Citrus Research and Development Foundation, Inc.

Annual Report to Florida Department of Agriculture and Consumer Services on CRDF Programmatic Activities

July 1, 2016 through June 30, 2017

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Prepared September 2017

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This report is directed to the Florida Department of Agriculture and Consumer Services (FDACS) to summarize CRDF activities in pursuit of solutions to HLB and other challenges to the Florida citrus industry. This report covers the Fiscal Year 2016-17, bridging the period July 1, 2016 through June 30, 2017. This narrative covers activities managed by the Board of Directors and via the Research Management, Commercial Product Delivery, and Industry Research Coordinating Committees.

Table of Contents

INTRODUCTION

Projects and activities highlighted in this report represent the ongoing portfolio of contracted work overseen by the CRDF. CRDF's Board of Directors is the governing body for the organization, and exercises its mission to develop and deliver solutions to HLB via the activities of three programmatic committees, highlighted in the accompanying diagram. The content of this report summarizes the progress and accomplishments of projects funded by CRDF through guidance from the Research Management Committee (RMC) and the Commercial Product Delivery Committee (CPDC), and building upon the prioritization and Gaps analysis conducted by the Industry Research Coordinating Committee (IRCC), illustrated below.

Upon Recommendation from these committees, the CRDF Board acts to approve priorities and funding of specific projects to meet the goals of developing a balanced management system for sustainability and success of the Florida citrus industry in the presence of HLB. While the immediate focus for much of the work is directed to growers and grove practices, the activities recognize the importance of science-based solutions that will enable the other elements of the Florida industry, from nurseries to harvesters, packers, to citrus processers and the important citrus fruit and products that they market.

Board members (highlighted in Figure 2 below) continue to aggressively pursue all avenues towards important short-term solutions as well as long-term, durable solutions that will allow Florida to regain its place as a world leader in citrus production, quality and health.

Figure 1. Programmatic areas provided by CRDF Committees

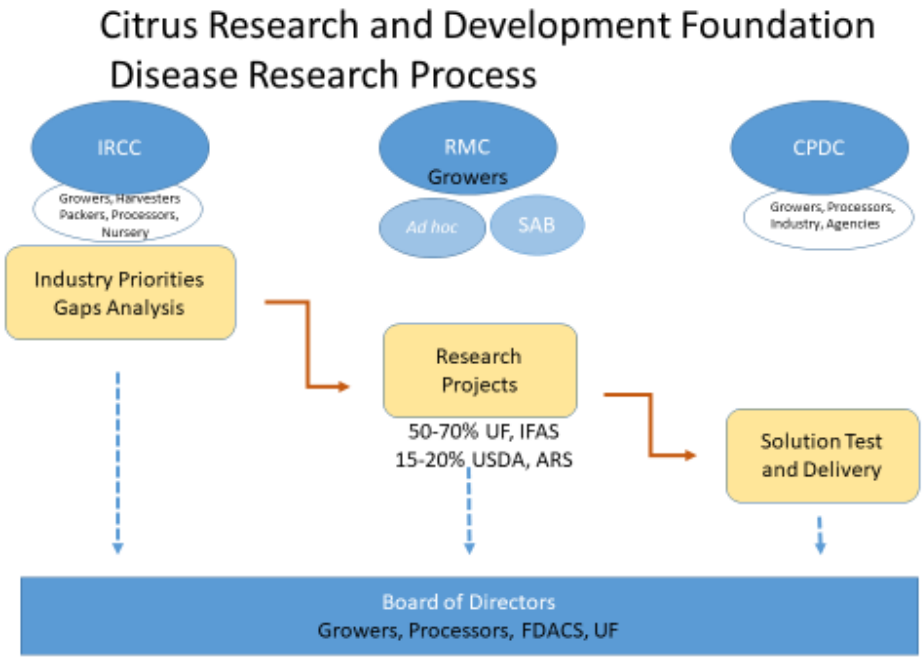
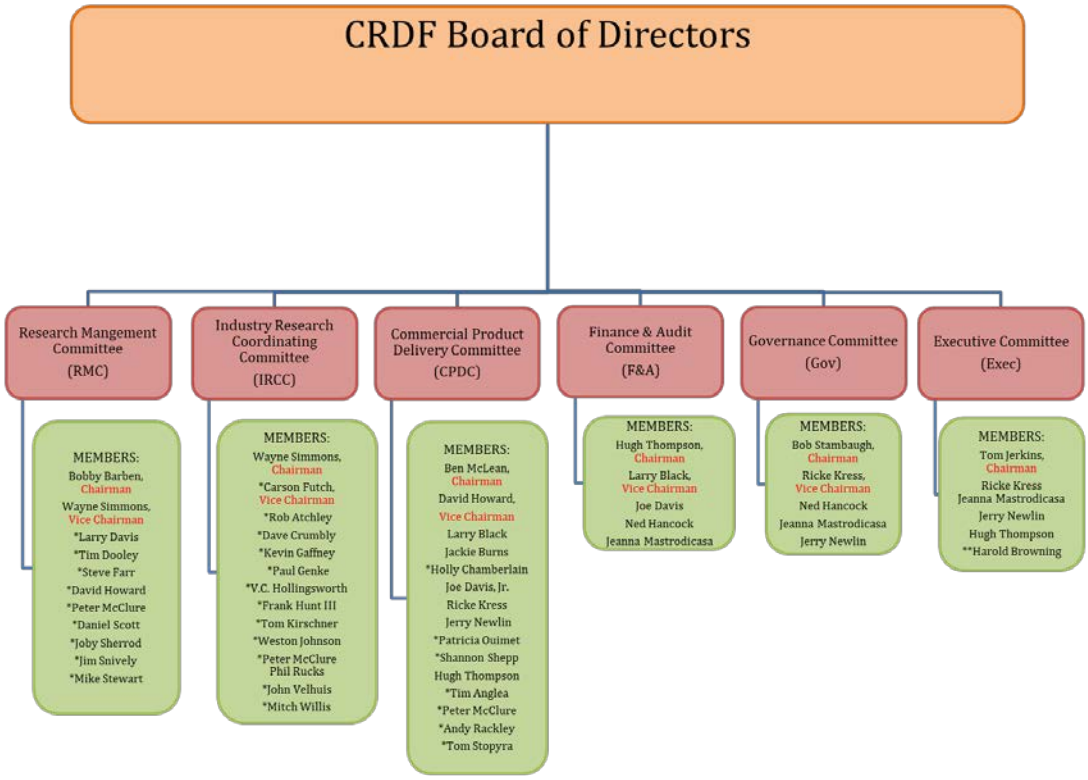


Figure 2. Organizational Chart for CRDF with Committee Structure



The balance of this report provides overview of the key topics of focus for CRDF efforts, and includes reference to specific projects within the portfolio. While most references are to activities during the recent fiscal year, necessarily the report alludes to prior progress leading to current project activities and progress. Projects are identified in this report by PI and CRDF project number. More details can be obtained on individual projects by searching the CRDF website at citrusrdf.org. The body of the report is organized according to interventions targeting the HLB pathogen Candidatus Liberibacter asiaticus; the insect vector, Asian citrus psyllid, Diaphorina citri; and the array of citrus host plants affected by HLB.

Candidatus Liberibacter asiaticus PATHOGEN INTERVENTION

Bactericides for HLB (CLas) Suppression

In early 2016, the bactericides oxytetracycline and streptomycin became available to Florida citrus growers under a section 18 emergency exemption. Projects funded by CRDF contributed to the data used to support this action, including a residue study (Nufarm 946C) and field studies (Richardson 16-022C). With the agricultural use of streptomycin and oxytetracycline, monitoring of resistance development in non-target bacteria is important and required by the US Environmental Protection Agency (EPA). A protocol for resistance monitoring in citrus groves was developed in consultation with the EPA and was implemented in 2017 (Triplett 16-009).

CRDF continues to work with growers to collect efficacy data to support a section 18 exemption renewal. Over seventy non-replicated, grower trials have been set up throughout Florida to evaluate grower applications of bactericides. New adjuvants are being evaluated for improved uptake of chemicals (Etxeberria16-023C) and new delivery methods are being evaluated including a CLaser-based instrument (Drouillard 16-025) and trunk injection (Minter 15-048C). Combination therapies including a combination of thermotherapy and bactericide applications are being studied in the greenhouse and field (Powell 910, Vincent 17-005) including a CRDF run field trial with a grower cooperator. To complement these studies, a project to develop a method of detecting and quantifying streptomycin and oxytetracycline using infrared spectroscopy methods, was funded (Tetard16-019C). These methods may be used in future studies to better understand the movement of the compounds throughout the plant.

