2017 (Cycle 3) USDA, NIFA, SCRI CITRUS DISEASE RESEARCH PROGRAM AWARDS
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INVESTIGATOR: Roper, C.; Borneman, JA.; Jassby, DA.; Rolshausen, PH.; Vidalakis, GE.; Maloney, KA, N.; Liu, HA.

PERFORMING INSTITUTION:
UNIVERSITY OF CALIFORNIA, RIVERSIDE
RIVERSIDE, CALIFORNIA 92521

DEPLOYMENT OF A SPECTRUM OF BACTERICIDES TO CURE AND PROPHYLACTICALLY TREAT CITRUS HUANGLONGBING

NON-TECHNICAL SUMMARY: Our research objectives are to design and identify bactericides that can cure/suppress or prophylactically treat Huanglongbing (HLB), and to target these bactericides to the phloem where the associated bacterium, Candidatus Liberibacter asiaticus (CLas), resides. We will develop two classes of bactericides, the first based on silver and sulfur nanoparticles, the second rooted in natural product discovery, mining anti-CLas compounds produced by microbes that inhabit HLB survivor trees in Florida. The difficulty with any anti-HLB formulation is optimizing its delivery to the phloem. In all cases, delivery of the bactericides to, and within, the phloem sieve tubes to kill CLas will be of paramount importance. Thus, we will perform detailed analyses of the phloem transit routes that a given bactericide takes when introduced through common application methods (trunk injection, foliar application or root applications). We will also continue to develop a promising, new petiole/branch delivery system for use in field trees. We will explore the chemistry of bactericides to optimize uptake by the sieve tubes. Because significant amounts of phloem plugging occurs in CLas-infected trees, we will evaluate bactericide transit pathways at the whole-plant level at varying stages of infection. This work on HLB-phloem transit routes will provide important information for us and for others in the HLB research community who are evaluating delivery of materials to the phloem. We will integrate this research with a robust extension and outreach program that will be coupled with an economic cost-benefit analysis structured around adoption of these treatments into commercial citriculture.

OBJECTIVES: The goals of this proposal are to facilitate phloem uptake and transport of bactericides that target the bacterium associated with citrus Huanglongbing disease, Candidatus

Liberibacter asiaticus.To accomplish this, we will obtain and use knowledge of citrus phloem architecture/transport and source/sink pathways in connection with developing nanoparticle and microbial natural product based bactericides. This project addresses priority number one outlined in the Request for Proposals: Development of therapies to kill or suppress Candidatus Liberibacter asiaticus (CLas) within trees or prevent CLas infection of healthy trees. In the larger scope it lies within the system components (page 5 of RFP): production- crop management; reduce environmental footprint; and food security and overlaps with consumers and markets: vitality of rural communities; impacts on urban systems.A major challenge in developing an effective anti-HLB formulation is optimizing its mobility and delivery to the phloem tissue where the HLB-associated bacterium, CLas, resides. Efficient transport of bactericides to specific, appropriate locations within the tree, especially the roots, is a crucial aspect of a functional anti-CLas delivery system. However, there is little detailed knowledge available regarding the fate of phloem mobile materials once they are introduced into trees using common delivery methods that are being used, such as trunk injection, soil or foliar applications. Although, one very recent (August, 2016) manuscript investigated retention rates of oxytetracycline in citrus trees following trunk injection. We will conduct florescent tracer experiments that map phloem transport pathways when materials are introduced through these application methods. This will yield important information about the routes bactericides travel when administered through delivery methods currently being utilized by growers and other funded SCRI CDRE projects (foliar or soil applications and trunk injections) as well as a novel petiole/branch feeding technique developed in this project.There is presently no cure for HLB or any suitable genetic sources of resistance to HLB. Management of HLB and its psyllid vector is difficult and expensive. Inspections, testing, tree and inoculum removal, biological and chemical psyllid control all contribute to the cost of production. The socio-economic effects of HLB are linked to the current unsustainable management strategies, such as constant insecticide sprays. This is economically unsustainable and harmful to human health and the environment. This project will contribute to the long-term profitability and sustainability of citrus production by providing novel, effective and sustainable strategies to manage Huanglongbing (HLB). To disseminate the fundamental discoveries and deliverable solutions created by this project, we will fully integrate the research with a robust array of extension and outreach components. Accordingly, our objectives are:

Objective 1: Optimizing formulations of chemical bactericides to facilitate phloem mobility
Objective 2: Harnessing the power of bactericides produced by citrus-inhabiting microbes
Objective 3: Testing bactericides for phloem mobility and as HLB therapies
Objective 4: Integration of Research and Extension

APPROACH: Objective 1: Optimizing formulations of chemical bactericides to facilitate phloem mobility. Initially, we will map citrus phloem transport patterns in detail using fluorescent tracer carboxyfluorescein (CF) and epiflourescent microscopy. Phloem transit will be assessed in healthy and CLas infected greenhouse trees following several tree delivery techniques. The first is a (i) novel petiole/branch feeding technique that we are developing in this proposal for use in citrus; (ii) direction injection (Arborjet); (iii) continual release injection; (iv) a soil drench and (v) a foliar spray application.Silver (Ag) NP synthesis: We will synthesize a range of AgNPs with different sizes using developed methods. In the first method, AgNPs are produced using a "green" synthesis methodology whereby Ag(NH3)2+ ions are reduced to Ag0

and subsequently form AgNPs by reacting the ions with a carbohydrate in a high pH environment. In the second method, silver acetate is dissolved in an organic solvent and refluxed with oleylamine. Regardless of the synthesis method, the AgNPs can be functionalized (made hydrophilic and, thus, more phloem mobile) by mixing them with a thiol-containing molecule. Sulfur (S) NP synthesis: Synthesis of SNPs will be based on the method developed by Chen et al. (2013). Copper (Cu) and Zinc (Zn) NPs: Several versions of CuNPs and ZnNPs are being developed for use against the bacterium Xanthomonas axonopodis pv. citri and CLas by other research groups. Thus, we will use CuNPs and ZnNPs as an important comparison in our studies. In vitro inhibition assays using NPs. L. crescens BT-1 is a culturable close relative of CLas and is identified as a suitable surrogate for the uncultivable CLas. We will use this system to establish the minimal inhbitory concentrations (MIC) and minimal lethal concentrations (MLC) for each nanoformulation.

