detection techniques under development in objective 1. Using special microfluidic chamber techniques, developed to study vascular bacterial pathogens, the mode of action of Zinkicide against Liberibacter and/or related bacteria will be determined.Objective 3. Residue analysis of trees treated in objective 2 will be done to determine the duration of effective concentrations in the tree. These residue concentrations will also be incorporated into standard toxicity assays for non-target organisms to provide information on the safety of the treatment.Objective 4. To determine the economic sustainability for the target audience (citrus growers) a benefit-cost analysis will be done to inform the growers on the most cost-effective way of using the treatment to get the best yield productivity return with the minimum input cost.The progress of each method will be evaluated at yearly stakeholder advisory committee meetings where progress on each objective will be presented to the entire research group and stakeholder advisors. Based on these results and the advice of the stakeholders, the plan for each objective will be assessed and modified as needed to ensure the most efficient use of resources towards the final goal of developing an effective management strategy for HLB.Feedback will also be acquired from grower outreach and extension events including field days and workshops to assess the value of the knowledge provided to the grower stakeholders

ACCESSION NO: 1005575 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10146 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23029 **PROPOSAL NO:** 2014-10146
START: 01 APR 2015 **TERM:** 31 MAR 2018
GRANT AMT: $2,096,540 **GRANT YR:** 2015
AWARD TOTAL: $2,096,540

INVESTIGATOR: Lorca, G. L.

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A NOVEL ANTIMICROBIAL APPROACH TO COMBAT HUANGLONGBING DISEASE

NON-TECHNICAL SUMMARY: Florida growers have assumed a total infection (symptomatic or asymptomatic) of all producing groves across the state. The strategies used to reduce the effects of HLB have not been effective. Novel strategies directed to eliminate the bacteria from infected trees, and to protect new groves are needed.The goal of this proposal is to assess the efficacy of this antimicrobial therapy in active HLB-infected groves. We have designed an efficient treatment plan that is cost effective and scalable, two aspects that will be

essential for any treatment discovery intended for large-scale application. Because recovery of HLB-infected plants in the greenhouse was rapid, we anticipate that the proposed therapy will deliver timely results in the mature, HLB-infected plants.. We will achieve this goal by (1) Optimizing the antimicrobial treatment in HLB-infected citrus seedlings in small scale field trials; (2) Evaluating the antimicrobial treatment in large-scale field trials. We will assess the economic, ecological, and environmental impact of each treatment; (3) Testing the antimicrobial activity of alternative organic compounds that have chemical scaffolds similar to those of the active compounds. We will have optimized the application of chemicals that are effective CLas antimicrobials.Our research team is working directly with citrus growers and groves in active production. The Stakeholder committee is comprised of large and small-scale growers, who will meet to discuss the progress and results of the project every 6 months. The results will be transferred to a broad audience through different publication media (such as the UF journal EDIS and industry publications) and presentations to grower associations.An effective antimicrobial treatment will have an immediate positive impact on the economic and social aspects of the citrus industry. The citrus industry, directly or indirectly, involves a large network of workers and companies currently operating in the state of Florida. Because the citrus industry represents a large portion of the Florida economy, the recovery of infected groves will have a substantial, positive impact on the economy in Florida as a whole.

OBJECTIVES: The goal of this project is to develop an antimicrobial treatment to cure Huanglongbing disease (HLB) in the field. We have designed a plan to efficiently carry out treatments, thoroughly evaluate results on a laboratory scale, and quickly translate treatment discoveries to groves in production. Upon completion of this project, we will have optimized treatments for field-scale applications of HLB-specific antimicrobials, including the analysis of environmental impacts (if any) associated with each of the proposed treatments. We will achieve the goal of this proposal by completing the following objectives. Objective 1. Optimization of antimicrobial treatment in infected citrus seedlings. Infected citrus seedlings will be used to determine the optimal dosage, dosing intervals, and the elimination rate for each compound, in a controlled environment. Seedlings will also be monitored for symptoms of plant toxicity throughout the treatment period.Objective 2. Field trials: application of the antimicrobial treatment and evaluation of environmental impact. Mature, HLB-infected trees will be treated in actively producing groves. Each compound will be analyzed for effectiveness in bacterial clearance and potential environmental impacts.Objective 3. Identify and evaluate the antimicrobial activity of natural compounds that have chemical scaffolds similar to the two effective compounds we have already identified. The significance is twofold; it may allow a faster translation of chemicals into field trials, and offer a plausible treatment option for organic growers.