Discovery of new bactericides against HLB is facilitated by an *in vitro* assay evaluating the effect of chemicals on the *C*Las surrogate organism *Liberibacter crescens* (Triplett 16-012C), a small plant greenhouse assay (Pelz-Stelinski 16-019C) and field evaluations. Field trials have been in place to evaluate bactericidal products available to growers like resistance inducers and botanical extracts (Curtis-934C, Booker-15-049C). Chemicals that may be repurposed for agriculture were field and greenhouse tested against HLB (Powell-910), and in laboratory and greenhouse experiments (Triplett 17-006C). New bactericides have been developed for use against HLB and citrus canker including a quaternary ammonium product that has been demonstrated to be a good alternative to copper applications (Santra 759) and a zinc-based bactericide that has also been found to be effective against canker (Santra 15-037C). Field testing against canker and HLB continues as does progress towards EPA registration. Another strategy being investigated is use of products that may act as plant defense "boosters". Some of these chemicals showed promise in early studies and are being further investigated for effective delivery (Wang 15-028).

An alternative to bactericides being investigated is the use of root colonizing bacteria to mitigate disease. Endophytic bacteria found on roots of "survivor" trees are being investigated (Wang 15-042), as well as antibiotic producing bacteria (Wang-916).

Two laboratories are funded to provide research and grower support through the diagnosis of leaf and psyllid samples by PCR analysis (Irey-15-032C and Batuman-15-034C). Another project with the potential to provide support to researchers is being developed to analyze the HLB bacteria within the tree and the psyllid host using fluorescent labeled *C*Las (Wang 16-005).

Citrus canker remains economically important to Florida citrus growers. In addition to bactericides, other methods of disease control are being investigated, including one project investigating the use of bacteriophages as a biocontrol method (Gonzalez 726). Cultural controls along with chemical and biological controls may be contribute to canker suppression. A research project addresses the disease control effect of a combination of chemical and cultural controls (Behlau 15-050C).

Bacterial diseases are the primary focus for funding by CRDF, but two fungal diseases impact citrus growers and require some investigation. An emerging disease of significant concern is the fungal quarantine pathogen, *Guignardia citricarpa*, the causal agent of citrus black spot (CBS). The biology of CBS in Florida is being investigated to help understand CBS epidemiology (Dewdney 15-005) and a project is in place to investigate management practices such as leaf litter biodegradation and fungicide applications to reduce inoculum, and an investigation into the mechanism of CBS resistance in sour

orange (Dewdney-715). Another important disease impacting Florida citrus is post-bloom fruit drop caused by *Colletotrichum spp*. being studied by Dewdney (16-010C). Objectives of this project are to investigate chemical control strategies, investigate the use of plant growth regulators to reduce the bloom period, and to develop a model to advise growers on optimum fungicide application timing.

Thermal Therapy to Reduce CLas Titer in Infected Trees

CRDF-funded research at UF and USDA previously has not identified how thermal treatment affects CLas acquisition by ACP feeding on treated trees. Discussions occurred on the need for this to be included in the MAC funding proposal addressing thermal therapy scale-up and research. Overlay of CLas acquisition testing on current field trials was suggested as a simple way to accomplish this goal. A project plan was developed by Kirsten Pelz-Stelinski of UF, IFAS, CREC and has been approved by CRDF, and subsequently approved for funding through the USDA MAC HLB program.

The objective of this project was to evaluate the effect of thermal therapy treatment on *Candidatus* Liberibacter asiaticus (Las) transmission by the Asian citrus psyllid (ACP). In May 2015, bioassays were completed to quantify the rate of CLas acquisition from infected citrus trees by Asian psyllids (ACP). These acquisition rates served as a baseline for acquisition prior to treating these trees with thermal therapy to reduce CLas infection. Thirty newly-emerged adult ACP were enclosed on individual branches of CLas-infected and uninfected (control) citrus trees using mesh sleeve cages. After one week, ACP were collected from the trees and placed in 80% ethanol. DNA from individual ACP was subsequently extracted and stored at -80oC. The rate ofC CLas acquisition by psyllids was quantified from these samples using nested quantitative polymerase chain reaction analysis (qPCR).

After initial acquisition bioassays were conducted, thermal therapy treatments were applied to CLas-infected trees in the experimental plot during the second week of July. Using a steam-generating machine, trees were heated to 55^{o}C for 30s. In approximately 4 weeks, the rate of CLas acquisition by immature and adult ACP was compared among infected trees receiving thermal therapy, untreated infected, and uninfected, untreated trees. CLas acquisition by adult psyllids enclosed on trees receiving thermal treatments did not differ significantly from acquisition by adult psyllids on untreated trees.

Based on these results, which indicated the thermal treatments applied during July 2015 did not reduce plant CLas titer or psyllid acquisition, a second thermal treatment was applied during late November 2015. In early January 2016, adults and nymphs were enclosed in mesh sleeves on trees for acquisition feeding approximately 5 weeks following treatments. Insect and leaf samples were collected after 10 d of acquisition feeding or upon adult emergence to assess adult and nymph acquisition, respectively. In addition, we have initiated a complementary laboratory study to evaluate the effect of thermal therapy on acquisition of CLas under controlled conditions.

In the field study, acquisition from treated trees did not differ from untreated trees. Furthermore, psyllids acquired CLas from symptomless tree that tested positive for CLas via qPCR. These “uninfected trees” later became qPCR-positive. Psyllid acquisition rates were positively correlated with leaf CLas titers, underscoring the importance of maintaining psyllid control even when CLas titers are low.

CRDF CPDC moved forward with plans to coordinate evaluation efforts of thermal therapy in conjunction with the USDA, APHIS HLB Multiagency Coordination (MAC) group. Building on the methods used to evaluate effects of other treatments (antimicrobials, soil amendments, etc.) on CLas and/or HLB and tree response, a before and after protocol was developed to document tree and environmental

conditions surrounding thermal treatments and a data plan for follow-up so that individual trials will be evaluated similarly and treatments can be compared. This protocol has been publicized on the CRDF web page so growers can do some self-assessments of their own thermal therapy trials and been implemented on a small scale with grower and research trials. The protocol became standard in the MAC funded CRDF project to evaluate thermal therapy scale-up. An overview of current field activity that the CRDF evaluation team has been engaged in follows.

Trees that have been evaluated are in varying stages of the decline due to HLB; most are heavily managed for psyllid control, nutrient applications, root health, etc. Evaluation of thermal therapy by the CRDF evaluation team has been completed. There are several enterprises operating field thermotherapy machines in Florida and at least two companies have been supported by USDA, APHIS, MAC to deliver additional thermal therapy to Florida for field trials. From 11 to 14 field trials have been evaluated with varying intensity and over varying periods of time. Different machines have been used delivering a range of temperature/duration combinations. Current data sets associated with these trials have been summarized. Results to date provide a description of the variation of measures and tree responses and all of these trials can be considered completed. Data analyses comparing pre- and post-treatment tree status have been provided, including yield, quality and other metrics. Overall, results have been unremarkable, as thermotherapy treatments only achieved short-term improvements in tree appearance and titer reductions that CLasted from 2 to several months. Reductions in fruit drop or increases in yield did not occur post treatment.

All of these trials were subjected to the protocol for evaluation as outlined per the approved work plan. The CRDF evaluation team also collected pre-treatment PCR bacterial measures, and other parameters. According to the protocol, periodic data collection following treatments assessed tree health responses as well as the specific impact on CLas bacteria. Having 14 locations under evaluation allowed us to drop some sites as we fulfilled the work plan and budget.

Additional trial evaluations were established to evaluate the new generation thermotherapy machines from Dr. Ehsani (UF, IFAS), Premier Energy, and Daniel Scott.

Asian Citrus Psyllid VECTOR INTERVENTION

Asian Citrus Psyllid Management and Citrus Health Management Areas

During FY 2016-17, CRDF supported ten projects directly addressing approaches to managing Asian citrus psyllid. Two of these projects are related to the topical area of RNAi/Psyllid Shield and thus are reported separately below. Several of the ACP projects reported here involve field trials of ACP management tools and their integration into citrus health preservation. The quarterly report for the period ending March 31, 2017 provided an update on the use of ACP suppression within CHMAs and the challenges faced by growers as ACP populations have increased, coincident with more unmanaged acreage. Two additional projects are pursuing novel interventions against ACP (Bonning 711 and Pelz-Stelinski 15-021). These projects are seeking alternative strategies to suppress psyllid populations as reported below.