Objective 2: Harnessing the power of bactericides produced by citrus-inhabiting microbes. Citrus samples will be collected in at least 5 FL groves. Tissues will be lyophilized prior to shipping to UCR. Similar samples from healthy trees (grown under ACP exclusion structures) will be used as negative controls. MS spectra from these controls will be used to subtract background plant metabolites and pull forward meaningful differences from the experimental sample set. To partner community level metabolic activity with microbes, it is necessary to conduct community level microbial analyses based on rDNA (bacterial) and ITS (fungal) sequencing. rDNA Libraries will be run on an Illumina MiSeq to obtain 300bp reads. Low quality and chimeric sequences will be removed using Quantitative Insights Into Microbial Ecology (QIIME). We will identify target compounds along with the microbes that map to the locations of those target compounds. Purifying the natural products in quantities large enough for testing in vitro and in vivo will require obtaining pure cultures of the citrus-associated microbes. Inhibition bioassays. The L. crescens inhibition assay will be used to guide isolation ofthe active compound from the crude extract using flash column chromatography and high-performance liquid chromatography.

Objective 3: Testing bactericides for phloem mobility and as HLB therapies. Ag, S or Cu NPs will be delivered to both healthy and diseased greenhouse trees using the delivery techniques that achieved the best systemic phloem penetration in the CF experiments in Obj. 1A. NPs will be applied to trees at a single high dose above the MLC determined in Objs. 1B. Trees that receive NP carrier buffer only will serve as the negative controls for the experiment. Application methods will include those described in Obj 1. All experiments will be repeated 3 times. We will determine the fate and distribution of NPs at the plant compartment level (roots, budwood and leaf petioles). The bark peels and the xylem sap will be weighed and then incinerated in a furnace at 550° C in air, filtered and injected into an ICP-MS for metal analysis. To obtain higher resolution on the distribution of the Ag, S or Cu between the phloem and the xylem, we will use scanning electron microscopy (SEM) coupled to energy-dispersive X-ray spectroscopy (EDX) to map the NPs inside the root, budwood or leaf tissue. The natural products that were inhibitory in our L. crescens inhibition assay in Obj. 2 will be assessed for their phloem mobility in greenhouse trees in FL using the same experimental design described for the NPs. Natural product presence in the vasculature will be monitored in budwood bark peels and xylem sap and whole root and leaf samples using MS.We will begin assessing efficacy of NPs and natural

products against CLas in greenhouse grown citrus trees. Curative treatments will be performed on infected trees obtained as described in Obj. 1 and will be delivered to trees using the delivery methods and concentrations that performed best in terms of delivery to the phloem. We will two criteria to assess bactericide efficacy over the course of 12 months: HLB disease severity and CLas titer. Disease severity will be assessed on a greenhouse tree scale of 0-3, 0: no symptoms/growth, 1: mild, 2: moderate, & 3: severe chlorosis/growth inhibition/leaf deformity. CLas titer will be determined using a qPCR method. Field trials will begin in year 3, focusing on the application method and formulations that performed well in the greenhouse studies. Field blocks will be setup as follows: Test blocks of 12 trees will be setup using a randomized block design. Blocks will consist of 12 trees/block, 6 of the trees will be graft-inoculated with CLas (in year 2) and the other 6 will not (uninfected). Three of the graft -inoculated trees will receive the bactericide being evaluated, 3 will not. Three of the uninfected trees will receive the bactericide being evaluated, 3 will not. The blocks will be replicated 5 times. Two different application methods (determined from Objs. 1 and 3) will be tested. The 3 year-old trees would receive bactericide applications beginning in year 3 and evaluated over a 2-year period with repeated applications of bactericide. This experimental design will allow for simultaneous evaluation of curative and prophylactic effects of each bactericide under evaluation. Trees will be separated into 4 quadrants and each quadrant will be rated on a 0-5 scale indicative of how many limbs are showing symptoms (0=no limbs-5= all limbs).Objective 4: Integration of Research and Extension. We will develop a robust extension and outreach plan with the following end-users as our target audiences. For extension activities, our target end-users are county extension agents, grower stakeholders and academic/industry researchers. For outreach activities, our target end-users are non-commercial citrus growers and undergraduate students in agricultural sciences. Economic analysis: We will perform empirical analyses of the costs and benefits from adoption of identified bactericides that will cure or prophylactically treat CLas infection so we may best inform growers about the potential gains from these practices, reduce uncertainty about them and, when cost effective, increase adoption, contributing to long-term profitability and sustainability. The perennial nature of citrus orchards, the incidence of HLB and presence of ACP, and their relative life expectancy suggest decisions regarding planting and care can have effects that linger long into the future. In these situations a dynamic economic model is appropriate as it captures these temporal interactions. The model simulates investment decisions under alternative control practices, accounting for differences across growing regions. The simulated results on costs and benefits under current cultural practices and alternative control practice scenarios can be compared to estimate the costs and benefits to citrus growers from adoption not just today, but into the future.

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PROJ NO: GEOW-2016-10983 **AGENCY:** NIFA GEOW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2017-70016-26142 **PROPOSAL NO:** 2016-10983
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GRANT AMT: $1,821,197 **GRANT YR:** 2017
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INITIAL AWARD YEAR: 2017

INVESTIGATOR: Duan, Y.