APPROACH: Objective 1. Determination and optimization of the antimicrobial treatment in infected citrus seedlings. For the proposed studies, we will use sweet orange (Citrus sinensis) "Valencia" seedlings. To accomplish this objective we will:1.1. Evaluate the toxicity of the selected chemicals on sweet orange seedlings. Different concentrations of each chemical will be tested.1.2. Evaluate the antimicrobial efficacy of the selected chemicals. The evaluation of the antimicrobial efficacy in plants will be performed by following the remission of HLB symptoms as well as the viability and titer of 'Ca. L. asiaticus' in tissue samples collected from new growth.1.3. Determine the stability of each compound within the citrus plant and identify

residues in plant and fruit tissue as well as soil samples following treatment. The distribution, stability, and turnover of each compound will be determined in both plant tissues and soil samples. The data collected will be used to address environmental concerns and ensure compliance with EPA regulations.Objective 2. Field trials: application of the antimicrobial treatment and evaluation of environmental impact. This objective will be divided into two phases:2.1 Small scale field trial. Based on the results of the greenhouse experiments, we will test the most effective treatments on infected trees maintained by the Horticulture Science Department at the University of Florida main campus.2.2. Large-scale field trial. The chemicals found to be the most effective for treatment of HLB in the small scale field trial, will be tested in large orchards. Multiple orchards will be tested during the large-scale field trials with different varieties of HLB-infected citrus trees; sweet orange trees (Citrus sinensis, "Valencia") and grapefruit trees (Citrus paradisi, "Ruby red" or "Flame"). On each of those groves we will perform:2.2.1 Evaluation of antimicrobial efficacy. The antimicrobial efficacy of each compound will be evaluated by following the presence and viability of 'Ca. L. asiaticus', by real time PCR.2.2.2 Evaluation of the impact of each antimicrobial treatment on fruit production and quality. Oranges from each field will be collected before and after treatment, and sent to the Citrus Research and Education Center (CREC) for quality analysis.2.2.3 Determination of the stability of each compound within the citrus plant and identification of any residues in plant or fruit tissue following treatment.2.2.4 Evaluation of the impact of each antimicrobial on the host and its associated microbiota. To this end we will define the effect of each antimicrobial on the citrus microbiome and characterize plant responses to each compound. We will also elucidate the detoxification pathways for each compound.Objective 3. Identification and evaluation of antimicrobial activity of natural compounds that share a similar chemical scaffold to the initial lead compounds. We will perform an in silico identification of natural products that interact with the microbial target proteins and in vitro characterizations of protein/ligand interactions. The identified chemicals will then be evaluated (in vivo) in citrus seedlings as described in objectives 1 and 2.The data obtained on each of the objectives will be exhaustively analyzed using different statistical methods.We will also perform an economic impact analyses. The economic component will include two primary activities. First, a traditional cost-benefit analysis of the compounds will be conducted to determine which compound has the highest net benefit per tree. The second activity will involve the construction of a dynamic bioeconomic model to determine the optimal (profit-maximizing) use of each treatment in the field.

ACCESSION NO: 1005657 **SUBFILE:** CRIS
PROJ NO: FLAW-2014-10119 **AGENCY:** NIFA FLAW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23027 **PROPOSAL NO:** 2014-10119
START: 15 MAR 2015 **TERM:** 14 MAR 2020
GRANT AMT: $3,338,248 **GRANT YR:** 2015
AWARD TOTAL: $3,338,248

INVESTIGATOR: Gmitter, FR, G.

PERFORMING INSTITUTION:
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DETERMINING THE ROLES OF CANDIDATE GENES IN CITRUS-HLB INTERACTIONS AND CREATING HLB-RESISTANT CITRUS CULTIVARS

NON-TECHNICAL SUMMARY: This project addresses the Citrus Production Systems priority of the SCRI/CDRE, specifically the development of HLB tolerant/resistant cultivars with acceptable horticultural and product characteristics. The current crisis in Florida citrus arising from the rapid spread of HLB disease and the subsequent decline in production threatens to spread to the other citrus producing states, as well. The US citrus industry is a multi-billion dollar contributor to the US economy, and its demise will have dire impacts not only on the communities built around this industry, but US citizens in general by removing an affordable and valuable nutrient source (citrus fruit and juice products) from our diets.The project will identify critical genetic factors controlling citrus responses to HLB among sensitive and tolerant/resistant citrus accessions, and define their roles. Through proposed over-expression experiments, and also using genome editing and transformation technologies, altered plants of commercial cultivars will be produced and tested for responses to HLB, ultimately yielding HLB-resistant lines free of transgenes. A multi-state Industry Advisory Panel, representing large processing interests, family farms producing fresh fruit, and industry organizations that interface with research, will provide guidance throughout the project on goals, objectives, and commercialization tactics. Interaction with industry at-large, as well as through an interactive website, will enable two-way communication on progress and new opportunities.The project will provide industry with new citrus cultivars exhibiting good horticultural performance, unaltered fruit or juice quality traits, and HLB-resistance, but free of GMO signatures. Consequently, commercialization should be unfettered by regulatory concerns or consumer resistance. The citrus industry can be secure, and social, environmental, or economic consequences of HLB-associated collapse of the US citrus industries can be avoided. Finally, US citizens will be well served by the continued availability of the affordable and valuable nutritional contributions that citrus fruit and juice products make to their diets and overall well-being and health.