Coincident with the maturation of several projects in this sector of the portfolio, the National Academy of Science (NAS) Comprehensive Review of the CRDF composite portfolio is engaging in evaluation of the vector component. A forum was conducted in July 2017 to bring funded researchers together to present and discuss their approaches and the results from work to date on the biology,

ecology, behavior and management of ACP. As with other topics within the HLB/ACP system, new project initiation will be developed following the evaluation and report of priorities from the NAS review team.

Field trials were installed during the reporting period in Project #16-020C (Christopher Vincent) to evaluate the additive value of ACP-repelling dye in applications of kaolin clay treatments. This new project is taking progress from a former project (#860 Sharma) and conducting field trials. The dyes selected in the former project have been optimized for inclusion in formulated kaolin clay and are being applied to determine the additive value of ACP deterrence and the ACP reduction potential of the clay by itself. No progress report for the current quarter was available at the time of this report.

Project 16-011C (Robert Adair) continued to evaluate the role of metalized reflective mulches in deterring early infection by ACP following planting of new trees. Building on work conducted earlier in SW Florida by Phil Stansly (447), this project has neared completion of the first full fruit harvest. An extension to this project was approved in 2016 to capture yield results and to develop the economics of use of this material. The June 2017 progress report indicated two metrics associated with use of metalized reflective mulch. 1) Fruit Crop Yield and Quality- The first crop yield data collected from 33-month old grapefruit trees planted into beds covered with MRM produced almost double (90% increase) the boxes per acre versus trees grown on bare ground as a grower standard. Likewise, the MRM fruit were significantly higher in juice volume, juice weight, and fruit weight versus the grower standard. Results for the compost treatment were intermediate with a 34% increase in yield.

2) Tree Growth and Pest Populations- The MRM treatment continues to offer lower ACP counts for all life stages (eggs, nymphs and adults) based on weekly scouting of 20 trees per treatment and greater growth rates determined by tree caliper, height and canopy volume. Coincidentally we saw fewer Diaprepes Root Weevil (DRW) adults in the canopies of trees with MRM.

Bob Adair reported that a detailed spreadsheet was developed to track all the caretaking expenses for the entire trial period, which includes all spray foliar spray applications, herbicide treatments, fertilizer applications, soil drenches as well as the associated materials has been maintained and updated as applications were applied. Similarly, the installation costs for the MRM and compost applications were documented. The economic returns from the first harvest for the MRM treated trees far exceeded the other two treatments (bare ground and use of organic compost) even when the installation cost of $223 (amortized over three years) for the MRM was factoring into the cost of production. The net return per acre for the MRM treatment was $106, whereas the trees grown in the bare ground treatment suffered a $709 loss.

Project 15-024 (Lukasz Stelinski) is approaching the end of its two-year cycle, addressing the details of ACP movement and how that can be incorporated into suppression strategies, including pesticide application programs. Progress reports from this project indicate that barometric pressures can influence ACP movement, particularly when pressures are fluctuating. Stable barometric pressures did not impact ACP movement, while increases in barometric pressure led to ACP dispersal. Dispersal also was increased with increasing temperature, as would be expected. Flight threshold temperatures and other details from this study can be incorporated into predictive models to assist in timing of sprays.

The impact of windbreaks and the grove architecture (solid new planting versus resets) was an aspect of this project. ACP populations were lower around edges of groves when windbreaks were present,

although this was not true for psyllid natural enemies. Over the two year period of the project, more ACP were present in young plantings of solid-set same-aged trees as in blocks where young trees were interspersed within a standing bock. This was noted across four locations and three varieties. The PIs indicate that this is the first report to demonstrate that the planting strategy of new trees in orchards may impact the populations of a horticultural pest.

Project 858 (Santra) completed a three-year project on composite polymner films as a deterrent to ACP feeding. Nearly 20 formulations of unique synthetic polymers were evaluated in this study, with a major emphasis on their impact on plant health. Phytotoxicity and other plant impacts were measured in greenhouses before seedling tree and field trials were conducted. Low incidence of infection in field trials in general prevented definitive evaluation of the utility of this approach to managing ACP populations. Rainfastness also was evaluated with the various formulations under simulated conditions in the greenhouse. While no formal follow-on project is active, work continues to determine the value of this approach to ACP management, perhaps integrating these results with the use of kaolin clay ACP treatments.

ACP research projects focused on developing knowledge towards interventions continued during this period as well. These projects included 15-021 (Pelz-Stelinski), which has as an aim the correlation between CLas transmission and the ACP immune system status. Potential points of intervention may result from this work. Likewise, Bonning (711) is searching for endotoxins from Bacullus thuringiensis (BT) bacteria with activity against ACP. Widely used against other insect groups, this approach has led to discovery of 2 endotoxins to which ACP appear to react. While this project is nearing its end, two circumstances indicate that this work will continue. First, Dr. Bonning has been hired by UF, IFAS, Department of Entomology into an Endowed Chair position, and thus will be more integrated into Florida-based work on HLB than from Iowa State. In addition, Dr. Bonning was awarded a USDA, NIFA, SCRI Citrus Disease Research Project in the most recent cycle, providing continuity to her current project from CRDF.

Dr Lukasz Stelinski (15-038C) is bringing the current phase of resistance monitoring to closure as the quarter ends, and will report the results of this work. A repeatable method was established and has been published for use by others monitoring ACP population susceptibility. Dr. Stelinski reports that field susceptibility to pesticides being monitored in 2016 remained stable, while in 2017, reduction in field susceptibility appeared in several locations.

The current project focused on providing recommendations and support to CHMAs in Florida (Rogers 15-035C) is contemplating the next phase to respond to lower participation in ACP management sprays, as well as to determine how CHMAs can be instrumental in other aspects of citrus health management in the presence of HLB. Discussions also are underway at USDA, APHIS and FDACS to determine program priorities for 2017-18 in the CHRP and CHMA program areas. Further details will emerge in the next 30-60 days.

Follow-up evaluation of the herbicide Reglone field trial was conducted during Q3 of 2016-17 by the PIs (Futch 16-027C). The studies were established at two locations, Wauchula (March 22) and Avon Park (March 23). The 100 GPA application increased defoliation as compared to the 50 GPA application due to better spray coverage. However, by 20 days after application at the Avon Park site, trees began to show signification regrowth with lots of new vegetative growth beginning to appear. The Wauchula site had significantly less regrowth but the general tree health was much poorer at this location as compared to Avon Park.

From visual observations, Reglone did not have sufficient translocation within the plant to kill the tree in place; thus this product will not provide sufficient injury to cause tree death.

RNAi for Psyllid Control

RNA interference, or RNAi, is a biological process in which double stranded RNA molecules specific to a targeted gene are introduced into a biological system that results in gene silencing of the targeted genes. Since the 1990s, there has been a growing body of research demonstrating that RNAs can silence the complementary messenger RNA sequences in many organisms including psyllids and other insects. Advances in understanding of the Asian Citrus Psyllid (ACP) genome, along with a growing ability to affect changes in proteins and transcripts through RNAi has intensified efforts to target RNAi to specific psyllid genes.

Since 2010, CRDF has funded 15 projects totaling $4.8 million directed at RNAi-based psyllid control. The goal is to develop a robust ACP control strategy and products using dsRNA molecules that can be deployed in an area wide program.

If successful, RNAi could potentially represent a more sustainable, less costly, and more specific ACP control method than the use of broad spectrum chemical pesticides. If RNAi could be applied exogenously, the method of control has the potential to be more timely than transgenic approaches that permanently alter the citrus tree's genetic material. This could result in a significant reduction in ACP populations, increased tree life and productivity, and lower overall costs associated with psyllid control and HLB treatment.

What Have We Learned?

Since 2010, several projects have been funded by CRDF and other funding agencies to identify specific gene targets within the ACP that, if silenced, would result in the death of or control of ACP and reduction in the spread of HLB.

In 2010, CRDF funded project 013 (Powell), a three-year study examining the physiology, biochemistry and inhibition of food digestive enzymes in adult and nymph psyllids. It targeted two enzymes (trypsin and cathepsin) used by ACP to digest food in their midgut. The evaluation protocol included use of an artificial feeding system on adult psyllids only. This involved setting up and maintaining a psyllids colony for feeding the dsRNA molecules. The study demonstrated activity of RNAi targeting the ACP in an artificial feeding system for adult psyllids.