PERFORMING INSTITUTION:
ARS-USDA
950 COLLEGE STATION ROAD
ATHENS, GEORGIA 30605

IDENTIFICATION, ASSESSMENT AND DELIVERY OF ANTIMICROBIAL COMPOUNDS FOR THE MANAGEMENT OF CITRUS HLB

NON-TECHNICAL SUMMARY: The devastating effects of the bacterial disease known as huanglongbing or HLB have severely crippled the citrus industry in the US by both increasing the production costs and dramatically decreasing revenue. Florida citrus production dropped from over 240 million boxes in 2003/04 to below 100 million boxes in 2014/15 (USDA-NASS) while per-acre spray cost increased fourfold during that period. Not only are 80% of trees in citrus operations infected by HLB in Florida but HLB is impacting citrus production in other states as well. The development and full deployment of any cost-effective strategies that can control the Las bacterium or mitigate the effects of HLB in the United States are vital to the survival of the citrus industry. This makes the topic of chemotherapy an important one amongst shareholders in the citrus industry because unlike other plant bacterial diseases, no resistant citrus cultivars are currently available for the control of HLB.The project presented here will identify and assess the most cost-effective treatment for HLB control by 1) deploying an inexpensive and accelerated screening procedure that greatly enhances our ability to find novel, active compounds through the incorporation of chemical libraries often involved with medicinal drug discovery; 2) evaluating pre-selected effective chemicals to determine the optimum application times and effective dosages for a field setting; 3) determining if the fruit quality and quantity in treated HLB-affected trees can recover to healthy unaffected tree levels; and 4) assessing the practicality of the treatment options from the growers perspective by providing an economic analysis of chemotherapy methods; all while 5) continuously communicating project advancements with the stakeholders to facilitate the acceptance and integration of new chemistries into growers' management programs. Upon completion of the objects presented, we will have ascertained the most successful antimicrobial for the control of HLB based on the ability of the chemical to 1) eliminate/suppress Las and restore an infected tree to a healthy, productive state;2) be excluded from the fruit and juice upon harvest; and 3) be economically viable in regards to large-scale field application. In addition, we will also have identified several new potential antimicrobials warranting further investigations in field trials. Overall, this will

allow us to reach our ultimate goal of providing actionable recommendations to the growers regarding advancements made in chemotherapy by providing the critical research necessary before products can be made commercially available for the treatment of HLB.

OBJECTIVES: Our overarching goal is to revitalize the citrus industry across the United States by finding a sustainable solution that eliminates the spread of huanglongbing and restores HLB-affected trees back to a healthy, productive state through the discovery and application of antimicrobial compounds that either kill or suppress Candidatus Liberibacter asiaticus (Las). The objectives listed below aim to define chemotherapy treatment methods that will reduce Las titer in areas such as Florida, where the disease is endemic, as well as protect new plantings in groves not yet infected in Florida, Texas and California.

Objectives:

1) Perform a high throughput screening of small molecules effective against HLB via our newly developed pipeline.
2) Determine the optimum application times and effective dosages of select antimicrobial through field trials.
3) Evaluate the fruit quality and quantity post treatment and perform a chemical residue analysis.
4) Evaluate the economic viability and performance of proposed treatments.
5) Inform stakeholders of the project's results and provide actionable recommendations for HLB therapy with cost effective protocols.

APPROACH: High throughput screening of small molecules. Two small molecule libraries, one from the Torrey Pines Institute for Molecular Studies (TPIMS) and the other from Chembridge Research Laboratories, will be screened for chemicals effective against Las. In order to facilitate the screening of these large chemical libraries (totaling over 30 million compounds) on HLB-affected plants and overcome the time/space limitations of previous approaches, we have developed a high throughput screening method that utilizes a two-prong approach for assessing the chemicals. For the first part of this approach, detached periwinkle leaves from infected plants (a HLB model system) are placed in 96-well blocks containing the chemicals to be tested for a total of 14 days. A sample consisting of ½ of the leaf tissue is taken prior to being immersed in the chemicals and the remaining ½ is taken at the end of the immersion period in order to test for the Las titers using real-time PCR. Each chemical treatment is performed in triplicate in order to help eliminate any false positives/negatives associated with sampling. The effectiveness of each chemical can then be deduced from the change in level of Las at these two time points, with an increased Ct value indicating a lower level of bacteria in the sample. The second part of the system involves the testing of the chemical on the growth of Liberibacter crescens, the closest cultivable relative to Las currently known. Optical density measurements taken at the initiation of the experiment and 10 days post-chemical exposure will be usedto determine the level of growth inhibition for each chemical assayed. The Chembridge library was screened using Xylella fastidiosa, as a surrogate bacterium to conduct a functional analysis of the key virulence genes in Las. This screen yielded three compounds that affected the pathogenicity of the Las complimented pilG mutant. These three compounds, along with any chemicals identified from the TPIMS library mentioned above will be evaluated in the

greenhouse for efficacy against Las and phytotoxicity using the traditional graft analysis previously published. Field analysis of a chemicals effectivenessIn addition, a field analysis of Validoxyamine A, Aliette 80WG, and Carvacrol will be conducted using a randomized block design to investigate the effects of chemotherapy on HLB infected citrus trees. Trials will be conducted on two citrus cultivars (grapefruit and sweet orange) at locations in both Florida and Texas with Las titers being measured at set time points along the treatment schedule using real-time PCR. Treatments will then be compared via statistical analysis to determine their effectiveness. The recovery of the tree's health will also be assessed using tree appearance, trunk diameter, and canopy volume/density.Juice and fruit analysisBecause antimicrobial compounds may have unwanted side effects for the consumers, their presence in the fruits and juices will also be analyzed using a macro matrix solid-phase dispersion method to extract the antimicrobial chemicals from the citrus juice samples in conjunction with gas chromatography-mass selective detection to define the chemicals and quantify the residues. The flavor and nutritional quality of the fruits and juices will also be analyzed using an electronic tongue and high performance liquid chromatography along with HPLC-mass spectrometry, respectively. Economic analysisThe economic viability and performance of proposed treatments are the ultimate factors determining whether the treatment will be adopted by growers so we will assess the costs and benefits of the different control methods at the grower level as well as the economic impact of the optimal control strategies at the industry level, accounting for both increased yield and enhanced quality of the fruits.

ACCESSION NO: 1011716 **SUBFILE:** CRIS
PROJ NO: IOW05497 **AGENCY:** NIFA IOW
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** TERMINATED
CONTRACT/GRANT/AGREEMENT NO: 2017-70016-26050 **PROPOSAL NO:** 2016-10971
START: 15 JAN 2017 **TERM:** 14 JAN 2020
GRANT AMT: $2,476,099 **GRANT YR:** 2017
AWARD TOTAL: $2,476,099
INITIAL AWARD YEAR: 2017

INVESTIGATOR: Bonning, B. C.