OBJECTIVES: The long-term goal of this project is the development of HLB-tolerant or resistant citrus cultivars, using genes from citrus and its close relative and free of GMO signatures, to support the survival and revival of the US citrus industry, and thus to avoid the economic losses, environmental degradation, and sociological consequences of its potential HLB-induced demise. Such new citrus scion and rootstock cultivars must not only tolerate or resist HLB. They must meet the horticultural performance expectations of growers, and yield fruit and juice products that meet the expectations of packers, processors, and most importantly consumers. Finally, they should be developed using contemporary genetic technologies and approaches in such a way that the cultivars will be free of GMO signatures, thus removing the impediments to their utilization and commercialization associated with regulatory requirements or consumer concerns and reluctance to purchase GMO citrus fruit or juice products. Achieving this goal will support the continued existence and expansion of the US industry, thus avoiding

the calamities described above and ensuring an abundant and inexpensive supply of nutritious citrus fruits and juice for the public. Objectives:1. Validate candidate gene expression in inoculated citrus through RNAseq.2. Identify sequence polymorphisms in candidate genes from citrus accessions with different responses to HLB and dissect the gene structure and genomic organizations of candidate genes.3. Understand the roles of candidate genes by over-expressing them in HLB-susceptible citrus cultivars.4. Develop CRISPR-mediated technologies for development of non-transgenic HLB-resistant citrus.5. Precision editing of candidate genes for producing HLB-resistant citrus.6. Outreach and disseminate project results to stakeholders and the public.

APPROACH: Methods:1. Various HLB-tolerant, -resistant, and -sensitive citrus cultivars and near relatives will be graft inoculated with CLas-containing budwood sources. Leaf samples will be harvested at inoculation time and bi-weekly thereafter. RNA will be extracted using commercially available extraction kits, rRNA removed using RiboZero or equivalent, and libraries prepared and samples barcoded to enable pooling prior to Illumina HiSeq runs, to validate candidate gene expression via RNA seq approaches.2. For each candidate gene, the transcribed region and 2-kb upstream and downstream of the transcript will be extracted from the rough lemon and Poncirus genome sequence and used as the input template for capture probe design. Illumina sequencing libraries will be prepared and enriched for candidate genes using the above-described SureSelect system. The citrus genotypes used will include most of the citrus species, cultivars, or citrus relatives that had been evaluated previously for HLB responses and several dozens of additional citrus genotypes that are being evaluated. Enriched libraries will be sequenced on the HiSeq 2000. Sequence reads will be aligned to the citrus genome sequences. Sequence contigs for candidate genes will be aligned with mRNA sequences from Objective 1 to understand gene structures and aligned with the available citrus genome sequences to understand the candidate genes' genome-wide organization and evolution.3. Candidate genes will be cloned into transformation vectors, which will be used to transform and regenerate sweet orange and grapefruit plants. These plants will be identified, and confirmed, then characterized for gene integration and expression. Once confirmed, plant will be inoculated with CLas and their relative sensitivity or tolerance of HLB disease will be determined by comparing growth, symptom severity, and CLas titer between transformed and control plants. Finally, those plants with apparently better resistance or tolerance will be entered inti field trials.4. CRISPR cassettes will be designed and employed for citrus transformation. Resulting modified plants will be characterized, as above, for their resistance or tolerance to HLB disease.5. Target genes and sites for editing will be determined, based on the criteria of being highly expressed in HLB-susceptible citrus genotypes but little expressed or down-regulated in HLB-resistant/tolerant Poncirus and rough lemon. gRNAs will be designed based on the genomic sequences from Objective 2. Agrobacterium transformation will be used to deliver CRISPR cassettes into orange and grapefruit, and plants regenerated. Induced genome sequence changes will be validated, gene expression levels will be determined, and plant responses to HLB disease will be characterized as above, in greenhouse and field environments.Efforts:Throughout the course of the project, information generated will be submitted for scientific publication. Further, progress in achieving the goals established will be reported through our website, as well as at conferences, scientific meetings, and citrus industry forums.Evaluation:Progress will be assessed by comparing the timelines established for each individual objective and outcomes as they are realized. We will publicize the CRISPR technologies we develop through scientific publications, oral and poster