Also in 2010, CRDF funded a four-year project 330 (Shatters) that examined the ACP feeding mechanisms as a means of blocking psyllid feeding on citrus. Chemical and molecular analyses of the isolated ACP salivary sheaths were conducted to determine structure and mode of synthesis, to identify specific salivary proteins secreted, and to construct a model describing the ACP feeding process. A number of enzymes present in the salivary sheath were discovered, two of which can be targeted to block sheath formation. An additional 6 proteins were identified based on their potential to bind to salivary sheath subunits. A continuation of the study (Shatters 330-1) was launched in 2013.

In 2011, CRDF initiated and provided funding for an RNAi InnoCentive™ Contest, a "crowd sourcing" approach to generating new candidate RNAi molecules against ACP. Under 401

(Powell), a one-year project, these candidates would undergo head-to-head testing to discover the most potent activities. Based on results of that contest, 14 RNAi gene targets from the Asian citrus psyllid genome were identified by a selection committee as candidates for further evaluation. This selection was made from a screen of 50 ACP gene targets pre-selected by the committee from approximately 100 submissions, using the protocol developed by Powell and Shatters, and based on artificial diets for adult psyllids containing dsRNA matching the sequence of a portion of these genes to observe toxicity. A method to test the nymphal response was still needed.

Also starting in 2010, parallel foundational RNAi research was conducted to understand pathogen/vector interactions at anatomical, molecular and cellular levels in CLas/ACP and other model systems.

Research by Falk (122) from 2010-2012 studied a very similar disease in tomatoes caused by the related gram-negative bacteria (Candidatus Liberibacter psyllaurus), vectored by the tomato/potato psyllid (B. cockerelli). It identified and then used specific psyllid RNA sequences to induce RNAi activity in recipient psyllids, and to assess and identify RNAi responses in the psyllids. This study used recombinant virus delivery.

Follow-on research by Falk from 2012-2015 (530, 531) used B. cockerelli as a model for ACP, and evaluated RNAi in psyllids. High throughput sequencing and bioinformatics, combined with virus-based biological control strategies, were used to clone hundreds of B. cockerelli sequences and specific messenger RNAs were targeted via injection and oral acquisition. The study demonstrated that RNAi effects, even mortality, can be induced within recipient psyllids, and built from that experience to move towards how best to apply this technology for psyllid control, using B. cockerelli and herbaceous plants as a system. In year 2 of the study, the research team evaluated transgenic potato plants for quantity and quality of different forms of RNAs present in the phloem, and the efficacy of specific sequences against B. cockerelli.

Research by Brown (U. Arizona) from 2010 to 2013 (021) acquired basic information about the molecular and cellular basis for CLas/ACP interactions, focusing on the gut and salivary glands of adult and immature psyllid to identify candidate genes with an emphasis on RNA-mediated gene silencing. Adult psyllid library comparisons were made between ACP and the potato psyllid, and feeding studies were undertaken to optimize an artificial feeding assay for potato psyllid.

Brown conducted a parallel study from 2010 to 2013 (038) that evaluated potential candidate genes for their negative effect on ACP. The study used feeding assays in which dsRNA molecules synthesized in vitro were included in the growth medium.

Research by Brown from 2012-2104 (510) continued examination of the molecular and cellular mechanisms that drive psyllid vector - Liberibacter interactions in the transmission pathway. The focus was on key anatomical interfaces (mouthparts, glands) where Liberibacter resides prior to and during plant inoculation, to clarify salivary gland secretions and molting fluid as pathways to transmission. The project sought to take advantage of recent advances in high

throughput sequencing and bioinformatics, and combined them with virus-based biological control strategies to develop new, complementary tools for use in controlling the ACP vector.

Research by Killiny (U Florida) from 2012-2014 (559) sought to understand the specific interactions between Clas and ACP to block its transmission. The study focused on molecular interactions to understand how the pathogen behaves within its vector, and then selected targets to disrupt the vector transmission process. The study performed an in vitro protocol to test molecules that may interfere with these interactions and cause a reduction in transmission and expressed these proteins in citrus to disrupt CLas transmission by ACP

Several promising targets have been identified, but one of the challenges has been how to most effectively deliver the RNAi molecules to the field.

In 2013, CRDF funded 618C (Dawson), an extension of the RNAi Innocentive™ project, in order to conduct in-plant characterization of dsRNA effects on all psyllid life stages and selection of targets to advance to field trials. Study objectives were to:

- Test the 14 selected dsRNAs separately for activity when expressed in plants using the CTV vector and evaluate their activity against both adults and nymphs feeding on citrus in caged greenhouse experiments.
- Identify the most active sub-fragments of the select dsRNAs based on psyllid mortality.
- Compare combinatorial effects of combining dsRNA sequences from multiple targets.
- Determine target and non-target effects of dsRNA in the psyllid, and
- Test effects of dsRNA on mortality of the brown citrus aphid, a related phloem feeding hemipteran insect. This information on cross-species effects was deemed to be necessary to characterize specificity of the toxic response, which is needed to advance the concept to a deployable strategy.

Through a series of CRDF-funded projects, the Dawson group at the University of Florida-CREC has shown that RNAi inducing sequences can be delivered to citrus trees using an infectious clone of Citrus Tristeza Virus (CTVvv), and when ACP adults and nymphs are fed on these trees, ACP reproduction was greatly reduced or prevented. Based on the hypothesis that this was due to larger food intake on plants versus the artificial diet, the CTV method was selected to test the effect of orally ingested dsRNA on nymphal stages because whole plants and excised leaves could be used. The tests included identification of the best sequences and combinations to target with dsRNA within the psyllid genome.

Psyllid toxicity studies used whole plant caged assays and excised leaf feeding assays with adult and immature psyllids. Delivery strategies were compared, including CTV expression, and exogenous application to rootstocks or to the scion in combination with penetrant facilitators. Data was collected on mode of toxicity, dsRNA longevity within the citrus tree and tissues, and analysis of cross-species toxicity on the Brown citrus aphid.

Results showed a reduction in survival of adult psyllids placed on RNAi expressing plants, but the effects on reproduction of the new generation of psyllids was much greater, likely because the nymphs are rapidly growing and need new protein synthesis, and due to their large uptake of phloem sap. Progress was made in identifying the best RNAi target sequences that can

prevent normal expression of specific genes in psyllids, based on an optimal compromise between efficacy and stability. There was also evidence of a possible reduction in acquisition/transmission of CLas.

Research by Stelinski (15-021) is examining whether pathogen or dsRNA exposure primes the ACP immune system to resist future infection by pathogens, including CLas, and whether this effect is multigenerational.

A mathematical model has been developed and is being refined to guide the design of area wide trials for both regulatory approvals and commercial use.

The Psyllid Shield concept recognizes that if RNAi is used to control the spread of HLB in new citrus plantings by controlling the population and spread of ACP as well as the acquisition or transmission of CLas, effects will be increasingly captured over larger scales in the environment from individual groves to area wide management. In 2014, CRDF funded project 932.1C (Keesling), a to conduct mathematical modeling to bring the necessary precision to a field trial design by accurately modeling the performance of the concept over different spatial dimensions, neighboring psyllid and disease pressure, and RNAi performance. Creating and refining the model based on existing research helped to identify research gaps in biological parameters, causes of psyllid movement, long-range movement of psyllids, and flush patterns based on tree age and species. This project ended in 2016 and is currently continuing with other funding.

In 2016, a project for a small replicated field trial was established with a commercial partner to validate the growth room data and to determine if a CTVvv together with RNAi can be used to specifically and economically control ACP with reduced or no use of traditional pesticides.

In 2016, CRDF funded 16-016C, a collaborative effort between CRDF and Southern Gardens to establish a small replicated proof of concept field trial over three years to replicate and validate the growth room data. Southern Gardens secured the necessary regulatory clearances, completed trial design, ACP mass rearing and tree inoculation, targeting a spring 2017 planting. If sufficiently positive results are obtained from this initial trial, the next step would be to establish multiple large-scale trials to validate the technology on a commercial scale to collect data for a full Section 3 registration.

CRDF continues to explore potential candidates for long-term commercialization of RNAi solutions for ACP intervention. Commercial partners will be needed for follow-on work to the phase one field trial described above. This includes support for a Phase 2 area wide “Psyllid Shield” field trial, as well as supporting regulatory, product development, Intellectual Property, and other work needed to bring products to market. CRDF continues to be prepared to facilitate, accelerate and incentivize corporate action and is prepared to provide regulatory, commercial delivery and other support, as appropriate, to candidate partners.