PERFORMING INSTITUTION:
IOWA STATE UNIVERSITY
2229 Lincoln Way
AMES, IOWA 50011

BT TOXIN-BASED STRATEGIES FOR MANAGEMENT OF DIAPHORINA CITRI AND CITRUS GREENING

NON-TECHNICAL SUMMARY: When an insect, the Asian citrus psyllid (ACP) feeds on the sap of a citrus tree, it can transmit a pathogen to the tree that causes citrus greening or Huanglongbing (HLB). As a result of this disease, citrus yields in Florida have dropped by 50% since 2008, and citrus in Texas and California is now threatened. Effective means of limiting the spread of ACP is the first line of defense against the spread of HLB into new areas in Texas and California and into new plantings in Florida. To manage HLB, citrus growers have repeatedly applied chemical insecticides to control ACP. Unfortunately, these pesticides preclude the use of biological control methods that use beneficial insects (natural enemies) to manage ACP populations and the elimination of insect natural enemies has led to an increase in populations of insects not previously known to be pests on citrus. It is clear that a more sustainable, less costly, and more specific control method is urgently needed to control ACP.Toxins from a soil-dwelling bacterium called Bacillus thuringiensis (Bt) have been used successfully to control some insect pests. On ingestion, the Bt toxin damages the gut tissue and kills the insect. Bt toxins are specific and provide a sustainable approach that can be used in combination with the best insecticide programs or with biological control agents. Having identified Bt toxins that have toxicity against ACP and having developed a method to further improve toxicity against such sap-sucking insects, the goal of this project is to take the next step toward practical use by developing multiple delivery systems for the ACP-active toxins. To be ingested when ACP feeds on the citrus tree, the toxins must be in the plant sap. The systems to deliver the Bt toxins to the sap include microorganisms (bacteria, plant virus) that reside within citrus trees, and modification of the citrus plant itself to produce the toxin. The economic impact of these Bt toxin-based strategies for ACP control will be evaluated. Information about these new management approaches will be delivered through multiple sources to growers and to the public, and feedback solicited to facilitate adoption of Bt-based ACP control tools by citrus growers.

OBJECTIVES: The U.S. citrus industry has an annual economic impact of more than $11 billion. Fruit production levels have been significantly reduced in Florida however due to the effects of citrus greening or huanglongbing (HLB). The causative agent of this disease is a bacterium, Candidatus Liberibacter asiaticus (CLas) that is vectored by the Asian citrus psyllid (ACP), Diaphorina citri. The damage caused by HLB is threatening commodity price stability and affordability of citrus products and increasing the economic and environmental costs for insecticide-based management programs. New, efficient, and long-lasting tools are urgently needed to control the insect vector, D. citri, toward mitigation of this disease. Preventing D. citri transmission of the bacterium CLas is key to curtailing the spread of citrus greening. Based on our recent critical breakthrough that makes Bacillus thuringiensis (Bt) toxins effective against phloem-feeding insects our long-term goal is to enable an environmentally benign, Bt toxin-based approach for citrus growers to control ACP that works within an integrated pest management (IPM) strategy and minimizes psyllid resistance. To accomplish this, the specific goals of the proposed project are to: 1) further create ACP-active Bt toxins that suppress psyllid populations and thereby curtail transmission of HLB (resulting in increased effectiveness and reduced likelihood of resistance), and 2) develop delivery approaches suitable for each of three citrus-growing states (Florida, Texas, and California). These first two goals align with the practical needs of growers in developing appropriate toxin delivery systems for use in IPM. We will examine and test four distinct approaches for efficient Bt toxin delivery. Three delivery methods used with non-transgenic citrus are the CTV vector, phloem-inhabiting bacteria, and a transgenic trap plant. The fourth delivery approach will use transgenic citrus plants. Further goals are to: 3) evaluate the economic impact of the Bt toxin-based delivery strategies, and 4) deliver information to stakeholders throughout the course of the project. Grower attitudes toward adoption of these strategies will be positive due to targeted and sound insecticide application schedules that rely on an integrated approach and allow for other control measures, such as biological methods. The result will be a reduction in the environmental consequences of intense insecticide applications and direct economic benefits to growers by reducing the number of required annual sprays for ACP management and slowing the spread of HLB.

The objectives are to:

1. Identify and optimize additional ACP-active Bt toxins. We hypothesize that we can isolate and optimize wild-type Bt toxins with even greater toxicity against ACP than those identified to date. The use of multiple toxins will reduce the likelihood of psyllid resistance;
2. Evaluate toxin delivery strategies (CTV, phloem-inhabiting bacteria, and trap plants with non-transgenic citrus; and Bt toxin delivery via transgenic citrus plants). We will develop delivery approaches suitable for the three primary citrus-growing states by evaluating Citrus tristeza virus (CTV) vectors, phloem-inhabiting bacteria, transgenic trap plants, and transgenic citrus for their efficacy of Bt toxin delivery against D. citri. We hypothesize that different toxin delivery systems will be appropriate in different states.
3. Evaluate the economic impact of Bt toxin-based strategies using mathematical modeling and economic analyses to assess the impact of different delivery systems in different states. We hypothesize that Bt toxin use in ACP management programs will result in economic benefit to citrus growers.

4. Deliver information to stakeholders and seek input through outreach activities. We hypothesize that needs-based outreach activities via multiple channels will facilitate the adoption by growers of Bt-based technologies for suppression of D. citri.