presentations at scientific conferences/meetings/seminars and a website to be developed under this project. Upon request, we will also provide appropriate CRISPR gene cassettes for research use (with the authorization of a standard material transfer agreement) and for new citrus cultivar development (with a fully executed licensing agreement). The impacts of our technologies can be measured in several ways. 1) Short-term: The number of requests received for the CRISPR cassettes; 2) Medium-term: The number of applications of our CRISPR technologies as documented in scientific publications, and 3) Long-term: The number of new cultivars developed using our CRISPR technologies. The overall impact of the project will be assessed by conducting written, short-answer surveys at the beginning and at the end of this project to document industry responses to the development and use of citrus cultivars with genetically engineered HLB resistance and non-GMO HLB resistance. Changes in knowledge gain, perception, and behavior will be extracted by comparing the survey results between Year 1 and Year 5.

ACCESSION NO: 1005600 **SUBFILE:** CRIS
PROJ NO: KS603372 **AGENCY:** NIFA KAN
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2015-70016-23028 **PROPOSAL NO:** 2014-10154
START: 01 MAR 2015 **TERM:** 28 FEB 2017
GRANT AMT: $3,734,480 **GRANT YR:** 2015
AWARD TOTAL: $3,734,480

INVESTIGATOR: Brown, S. J.

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DEVELOPING AN INFRASTRUCTURE AND PRODUCT TEST PIPELINE TO DELIVER NOVEL THERAPIES FOR CITRUS GREENING DISEASE

NON-TECHNICAL SUMMARY: U.S. citrus growers have a critical need for grove-deployable management practices that keep healthy citrus from becoming infected and infected trees from becoming symptomatic. We present a systems-based pipeline approach delivering commercial, grove-deployable solutions using a novel therapeutic delivery strategy and citrus transgenics. A data integration and analysis platform combining existing complex -omics/biological data with molecular/cellular research will steer hypothesis-driven testing of inhibitors of multiple molecular pathways to provide solutions that can be optimized by combinatorial delivery to citrus. Research areas will include: 1) molecular interaction inhibitor discovery (gut membrane binding peptides, RNA aptamers and non-toxic chemical library screening) to block psyllid acquisition/ transmission of HLB and/or growth in the plant; 2) dsRNA delivery to induce psyllid RNAi responses that block HLB transmission or kill the psyllid (or both). The proposed delivery system has negligible environmental impact, is

economical in comparison to current control strategies and is highly tractable, allowing it to function as a delivery vehicle for different solution strategies. Co-delivery with bactericides (previously shown to be effective against HLB through laborious injection methods) will be evaluated as complementary methods of control. To translate these therapeutic treatments into long-term solutions, transgenic research will be initiated to produce interdiction molecules (peptides and dsRNA) expressed in the phloem. By engaging stakeholders in design and testing stages, and educating the public, we will deliver acceptable solutions that are applicable to citrus greening and extensible to a wide variety of related economically important pathogens of citrus and other specialty crops.

OBJECTIVES: Research:Improved understanding of HLB transmission and interactions in psyllid and plant phloem.Grove deployable solutions and suite of effective management practices identified to keep current citrus production, maintain high fruit quality, and prevent fruit drop.Extension/OutreachIncreased growers/public understanding of biotechnology methods of disease management.Researchers understand consumer attitudes towards biotechnologies.Increased awareness of novel therapeutic application technologies and products by industry segments.Industry understands economic feasibility and practicality of management recommendations.Students increase knowledge of bioinformatics, genome analysis, database construction: New data visualization tools.Understanding of industry/ consumer knowledge about HLB and its impact on U.S. citrus production.

APPROACH: Objective 1: Data integration / AnalysisCombine -omics data into single platform.Manually curate target gene sets in psyllid.Model biochemical pathways.Create visual digital library to access organ or organ system data.Objective 2:HLB Science to SolutionPerform PPI and proteomic studies on gut proteins.Mine interactome and screen dsRNA, RNA aptamer and nontoxic small molecule libraries for blocker molecules.Test candidate molecules in soil application, RNAi and transgenic plant.Test grove-deployable strategies.Conduct metabolomics analysis of treatment efficacy.Evaluate delivery strategies in greenhouse experiments.Objective 3 Engagement, Extension, EducationUtilize industry-relevant Extension and Engagement Methods to introduce emerging agricultural strategies to the citrus industryInvestigate consumer attitudes toward and acceptance of HLB reduction and prevention technologies to inform consumer engagement and outreach