CRDF continues outreach to other companies engaged in RNAi research and product development for potential collaborations. There are number of large agrichemical companies such as Monsanto/Bayer and Syngenta investing in RNAi research and product development, and acquiring smaller companies and technologies. There are also a handful of small

companies that are working on RNAi-related products for plant agricultural applications. Most of these companies appear to be pursuing foliar or other exogenous applications of RNAi, creating a niche for CRDF to pursue RNAi using CTVvv delivery. CRDF program management will continue to monitor and engage with these companies to identify opportunities for collaboration in areas relevant to CRDF.

Rear and Release Psyllids as Biological Control Agents – An Economical and Feasible Mid-Term Solution For Huanglongbing (HLB) Disease Of Citrus

The purpose of this NIFA-CAPS Project is to create attractive options for management of HLB by replacing the wild type insect vector (ACP) with a population that is unable to transmit the bacterial causative agent (CLas). Achieving this outcome will require progress in the following three areas of emphasis – An *Effector Mechanism*, A *Driver System*, and *Diffusion*. The current conditions threatening citrus production nationally require our key personnel to work concurrently on parallel technical plans and to accelerate the leading alternatives based on assessments by our team leaders, advisors and management. This research has established a broad foundational knowledge base of molecular interactions between host, pathogen and vector that is now contributing to additional NIFA-funded programs. Part of our outreach in the final phase of this program will be to integrate our progress with others focused on the HLB challenge and to extend the breadth of communications about new technologies for disease intervention such as the current study in progress by the National Academies, Board on Agriculture and Natural Resources, “A Review of the Citrus Greening Research and Development Efforts Supported by the Citrus Research and Development Foundation: Fighting A Ravaging Disease.”

To successfully use an Effector for insect replacement, we need to disrupt interactions required for the spread of HLB while adequately maintaining psyllid fitness. New actives discovered in this program that are specifically toxic to psyllids may be used for novel insect suppression technologies. While this is not the proposed population replacement, if genetic or other modes of conditional delivery can be developed then new forms of biological control will be feasible. For example, these assessments have suggested a near term application of this research for the protection of new solid block plantings from HLB. We continue to evaluate the “Psyllid Shield” control strategy. While it is not full insect replacement, it is based in part on research progress in the search for Effectors. CRDF has supplemented funding to model field results under various scenarios and has selected 5 RNAi sequences as field trial candidates based on the results of indoor experiments with caged insects. A key stakeholder partner identified by CRDF is investing in regulatory approvals necessary for field trials of this disease management concept.

Our team has updated project objectives and budgets for the remaining term of the funded work to synchronize our remaining cash flow with priorities set in the CLast Annual meeting. These budget amendments are being finalized with the respective institutions.

TECHNICAL PROGRESS

Effector Mechanism

Initial assessments have not identified the required variation in CLas transmission to occur naturally in ACP populations. However, the prospects for engineering a mechanism to achieve the desired phenotype are under active investigation. The effector is the content of the phenotypic change we aim to introduce. Candidate effectors are being identified through multiple parallel methods of investigation including bioinformatics, proteomics, yeast two-hybrid (Y2H), peptide-ligand and scFV-ligand libraries.

- There is a growing list of candidate effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns--immunoprecepitations and mass spectrometry). This workflow of the Effector team has already generated more high quality targets than can be analyzed in bioassays. In many cases loss of gene expression through RNAi is highly toxic to psyllids. We have only conceived of two tools to use to disrupt the Effector Mechanism, RNAi and competitive protein ligand inhibitors (proteins, such as scFV antibodies or peptides). Secondary metabolites or RNA aptamers are potential additional options.
- Transcriptome expression profiling: Extensive transcriptome data sets (the Transcriptome Computational Workbench; TCW) have been created from whole adults, adult salivary glands, adult guts, and nymphs infected or uninfected with CLas or CLso and are continually updated with datasets associated with published manuscripts and made available to researchers (www.sohomoptera.org/ACPPoP). Published manuscripts include; “Asian citrus psyllid expression profiles suggest Candidatus Liberibacter asiaticus-mediated alteration of adult nutrition and metabolism, and of nymphal development and immunity” in 2014, “Comparison of potato and Asian citrus psyllid adult and nymph transcriptomes identified vector transcripts with potential involvement in circulative, propagative Liberibacter transmission” in 2015, “'*Candidatus* Liberibacter solanacearum' and evidence for surface appendages in the potato psyllid vector’” published in PLOS ONE in 2016, and recently “Colonization and intrusive invasion of potato psyllid by *Ca*. Liberibacter solanacearum” in Phytopathology. A project studying the effect of temperature on infected and uninfected psyllids will utilize the TCW BcAN dataset. The goal is to learn the potential impacts of extreme temperature on the vector with the aim to better select effectors. Data revealed that extreme temperature impacts bacterial titer within the psyllid vector equally, suggesting the activity of specific gene(s) at non-optimal temperatures, not the amount of bacteria, plays a key role in altering transmission parameters. This hypothesis was confirmed from differential expression analysis, in that data did show that high temperature had a greater impact on psyllids, regardless of infection status, based on the larger number of genes that were significantly changed. Functional analysis of the genes mis-expressed at this higher temperature extreme are underway. Preliminary data suggests that functions known to be important in pathogen-host interactions are affected.
- Proteome expression profiling: During this report, TCW was updated to allow for protein sequence and corresponding spectral count data to be analyzed. In total (average of 3

technical replicates) 608 unique proteins were identified by mass spectrometer in adult whole body, gut and salivary gland tissues. Results showed that 55% (304 proteins) had modified changes in abundance due to Liberbacter presence or absence in adult whole body, gut, and salivary gland tissues. The predicted functions of these proteins were investigated using Gene Ontology and KEGG programs and results confirm that were previously reported regarding our invasion model. Additionally, comparative *in silico* analyses identified 22 genes that were modified by Liberibacter presence in both the transcriptome (expression) and proteome (abundance). To date 4 of these genes (previously reported 2) have been tested using RNAi with one showing a reduction in transmission and therefore a promising target for control.

- Yeast two-hybrid: Of the 109 matings and 83 co-transformations completed to date, 47 ACP, 35 CLas and 10 phage genes have been selected for additional analysis. Of those a total 31 ACP, 16 CLas, 6 phage, and 2 endosymbiont-associated genes were selected for further study. Those that showed interactions had a variety of functions initially reported in virus-host pathogen systems, and more recently, for bacterial pathogen-interactions with their hosts.
- Co-immunoprecipitation: In total, roughly 25 gene candidates have been selected and/or attempted for CoIP analysis. To date 5 ACP, 2 CLas, and 2 phage genes have been tested (3 replicates) using crude protein samples extracted from both infected and uninfected adult whole body tissues. Results showed that a bacterial and phage protein interacting with psyllid genes involved in endocytic processes. Another phage gene was shown to interact with a psyllid transporter gene. In all 30 interacting partners are recognized as important. Both psyllid genes were subjected to RNAi analysis and the bacterial and phage genes were subjected to qPCR experiments. One manuscript using Y2H and CoIP findings is in preparation from this project, with an expected submission date of August 2017.
- TEM/SEM studies: Micrographs revealed putative endocytic/exocytic processes, biofilms that require complex cellular communication, and an intravesicular bacterial lifestyle evidenced for the first time allowing for additional effector candidates to be explored. Two manuscripts describing these efforts were published CLast year.
- Validation of effectors: RNAi testing has been conducted for 31 different psyllid genes using the single-gene and/or stacked (multiple-gene) RNAi approach and either CLso-infected (born and reared) or -uninfected (introduced to a CLso source plant) nymph and/or adult psyllids to date. Genes are selected as 'transmission interference candidates' by literature review of other pathosystems, from 3 different expression profiles e.g. proteome and transcriptome, and/or protein-protein interaction (standard yeast-two hybrid, specific bait prey co-transformation and co-immunoprecipitation analyses). Thus far, positive results (reduced transmission) have been obtained for 12 genes in functional transmission bioassays. In addition, 3 other genes have caused some psyllid mortality. To better understand the impact of "gene stacking" in RNAi assays, four genes that have shown very promising results were selected for more extensive analysis. All possible dsRNA stacking combinations (15 total) where studied. Both persistence and gene knockdown were examined. Treated insects were tested immediately after a 3 day-dsRNA feeding period (oral delivery), followed by a 7d holding period on plants. Results

after the 3- day feeding from all dsRNA combinations showed gene knockdown (i.e., significant decrease in relative gene expression). Testing of insects after the 3-day dsRNA feed and 7-day holding period show the same level of knockdown but interestingly in more than a few combinations a gradual decrease in the level of knockdown, indicating a possible effect on persistence that could negatively affect efficacy of dsRNA treatments. Furthermore, preliminary results for one of the four genes in specific combinations shows an actual upregulation of the relative gene expression. Indicating not only a possible problem with persistence, but possibly some form of trans-activating transcription regulation. These results are preliminary, but warrant further study to better characterize this putative yet important phenomenon and shed light on how best to provide more effective combinations of RNAi candidates. Putative functions of the 12 genes strongly suggest an invasion model similar to other known bacterial pathogens, including endo-exocytosis involving at least a clathrin-mediated step. As a next step, additional genes are being selected for RNAi analysis based on these results.