APPROACH: Objective 1: Identify and optimize additional ACP-active Bt toxins. Isolation of D. citri active toxins Up to an additional 60 Bt strains will be screened in bioassays with D. citri for toxicity. Amplified strains will be checked for stability, crystal proteins purified and proteolytic profiles characterized. ACP toxicity assays will be conducted by membrane feeding assay using established procedures. The psyllids will be examined daily to monitor survival over a 7-day period. Five replicate bioassays with five ACP per treatment will be conducted. Relative toxicity of toxin combinations from individual strains will be estimated by comparing mortality rates of ACP exposed to Bt to mortality rates of ACP feeding on control diet, using one-way ANOVA, Tukey's test, and logistic regression analysis (Proc Logistic; SAS version 9.4; SAS Institute, Cary, NC). Purify individual toxins from Bt strains with toxicity to psyllids and test individual toxins for ACP toxicity. Purification of individual toxins is a multistep process involving solubilization of toxins and gel filtration column purification. Resulting fractions will be analyzed on SDS-PAGE gel, and those containing a single toxin will be pooled. Purified protein will be stored at -20°C. The median lethal concentration (LC50) values for purified individual Bt toxins will be determined by conducting bioassays as described above. Purified toxins with ACP toxicity will be separated by SDS-PAGE and stained with Coomassie Blue. Stained toxin bands from the gel will be cut out used for toxin identification by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Coding sequences for specific toxins will be identified by analysis of the genome of the relevant bacterial strain with reference to peptide mass fingerprinting results.Modify toxins identified with ACP gut-binding peptide for enhanced efficacy. We will add an ACP gut-binding peptide to selected toxins to further increase efficacy against ACP. Selected toxins will be modified with ACP gut-binding peptide 15 previously isolated in the Bonning lab. Modified toxin constructs will be expressed and purified for testing in ACP bioassays.

Objective 2: Develop and evaluate toxin delivery strategies. Having identified two wild-type Bt toxins with ACP activity, we will test two delivery options for efficient delivery of toxins to ACP (CTV, transgenic citrus) and develop delivery systems based on phloem-inhabiting bacteria and trap plantsCTV vector CTV constructs containing different Bt toxin derivatives will be introduced into citrus cultivars, and their impact on ACP survival, reproduction, and ability to transmit CLas assessed using techniques established in the Dawson lab. We will test the hypothesis that CTV delivery of an ACP-active toxin will efficiently suppress ACP populations on non-transgenic citrus. We will determine the positions from which to express the Bt toxins for both optimal activity against psyllids and optimal stability of the vector. We will utilize the CTV vector to quickly assess the efficacy of different Bt-toxin constructs and the value of the vector as a means to rapidly deploy effective Bt-toxin constructs in the field.Phloem-inhabiting bacteria We propose to deliver ACP-active Bt toxin to citrus trees via phloem-inhabiting bacteria. We will 1) conduct proof-of-concept experiments using a bacterial pathogen of citrus (Spiroplasma citri) for Bt toxin delivery, and 2) develop delivery systems using non-pathogenic bacteria isolated from phloem-enriched citrus tissues.Transgenic Indian curry leaf plant The Indian curry leaf plant (Murraya koenigii) is an alternative host for ACP. Transgenic M. koenigii plants

expressing the Bt toxin will provide an additional tool for management of ACP on the basis that trap plants can be of considerable value for IPM. We will follow published reports of Indian M. koenigii propagation for production of transgenic plants, but using plants derived the U.S.Transgenic citrus plant We propose to use Agrobacterium tumefaciens-mediated transformation of juvenile stem explants for production of transgenic Duncan grapefruit expressing Bt toxins using well-established procedures.

Objective 3: Evaluate the economic impact of Bt toxin-based strategiesThe economic analysis of Bt toxin-based psyllid control strategies will build on current modeling efforts and will involve three steps: 1) a farm-level analysis of how each effective Bt toxin and delivery method affects the costs to produce citrus for the grower, 2) an area-wide analysis of how each effective Bt toxin and delivery method affects the rate of spread of HLB and regional methods to manage HLB, and 3) a market analysis of how changes in citrus production and costs affect final market prices and quantities. From these data, the net benefits to consumers and producers can be estimated.

Objective 4: Deliver information to stakeholders and seek input through outreach activities. Our outreach efforts will focus on dissemination of project information through meetings with multi-state audiences, statewide biannual meetings and field days, and small informal and more frequent visits to key stakeholders and industry decision-makers identified in our social network analysis. We will develop web pages to disseminate new information on our existing websites. Building on our success in educational materials development for HLB, we will provide printed materials, laminated cards, DVDs, and video presentations at each meeting and on request. We will organize at least two annual meetings with our respective grower organizations or present our results at regularly scheduled annual grower meetings to receive feedback on our progress and advice on how to proceed.

PROGRESS: 2017/01 TO 2020/01
Target Audience: Nothing Reported Changes/Problems: January 2017 -- Grant transferred to Florida. What opportunities for training and professional development has the project provided? Nothing Reported How have the results been disseminated to communities of interest? Nothing Reported What do you plan to do during the next reporting period to accomplish the goals? Nothing Reported

IMPACT: 2017/01 TO 2020/01
What was accomplished under these goals? January 2017 -- Nothing to report -- Grant transferred to Florida.

PUBLICATIONS (not previously reported): 2017/01 TO 2020/01
No publications reported this period.

ACCESSION NO: 1011695 **SUBFILE:** CRIS
PROJ NO: SC.W-2016-10974 **AGENCY:** NIFA SC.W
PROJ TYPE: OTHER GRANTS **PROJ STATUS:** NEW
CONTRACT/GRANT/AGREEMENT NO: 2017-70016-26051 **PROPOSAL NO:** 2016-10974
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INVESTIGATOR: Luo, F.

PERFORMING INSTITUTION:
CLEMSON UNIVERSITY
CLEMSON, SOUTH CAROLINA 29634

SELECTION, MOLECULAR AND GENETIC ANALYSIS OF HLB TOLERANT/RESISTANT VARIANT CITRUS PLANTS

NON-TECHNICAL SUMMARY: Huanglongbing (HLB) is presently the most devastating citrus disease worldwide. Because of the extensive spread and a lack of effective control measures for HLB, Florida's $9 billion citrus industry has experienced a decline in production of nearly 75%. As a result, the industry is presently fighting for its survival. The other major citrus states are at high risk for a similar disastrous situation if suitable remedies are not forthcoming soon. Neither a simple cure nor successful management strategies have been identified even after 10+years of the disease presence in Florida. Previous efforts to create new HLB tolerant/resistant cultivars focused on conventional breeding or development of transgenic plants. Unfortunately, the approaches used in those projects are inadequate to meet the current crisis because of the lack of sources of resistance in citrus and time constraints associated with citrus breeding. Furthermore, genetic engineering presents many regulatory and public acceptance obstacles for transgenic citrus. On the other hand, more than 10 years of HLB and tens of millions of infected trees in groves across Florida provide an unprecedented opportunity and high probability to select resistant natural mutants. Field resistance or tolerance to HLB has already been noted amongst the citrus cultivars and their relatives. This provides circumstantial evidence that natural variation exists regarding HLB tolerance/resistance within the citrus gene pool. Exploitation of this natural tolerance/resistance may provide a key solution, within a short time frame, to the survival of the citrus industry, especially in Florida. This project addresses the "Development of tolerance or resistance in commercial citrus in all production areas with a focus on delivery of new cultivars [or rootstocks and scions] using all available strategies", which is one of the priorities set forth by the Citrus Disease Sub-committee. In this proposal, we present a systematic approach with emphasis on skillful selection of natural mutants to develop new HLB tolerant/resistance citrus cultivars. First, we will select variant seedlings and bud sports with greater HLB tolerance/resistance in citrus groves across Florida and test them in field trials. With the obtained HLB tolerant/resistant bud sports/variant seedlings and their susceptible siblings, we will be able to apply transcriptome profiling and comparative genomics to identify HLB tolerance/resistant or susceptibility related genes. Moreover, with the HLB susceptibility

related genes identified in our preliminary study and this project, we will develop new resistant varieties via gene editing using CRISPR-Cas9 on the identified target genes. The CRISPR engineered citrus plants do not include foreign genes and are currently not restricted in the US. Meanwhile, we will conduct active outreach and extension to disseminate our project results to growers, stakeholders and the public. The successful achievement of our goals and objectives will broadly impact the entire US citrus industry, by maintaining the viability of industries not yet fully impacted as in Florida, and by providing the plant materials that will encourage Florida growers to replant with confidence. The combined economic impacts of our US citrus industries will be measured in billions of dollars. The many communities throughout the country having economies built around viable citrus production will avoid the major disturbances that will come from the economic disasters that HLB incurs. Current approaches for HLB management are based on an over-reliance of chemical insect control, and more recently the widespread use of antibiotic compounds; the environmental impacts of these practices may prove to be unacceptable in the long run, and resistant cultivars can mitigate such damage. The selected citrus varieties with HLB resistance/tolerance are expected to be the most durable, eco-friendly and cost-effective solution to this devastating disease. The success of this project will set up a model for the citrus community across the globe to follow, and therefore solve the HLB problem.

OBJECTIVES: The goal of this proposal aims to develop new generation of citrus cultivars that not only survive, but are also profitable in the presence of HLB. Huanglongbing (HLB) is presently the most devastating citrus disease worldwide. Because of the extensive spread and a lack of effective control measures for HLB, Florida's $9 billion citrus industry is presently fighting for its survival from the crippling effects of billions of dollars in lost revenue. In recent years, we have observed field resistance or tolerance to HLB in some citrus plants in commercial groves and breeding orchards, providing evidence that there is variation regarding HLB tolerance/resistance in the citrus gene pool. In this proposal, we take advantage of existing genetic variations and deliver HLB tolerant/resistant cultivars with desirable fruit traits through the screening of natural mutant citrus plants or bud sports of commercially grown cultivars with a greater tolerance/resistance. The selected HLB tolerant/resistant citrus variants are expected to be the most durable, eco-friendly and cost-effective solution to the menace of this very devastating disease. We will pursue five interrelated research objectives in this proposal.

Objective 1: Select variant citrus plants or bud sports with HLB tolerance/resistance. Selecting natural mutant citrus plants or bud sports of commercially grown cultivars with a greater tolerance/resistance is the quickest method to obtain HLB tolerant/resistant citrus cultivars. Sources of natural tolerance/resistance to HLB that were once concealed within unaffected groves in Florida will become more and more apparent as the incidence of HLB approaches 100%. We propose to focus on investigating two existing sources of natural variants for HLB tolerance/resistance. The first source has arisen from volunteer seedlings in commercial groves in Florida that have resulted from years of natural fruit drop. We have observed that about 10% to 20 % of ca. 300 volunteer seedlings in the Scott grove (VGS) are thriving despite being surrounded by HLB infected trees. Our initial analysis has demonstrated that a subset of the seedling selected from the VG showed a sustained tolerance to HLB in greenhouse studies. The second source is the bud sports derived from millions of infected trees across Florida citrus groves. Our initial results with Ruby Red grapefruit were very positive and prompted us to extend our search in more commercial citrus groves across Florida. Co-PI Duan will lead one

postdoc (1 FTE/year) and one technician (1 FTE/year) to select mutant citrus and bud sports in south Florida and co-PI Gmitter will lead the selection in the central and north Florida with one postdoc (1 FTE/year) and one technician (1.5 FTE/year).

Objective 2: Identify citrus HLB tolerance/resistance-related genes for potential citrus genome editing targets through transcriptome profiling. Our preliminary studies demonstrated that transcriptome profiling of HLB tolerant/resistant citrus plants and their HLB susceptible siblings could identify genes that are related to HLB tolerance/resistance or susceptibility in citrus plants. In this proposal, we will profile the transcriptomes of 30 HLB resistant/tolerant citrus plants and their 30 susceptible siblings before and after HLB infection using RNA-seq. We will use bioinformatics and functional genomic analysis to predict a list of HLB tolerance/resistance or susceptibility related genes. The identified candidate genes will be experimentally verified in a wide range of citrus genotypes, such as different species of citrus or their relatives that display either tolerant/resistant or susceptible phenotypes. Co-PIs Duan and Gmitter will provide the RNA samples of citrus tree. PI Luo (0.11 FTE/year) and one postdoc (1 FTE/year) will perform bioinformatics and functional genomic analysis. Co-PI Cano and one postdoc (1 FTE/year) will perform pathway analysis of differentially expressed genes.