- DsRNA quantification and tracking: Experiments to detect and quantify the fate of dsRNA in plants and insects are underway. To date 200 ng total dsRNA was applied to seedling tomato plants for 24 hours, followed by allowing five adult PoP to feed on those plants for 24 hours, after extraction and northern blot showed low level detection of dsRNA with digoxigenin label RNA probes detected by chemoluminescence. Also, fluorescently labeled dsRNA fed to both seedling tomato plant and adult PoP has been tracked through the phloem in the roots of the intact plant and in the gut of whole PoP by fluorescent low power microscopy. This will be followed up a closer tracking of dsRNA with tissue sectioning. These studies are necessary for monitoring the persistence and location of dsRNA in plants and psyllids as well as non-target insects.
- Quantitative PCR (qPCR) analyses have been conducted on 14 genes (previously reported 9) to date, including psyllid, phage and Liberibacter genes. Expressional profiles were obtained from early instars (1st -2nd), late instars (4th-5th), teneral, and non-teneral adult psyllids. One Liberibacter gene showed significantly higher relative expression (3-6x) in later instars to adult life stages. This gene is putatively involved in cell motility and also showed significant interaction in Y2H studies. Another Liberibacter gene, putatively involved in cell communication, showed a similar expression profile (high expression in late instars and adults). Interestingly a phage gene with a putative function implicated in virulence in other well-known pathosystems, was significantly higher expressed (2.5x) in the early instar. Low expression profiles for two phage genes with putative functions known to aid in virulence in other pathosystems, suggest that these are not key effectors in the CLas/ACP system. In addition, preliminary data for a unique psyllid gene predicted to be a key factor in invasion, shows differential expression between infected nymphs (1.3 fold increase) and adults (0.7- 0.03 fold decrease). Similar studies for 4 additional psyllid genes are in process both PoP and ACP to study relative gene expression in infected psyllids vs healthy psyllids. Work was completed to add 2 additional reference genes to add validity to the qPCR for the psyllid studies due the high level of variability in experimental conditions. Preliminary data indicates both up and down regulation varying with gene but also with life stage, pointing towards the relative importance of specific genes and life stages for further targeting efforts. In another experiment, expression

profiles were obtained from adult psyllids having access to a Liberibacter source after a set period (0, 2d, 4d, 8d, 12d, 16d, 20d, and 24d). The AAP experiments were set-up and psyllids collected (3 replicates). Gene expression studies were conducted by qPCR with 12 PoP genes to date, all implemented in invasion. Five of these genes show a steady decrease in relative gene expression over the entire time course. Two showed a steady increase. The others showed inconclusive results and a shorter period time course was set up as above only collecting insects at 0, 3, 6, 12, 18, 24 and 36 hours. Four of these PoP genes were analyzed by qPCR to date. The conclusion is that their gene expression is effected very little if not at all by the onset of CLso infection over time.

- TCW databases, website, and interface for all bioinformatics: Database annotation updates are critical for completing the remaining transcriptome analyses needed to complete manuscripts anticipated during the CLast year of the project, and for annotating the comparative RNAseq data sets upon completion. CLast quarter it was determined that quality of the data were not optimal because the computation of the read counts was inadequate. Therefore C. Soderlund and M. Willis (Gang lab, WSU) worked together to determine appropriate parameters for the mapping. The TCW databases were rebuilt with the new data, given a December 2016 update archive. A study to determine candidate transcripts that influence psyllid speciation is ongoing and one manuscript “Comparative transcriptomes of Asian Citrus Psyllid and Potato Psyllid” (C. Soderlund, T. Fisher, M. Willis, R. He, D. Gang, and J.K. Brown) was published, May 2017. To date, orthologous pairs were computed using the TCW bi-directional best-hit (BBH) algorithm with e-value 1E-05 and the restriction that the alignment must overlap at least 50% of the shortest transcript, which resulted in 8892 pairs. A comparison of the 5’UTR, CDS, and 3’UTR showed percent similarity of 60, 73, 59 and percent GC-content of 24, 34.5, 23.5, respectively. The codon analysis shows an overall 24.4% synonymous codon and 33% non-synonymous pairs, where the synonymous codons were further broken down into 10.4% 2-fold and 13.4% 4-fold degenerate. The transition differences of the three codon positions are 9.3%, 6.7% and 18.6%; the transversion differences of the three positions are 9.8%, 8.7% and 16.0%. Using TCW, the alignments were written to file for analysis by the KaKs calculator program using the method=YN, and results loaded into TCW. There were 8850 with a p-value, where 7295 have KaKs<1 and 1553 have KaKs>1, with KaKs quartiles Q1 0.02873, Q2 0.11531, Q3 0.6708. The third objective is to map the Dc transcripts to the Dc genome sequence. Initial results showed multiple ambiguities when using blast results. A more strenuous approach was developed to use dynamic programming to get the best matches and data analysis is in progress.
- RNASeq time-course: 60 RNAseq ACP libraries were constructed from two treatments (ACP_CLas-infected vs uninfected), 5 stages (instar 1+2, instar 3, instar 4+5, teneral adult and adult) and 6 replicates, and these libraries were pooled with 12 libraries barcoded in a lane for 150 bp paired-end sequencing and 1,960 million reads totaling 296 G bp data were generated. Samples were provided by Dr. Kirsten Stelinski, collaborator. The data assembly, mapping and annotation are in process.
- Endosymbiont genome sequencing: The psyllid ovary samples from 4 psyllid species: ACP, PoP-central, PoP-western and Carrot psyllid, were used for making 4 Illumina genomic libraries and pooled on a lane for 150 bp PE-sequencing. In total, 314 million reads were

produced, and total bases are 47.4 G bp. The new longer read data will be integrated with the existing data (100 bp PE reads) to help improve the assembly qualities of the endosymbiont genomes. The assembly of contigs for each genome has been completed and annotation are in progress.

- Transcriptome and gene expression analysis of Asian citrus psyllid in response to *Ca.* Liberibacter asiaticus: RNA-seq libraries were constructed from *C*Las-infected and *C*Las-free ACP samples of three different developmental stages (nymphal instars 1-3, nymphal instars 4-5, teneral and post-teneral adults). With 150 bp paired-end sequencing on the Illumina Hiseq2500, 152 Gb of sequence data were generated from 56 million reads per library/replicate, which was assembled into 34,122 contigs with 18,827 (55.2%) being annotated, which were then further analyzed for potential functional classification and potential roles in infection. The results suggested that gene expression in different developmental stages did not respond in the same manner to *C*Las infection. With more contigs being up or down- regulated, nymphal instars 4-5 showed a more sensitive response to *C*Las infection than nymphal instars 1-3 and adults. A comprehensive analysis of the transcriptomes revealed vector life stage differences and differential gene expression in response to *C*Las infection, and identified specific genes with roles in nutrition, development, immune response and transmission pathways.
- Two classes of peptides that may stop the Asian citrus psyllid's ability to acquire/transmit (AcTrans) CLas have been identified in functional assays. One set of three hexameric peptides significantly reduced the psyllids subsequent ability to acquire/transmit (AcTrans blockers) the '*Candidatus*' Liberibacter asiaticus (CLas) bacterium when fed to psyllid nymphs. Two separate bactericidal peptides that kill CLas within infected leaf tissue when these peptides are taken up into the leaf vascular tissue have also been identified (they are mobile in the leaf vascular tissue and reduce leaf bacterial titer by greater than 80% in 7 days). In the previous report, preliminary results showing that by combining both AcTrans blockers and bactericidal peptides were presented showing induction of greater than 95% mortality in developing psyllid nymphs and while none of the surviving nymphs successfully acquired the CLas bacterium. Further replicated studies show similar results with high psyllid mortality and no complete acquisition (defined as movement into the salivary gland) of the bacterium by the surviving psyllids.
- Single AcTrans blocker peptide experiments have been initiated in combination with the antimicrobial peptides to determine the minimal combination that has the desired effects on psyllid mortality and acquisition. One of the three AcTrans blocker peptides was identified as having the most significant effect on psyllid mortality and CLas acquisition. Transgenics are being initiated with a combined expression of this AcTrans and the antimicrobial peptide.
- A topical application strategy has been developed that could be deployable in the field. Its effectiveness in delivering organic antimicrobial molecules of MW of at least 600 MW and also in systemic delivery of dsRNA molecules of up to 300 bps was demonstrated (a MW much larger than previous peptides). A 10 g batch of this peptide has been synthesized and demonstrated to have the same antimicrobial activity as the previously synthesized peptides. Plant application experiments are now being conducted.

- A transgenic citrus strategy has been developed that will produce a single phloem-cleavable peptide that, when processed, produces the desired smaller and biologically active peptides (both AcTrans blockers and antimicrobial). This system utilizes an already identified peptide cleavage system within the phloem of citrus. Plants have been produced and are currently being evaluated in the greenhouse for expression.
- Single chain antibodies targeting surface antigens on CLas have been created that interact with 12 different predicted surface epitopes. These antigens include the major outer membrane protein OmpA, two flagellar antigens, and the capsular polysaccharide synthase, and two pili components. Some of these have been expressed in transgenic citrus and others have been expressed and purified using a 6X histone tag strategy. These will be used for laboratory bioassays developed to study acquisition and CLas survival. Citrus rootstocks expressing two scFv have been made at Fort Pierce. A scFv selected to bind a surface exposed epitope of TolC = NodD (secretory pore) and a scFv selected against InvA, a protein produced by CLas believed to prevent apoptosis of infected cells. Multiple scFv selections have been introduced in citrus and multiple transformation events (~400 in all) are currently under evaluation for their effect on CLas survival in the plant, and acquisition/transmission by the psyllid.

Driver System

A new trait will not spread efficiently upon release within an existing population without a genetic bias of some kind. The driver is the medium of spread of the introduced phenotype--lack of CLas transmission. The drivers under investigation are viral, endosymbiont and chromosomal.

- From sequencing worldwide collections of *D. citri* and bioinformatics analysis, several potential candidate viruses have been discovered that might be useful for paratransgenesis delivery systems for inducing desirable traits in *D. citri*. Efforts continue to develop some of these for use as tools in this project.
- DcPLV was the first *D. citri* virus identified by us, in *D. citri* from Taiwan, China, and Brazil, but not yet from any U. S. collected *D. citri*. DcPLV is a novel insect virus with an unusual genome organization. DcPLV has a positive-sense ssRNA genome of 10,222 nucleotides and contains a single ORF coding sequence of 8,757 nucleotides. Attempts to clone the entire genome obtained through the extension overlap PCR strategy as cDNA using different strains of *E. coli* cells including JM109, DHB10 and MDS but have not yet been successful. The hope is to use cultured cells to recover infectious viruses. This bacterial free strategy was recently applied to create infectious forms of a virus in the genus *Flavivirus*.
- Attempts to identify promoters to drive transcription of introduced sequences in *D. citri* by cloning putative promoters for *D. citri* actin and tubulin genes into a GFP reporter construct are in progress. This is important for delivering cloned viral constructs, such as for DcPLV, into *D. citri.* These promoters will be tested by detecting GFP expression following injection of the promoter-GFP constructs into *D. citri* insects and insect cells. This system is also being used to test the efficacy of previously described hemipteran and

viral promoters. Good success in culturing cell from the hemipteran, *Anasa tristis* has been achieved.

- Another virus originally discovered early in this work was *Diaphorina citri* Reo-like virus (DcRV). We have colonies of *D. citri* (Hawaiian collection) infected with DcRV in the UC Davis CRF. We have transmitted DcRV to naïve California *D. citri* and found that the DcRV is transmitted through eggs to progeny.
- With respect to the bacterial driver system a goal for the project going forward is to develop new methodology to increase adult survivability post embryo injection. Problems arise after injected embryos hatch, since first instar nymphs prove difficult to handle and survival to the adult stage is minimal. It is hypothesized that to increase survivability of injected ACP to adulthood it is necessary to decrease ACP embryo and instar handling as much as possible. New methodologies for injecting and handling embryos were developed during the past quarter.
- To collect ACP eggs, small swingle seedlings in "cone-tainers" are inoculated with 10-15 pregnant ACP adults. Cone-tainer seedlings are selected preferentially for young flush suited for oviposition sites. ACP are returned to colony cages after approximately 3 hours or 1.5 hours or less after the time of oviposition.
- Flush with eggs is clipped from the plant and washed in 0.02% nonidet for one minute, and rinsed in DI water. After air-drying eggs are transferred with forceps to a 0.5mm strip of double stick 3M tape attached to a coverslip. that is then placed within in a petri dish with moist filter paper.
- The cover slip with eggs are placed on a micro manipulator to be injected. Halocarbon oil can be used on the side to prevent clogged needles.
- After injection eggs are placed into a petri dish and placed in an incubator and monitored. Three days after injection eggs and tape are dusted in potato starch to prevent them from getting stuck in the tape after hatching.
- On day 4 newly emerged ACP nymphs are transferred to fresh flush on cone-tainer seedlings covered with a mesh cylinder to prevent emerging ACP from escaping. Plants with ACP nymphs may then be transferred to an environmental chamber or green house.
- Cone-tainer seedlings with transferred ACP nymphs are monitored for 14-21 days until adults begin to emerge.
- Injected ACP must be separated and sexed before they reach sexual maturity. Each ACP can be sexed under a dissection scope in a small glass vile.
- Virgin wild types should be paired with injected adults for mating. Each pair can be placed on cone-tainer seedlings and allowed time to mate and lay eggs. These eggs should be allowed 14-21 days for full development before screening.
- The first new method of rearing injected ACP from instar to adult focuses on allowing newly emerged ACP nymphs to crawl from cover slips directly to fresh flush to help decrease handling at the first instar nymph stage. The only major modification made to the initial standard operating procedure was to attach cover slides to fresh flush where nymphs were allowed to crawl directly onto flush without being handled. In this method cone-tainers were not a viable option due to their small size, orange jasmine (Murraya

Exotica) was used for ACP nymph development. This method allows nymphs to crawl to a feeding site of their choice.

- In greenhouse assays, 226 nymphs and 0 adults emerged from the 381 embyros not injected, representing 59.3% egg hatch. Egg hatch was lower (29.1%) when embryos were injected, with 65 nymphs emerging from the 223 embryos injected. No adults emerged from either treatment.
- When ACP development occurred in incubators, rather than a greenhouse, egg hatch rates were 59.3% (294/525) and 3.2% (3/95) among non-injected and injected embryos, respectively. One adult emerged from the non-injected embryos.
- The second new method of rearing injected ACP from instar to adult involves injecting ACP embryos directly on the flush in the original oviposition sites, and development of this method is in progress. In this method embryos were left in original oviposition sites and injected accordingly. This method decreased handling of the ACP embryos and nymphs, and uninjected controls are promising. However, ACP embryos are clustered and thus difficult to inject individually and further testing is required. Of 131 embyros not injected, 105 nymphs and 5 adults emerged, representing 80.2% hatch and 3.8% survival to adulthood, respectively. No nymphs emerged from the 9 embryos that were injected.
- A third method for rearing ACP for injection forces oviposition without the need to transfer the eggs, or eggs+flush for injection, groups of males and females (2 females and 3 males) were transferred to small Petri plates with 1% agar with and without 20% sucrose and observed oviposition. An extract of new flush (the flush was ground using TissueLyser II) was added as a treatment to a set of dishes to see if the 'flush extract' would induce oviposition. After 8 days, some mortality was observed in the absence of sucrose, indicating that adults will feed on the agar (*D. citri* typically die after 3 d without feeding). After 7 days, up to 20 eggs were observed in untreated dishes containing 20% sucrose. The following week, eggs started to hatch and nymphs successfully fed on the agar. This suggests that this method could be explored to circumvent the problem of transferring groups of eggs with flush to a different surface before the microinjections, and avoid the difficulties associated with egg clustering. Agar can be easily excised and placed on a coverslip, then removed to a fresh bed of agar. Following egg injections and hatch, nymphs could be recovered and transferred to plants. This possibility is currently being investigated.
- While nymphal hatch rates for uninjected embryos were high when eggs when retained on flush, successful individual egg injections have remained difficult for both the Pelz-Stelinksi and Handler labs due to egg clustering and the inability to properly position the micro-needles for individual injections. In addition to the agar plate method under development by Pelz-Stelinski, the Handler lab has initiated protocols for DNA delivery to eggs maintained on flush by biolistics bombardment with micro-pellets coated with DNA. This is a method routinely used for plant tissue gene-transfer, with just a few reports of successful transformation of insect embryos. The most effective of these efforts was reported in 2008 where Drosophila was successfully transformed at rates of 3-4% using a Bio-Rad PDS-1000/He biolistic instrument with the Hepta-adapter. The lab has obtained a previously used PDS-1000/He instrument and have been repairing and retro-fitting it for testing, as well as testing protocols for preparing eggs on flush for treatment. This