Objective 3: Identify genetic variants that are associated with HLB tolerance/resistance in variant citrus plants. The greater tolerance/resistance of the progeny of Duncan grapefruit has been confirmed in Duan's lab. Our preliminary re-sequencing study showed that there are genetic variants existing in HLB resistant/tolerant Duncan trees. In this proposal, we will re-sequence six tolerant/resistant and six susceptible sibling plants and their parents. Bioinformatics and comparative genomics analysis will be used to identify HLB tolerance/resistance or susceptibility related segment deletions, copy number variations (CNVs), structural variations (SVs), SNPs, and Indels in each citrus line. We can thus identify genes with moderate/high impact variants. The identified target genes will be experimentally verified in a wide range of citrus genotypes, such as different species of citrus or their relatives that display either tolerant/resistant or susceptible phenotypes. Co-PIs Duan and Gmitter will provide the RNA samples of citrus tree. PI Luo (0.11 FTE/year) and one postdoc (1 FTE/year) will perform bioinformatics and comparative genomic analysis. Co-PI Cano and one postdoc (1 FTE/year) will perform pathway analysis of genes with high/moderate impact variants.

Objective 4: Engineer HLB tolerance/resistance using genome editing technology.We will knock down or knock out genes that are involved in citrus susceptibility to HLB, obtain loss-of-function mutants, and generate mutant citrus plants with HLB tolerance/resistance. We propose to use a CRISPR/Cas9 system that has been tested in citrus and to edit six to 12 genes in sweet orange and grapefruit for HLB tolerance/resistance. We propose to edit genes in sweet orange and grapefruit simultaneously. This is necessary because sweet orange and grapefruit are likely to have different alleles at these gene loci, and both are highly susceptible to HLB and critically important to the U.S. citrus industry. Due to the dire situation, we cannot afford the time to wait from results from sweet orange and then apply the technique to grapefruit, or vice versa. Co-PI Gmitter will lead one postdoc (1 FTE/year) and one technician (1.5 FTE/year) and focus on editing genes in sweet orange, while Co-PI Deng will lead one postdoc (1 FTE/year) focus on editing genes in grapefruit.

Objective 5: Outreach and dissemination of project results to stakeholders and the public. Outreach and dissemination of project results will keep stakeholders and the public informed and engaged with our research, which is critical to the success of project. We will assemble a stakeholder advisory committee and hold regularly committee meetings to seek inputs and advice to guide our research and outreach activities. Project results will be distributed to stakeholders and the general public through a variety of communication channels, such as meetings, events, field days, workshops, webinars, website and print materials. Summaries of results will be put into non-technical language and disseminated through multiple venues as listed above. University extension specialists and local farm advisors will be engaged to use these materials to inform their stakeholders. Dr. Polek will work with California and Florida's certification programs to get resulting material into a testing and clean-up program. Certified material will be propagated and planted into evaluation blocks. We will create a website to allow growers to report the variant citrus plants they observe and disseminate project progress/results to growers. Working closely with the project team, co-PIs Polek and Alabi (0.083 FTE/year) will lead the outreach and dissemination of project with one technician (0.33 FTE/year) and one staff (0.083 FTE/year for four years). PI Luo (0.11 FTE/year) will lead a student (0.1 FTE/year) to develop and maintain the website.

APPROACH: Methods 1. Methods for Objective 11.1. Select, propagate and evaluate HLB tolerant/resistant scion. More graft inoculations will be performed on additional volunteer Ruby Red grapefruit seedlings from the Scott grove. The new plants will be grown in pots in the greenhouse located at the US Horticultural Research Laboratory under standard management protocols. Ten propagated plants from each VG plant will be inoculated via graft or psyllid inoculation with grapefruit plants from a commercial nursery serving as controls. The inoculation process will be repeated three times with a total of thirty propagated plants for each VG plant. The tolerance/resistance of individual plants will be assessed by monitoring symptom severity in addition to Las titers. The assessment of the trees will be performed at time 0, 3 months, 6 months, 9 months and 12 months after graft inoculation. The best performing line(s) will then be subjected to field trials. 1.2. Bud sport selection and evaluation. We propose more extensive searches of bud sport and controlled inoculation. In addition to our own searching, we will work with extension agents and growers to collect potential bud sports from commercial citrus varieties. The number of new plants from each sport will be prepared, inoculated, and tested as mentioned above. To accelerate the evaluation of tolerance/resistance, we also propagate the selected bud sticks on HLB-affected and symptomatic Duncan plants (recipient) via bud stick grafting, the resistant/tolerant bud sports should grow out and remain vigorous growth along with low or high Las titers. The sports with greater tolerance/resistance will be integrated into field trials.1.3. Field trial of selected trees for HLB resistance.

Once the most resistant/tolerant plants have been verified and propagated, we will conduct field trials in Florida to ensure the productivity of the selected trees in an area where HLB is endemic. The quantity and quality of the fruit produced during the field trial will also be evaluated by determining the overall fruit drop and conducting routine juice analysis (Brix, acid, percent juice).2. Methods for Objective 22.1. Transcriptome profiling design and experiments.

We will profile the transcriptomes of 30 HLB tolerant/resistant citrus trees and their susceptible siblings before and after HLB infection. All the transcriptomic experiments will be performed with appropriate biological replication.2.2. Bioinformatics and functional genomics analysis of transcriptome data.

Sequence reads will first be quality checked, then used to construct transcriptome. We will then perform the DE (differentially expressed), alternative isoform difference gene functionality, co-expression network and pathway analyses. 2.3. Experimental verification of differentially expressed genes. We will experimentally verify the expression of genes and isoforms found to be differentially expressed in citrus lines as well as in other HLB tolerant/resistant or susceptible citrus lines.3. Methods for Objective 33.1. Resequencing design and experiments. We will re-sequence six tolerant/resistant and six susceptible sibling plants and their parents.3.2. Bioinformatics and comparative genomics analysis of re-sequencing dataAfter cleaning the raw reads (remove the low quality reads and adaptors), SNPs, Indels, SVs and CNVs will be identified, annotated and compared. 3.3. Experimental verification of genetic variations. Candidate SNPs, Indels, SVs, CNVs and segment deletions will be validated using PCR followed by sequencing.4. Methods for Objective 44.1. Choose target genes and target sitesWe propose to focus on 6 to 12 genes that are likely involved in HLB susceptibility in sweet orange and grapefruit.4.2. Design gRNAs and construct expression cassettes. Guide RNAs (gRNAs) will be designed based on the whole genome sequence of sweet orange in two Citrus genome databases and the grapefruit genomic sequences from Objective 3.4.3. Test the functionality of expression constructs using a transient expression system. Expression cassettes containing gRNAs and other essential components will be first tested to find out if they will function, i.e. induce mutations, in citrus. This will be done using a transient expression system.4.4. Deliver expression constructs into citrus and regenerate mutant plants. Agrobacterium-mediated transformation will be performed using the protocol that has been optimized by Gmitter's team. Complete plantlets will be grown in a growth room and then in a secured greenhouse. 4.5. Validate sequence changes in putative mutant plantsTotal genomic DNA will be isolated from mutant lines and amplified in high fidelity PCRs with specific primers. The amplified products will be cloned and at least 30 randomly selected colonies per construct per citrus line will be sequenced. The sequences will be aligned with the genomic sequences of sweet orange and grapefruit to identify and verify nucleotide deletions, insertions, and/or changes. 4.6 Analyze gene expression levels in mutant plants. Total RNA will be extracted from mutant and wildtype plants at multiple time points after Las infection. qRT-PCR will be performed on available real-time thermal cycler. The relative quantification of gene expression levels will be calculated.4.7. Assess HLB resistance of mutant plants. Mutant lines with confirmed changes in nucleotide sequence and gene expression will be propagated to produce clonal plants for Las inoculation and HLB tolerance/resistance assessment. For each mutant line, two clonal plants will be mock-inoculated with HLB-free buds, and six clonal plants inoculated with HLB-positive buds. Mutant lines with no or mild HLB symptoms will be identified for field testing.4.8. Field testing of mutant plants. Mutant lines with confirmed changes in nucleotide sequence and gene expression, and increased HLB tolerance/resistance will be planted in a secure citrus grove (USHRL, Pico's Road Farm). Mutant and wildtype plants will be arranged in the field in six completely randomized blocks and measured for shoot growth and rated for HLB severity as

above described. Shoot growth (diameter and length) will be measured every three months, and HLB severity will be rated every three months.5. Methods for Objective 55.1. Organization and engagement of stakeholder advisory panel. A stakeholder advisory panel will be organized to provide oversight on the relevancy of progress made by the research team. This panel will include university scientists, industry organization members, growers, and nurserymen. An annual meeting will be held. The panel will evaluate the progress against the proposed milestones of each objective.5.2. Dissemination of project outcomes via print, electronic and social media. Written materials will be disseminated via the project website, and other industry websites. In addition, articles and press release will be written and submitted to industry magazines. Project outcomes and events will be shared via Twitter and other social media platforms.5.3. Organization of seminar and workshops. Working group will be organized for university extension specialists and farm advisors Project team members will be available to speak at local grower seminars and workshops organized by these extension personnel.5.4. Industry meetings and events. The outreach team will give presentations in industry organization meeting in each citrus producing state, thereby reaching a large audience of stakeholders.5.5. Field Days. Field days will be organized for the growers and nurserymen to judge overall health and vigor of the tree, and evaluate the fruit characteristics and qualities.5.6. Project Website. A project website will be developed at the beginning of the project. Specifically, we will have a webpage allow growers to report the variant citrus plants for our team to evaluate.

Contact PI / Project Leader Information:

Name: GROSSER, JUDE WILLIAM

Other PI Information:

GMITTER, FREDERICK;BOWMAN, KIM D;CANO MOGROVEJO, LILIANA MARIA;VASHISTH, TRIPTI;ALBRECHT, UTE;WANG, YU;NIU, YUE

Awardee Organization:

Name: UNIVERSITY OF FLORIDA
City: GAINESVILLE
Country: UNITED STATES

AN INTEGRATED APPROACH TO THE ACCELERATED DEVELOPMENT OF ROOTSTOCKS THAT IMPART HLB TOLERANCE TO TREES GRAFTED WITH COMMERCIAL SCIONS

Agency: NIFA

Project Number: 2017-70016-26328
Contact PI / Project Leader: GROSSER, JUDE WILLIAM
Awardee Organization:

Abstract Text:

The goal of this project is to develop and deploy new HLB tolerant or resistant rootstocks to ensure the future vitality of the US citrus industry. This will be accomplished through partnership of two long-term, successful comprehensive citrus rootstock breeding programs, at the UF-CREC and the USDA-ARS-USHRL, in combination with a team of accomplished specialists to identify genetic, transcript, metabolic and physiological differences between HLB-tolerant and -susceptible rootstocks to uncover tolerance mechanisms and to develop efficient selectable markers that will facilitate the breeding process. The project will utilize extensive collections and existing field trials with novel and diverse citrus germplasm planted throughout Florida that have seen increasingly intense pressure from HLB, and thus have undergone a natural screening process. The germplasm resources and the team's collective experience in its evaluation provide a very solid basis for advanced breeding, directly targeting the industry's need for HLB tolerant trees through rootstock effects on scion performance. New rootstock candidates will be created using complementary HLB-tolerant parents already shown to transmit this characteristic to offspring. Candidate rootstocks will be identified in greenhouse and field studies, and then planted in replicated field trials for evaluation of tree survival and overall performance, fruit yield, and quality. This information will be made widely available through interaction with industry partners and project outreach, to support HLB-tolerant rootstock selection decisions for new plantings. Finally, candidates selected for release will be channeled through state and national agencies responsible for certified nursery production programs, and extensively propagated to ensure wide availability.

Project Terms:

base; Breeding; Characteristics; Citrus; Collection; Development; Ensure; Evaluation; experience; field study; Florida; Fruit; Future; Genetic; Goals; Industry; industry partner; Metabolic; novel; Nurseries; offspring; outreach; Parents; Performance; Physiological; Plants; pressure; Process; Production; programs; Resistance; Resources; screening; Solid; Specialist; Transcript; Trees