includes coating the flush with 1% agar to support the eggs during bombardment. The agar overlay using Swingle flush gave considerably better support for ACP development than C35, and was not significantly different between agar treatment and controls when reared within an incubator (0, 18 and 20% adult development, on C35, Swingle + agar, and Swingle control, respectively). Although control survival was better in greenhouse rearing (29% adult emergence), maintenance of incubator conditions are simpler and more consistent, and therefore preferable. Tests for biolistics-mediated gene transfer in ACP embryos will now be initiated on Swingle flush with eggs overlayed with 1% agar and subsequent rearing in incubators.

- Transinfections with non-native hemipteran *Wolbachia* are ongoing. Development of co-infected ACP requires quantitative identification of each strain from the psyllid. While qPCR can be used to conduct whole body quantifications, localization of the new Wolbachia strains is also necessary because the amount of genetic material from foreign Wolbachia is low in individual tissues. Therefore, primers have been constructed primers for fluorescent in situ hybridization (FISH) to identify the unique Wolbachia cells in tissues. The effects of individual Wolbachia infection (wDi, ST-173) and co-infection on the survival and transmission capacity of CLas-infected vs. uninfected ACP is currently underway.
- Efforts continue to streamline the process for generating insects that carry reciprocal translocations to facilitate a chromosomal gene drive system for population replacement in the psyllid. Several chromosome translocation-based drive elements have been generated in Drosophila.
- A paper describing how to generate chromosome translocations and their actual properties has been submitted for publication. The manuscript has been posted online at bioRxiv.org, http://biorxiv.org/content/early/2016/11/17/088393. As discussed in previous updates, efforts continue to streamline the process for generating insects that carry reciprocal translocations. The original work used a three-transgene approach that required a number of generations and crosses to generate translocations. A number of translocations in Drosophila have now been generated using a two-transgene approach that involves two strains, one expressing Cas9 and a second line expressing a guide RNA that cleaves both transgene-bearing chromosomes. Both of these have an unanticipated fitness cost and do not drive. It is hypothesized that expression of Cas9 remains present in these animals, and is expressed in the germline in each generation. Cas9 has been shown to have effects in other systems, and thus it is possible that this expression lowers fertility in either males or females. To eliminate this expression we took the translocation strain and injected guides into its germline that are designed to cleave and destroy Cas9. Progeny from this injection are now being tested for the presence of functional Cas9. Those that pass this test will be retested for drive.
- Great caution must be used in making the determination that any one of the lines scored as positive for being a translocation based on marker rescue with the Cas9 system. This is because, for still unclear reasons (being explored using PCR and sequencing), the act of cleavage sometimes results in marker activation that does not correspond to generation of an actual translocation. This was observed in several drive experiments. The only way to ensure that a putative translocation is really a

translocation is to outcross heterozygotes and look for the 50% embryo lethality that must occur if the translocation is present. This is then followed by PCR of a number of lines to confirm. PCR confirmation works well.

- As a third approach, attempts to generate a translocation directly in the germline of an injected embryo are underway, as this would simplify the process even further. Homologous integration of both constructs in an injected embryo has been successful, but these appear not to be forming translocations when they integrate. It is not understood why the homology arms are not (at least most of the time) being used in the insertion process. One possibility is that the integration events occur at cell cycle stages when NHEJ or other repair pathways dominate over HR. To overcome this potential problem, Cas9 is being expressed under the control of S/G2 phase controls using cell cycle-dependent degradation elements.
- Efforts continue to generate psyllid cell lines. This approach involves trying to simplify the process of immortalization by taking cells from embryos and introducing oncogenes and cell death inhibitors, and/or inactivating tumor suppressor genes. These approaches can allow cells to survive and proliferate even in the absence of some growth regulators that would normally need to be present, but that are unknown.
- A number of constructs designed to either overexpress or knockdown or knockout the expression of specific genes involved in cell death and cell proliferation using psyllid-derived reagents have been generated. Sequences that should encode the psyllid ubiquitin promoter or the baculovirus IE3 promoter are used for overexpression. For the Crispr/Cas9 targeting of specific genes fluorescent reporters are being used under the control of a ubiquitous promoter, linked to homology arms for the genes being targeted, components of the hippo/warts pathway.
- Efforts continue to generate transgenics using adult injection, thus far without success. Early efforts focused primarily on females. In the CLast cycle work was initiated on males, which is continuing.
- Work is in progress to have cleavage-based gene drive elements working in the fly by the end of the final quarter. While these are predicted to have low or intermediate threshold drive characteristics, they have the advantage that all components of the drive element are present in a single construct, and many different genes can in principal be used to generate these elements.

Diffusion

Once a nuPsyllid population is developed, its successful use will depend on series of factors based on the overall phenotype and fitness of the population in the environment and most importantly, will depend on human adoption, including the behavior of regulatory agencies, growers and consumers. All of these attributes must be modeled accurately for a nuPsyllid release to be used effectively. As for any other innovation, diffusion is the rate of change. Several aspects of the technical and communication plan can be addressed most effectively only when an actual candidate nuPsyllid is available for release. The ability to rear, release and monitor psyllids has been initiated and is of immediate use in HLB disease management applications outside of this proposal.

- There is a substantial effort to rear and release any type of nuPsyllid under development:
    - Florida, Texas, and California will each develop and maintain its own colony to provide nuPsyllids for initial greenhouse studies and pilot field releases within its borders. The decision as to where to house nuPsyllid colonies within each state will be likely have to be made at several administrative levels.
    - Regulatory agencies will likely require that nuPsyllid colonies be housed in a controlled/quarantine facility. Potential sites in each state were identified.
    - An estimated population size for a nuPsyllid required for testing cannot be provided until the driver mechanism is selected. The effector mechanism may have associated fitness costs, as well, and these will have to be figured into rearing effort estimates.
    - The initial plan is to piggyback nuPsyllid rearing efforts onto that of the existing parasitic wasp programs (*Tamarixia*) for initial testing with care to control for *Tamarixia* contamination.
- Induction of foliar volatiles: The development of 'super-stimuli' which are strong behavioral elicitors, may provide a means of boosting the efficacy of synthetic attractants by enabling them to outcompete background stimuli. Plant pathogens elicit the production of super stimuli in their host plants to make infected plants more attractive to insect vectors; examination of pathosystems may reveal the identity of potentially useful super-stimuli. Of significance to the CLas-ACP-citrus pathosystem, Dr. Lukasz Stelinski (UF) and his associates have shown that CLas-infected foliage emits the volatile signaling compound methyl salicylate, and that it acts as a super-stimulus in attracting uninfected ACP to CLas-infected trees. The emission of methyl salicylate (MeSA) is governed by the production of salicylic acid (SA), an internal signaler that is induced by pathogen infection. The Stelinski and Sétamou (TAMU) labs are developing scent attractants containing methyl salicylate.
- Another important attack/stressor signaler system in plants is the jasmonic acid/methyl jasmonate (MeJA) system. Exogenous application of MeJA to potted CLas- and CLas+ Valencia orange trees significantly altered volatile emission both quantitatively and qualitatively. In behavioral assays, ACP significantly aggregated at higher levels of MeJA-treated foliage.
- Exogenous applications of salicylic acid to CLas- and CLas+ Valencia trees resulted in: 1) Emission of a quantitatively greater amount of volatiles; 2) Production of high levels of MeSA, with this compound comprising 50% of the total amount of volatiles emitted; and 3) Absence of indole, E-jasmone and other compounds in the foliar odor induced by the application of MeJA.
- An emulsified wax carrier (SPLAT, ISCATech, Inc.) can be used to convey MeJA to citrus foliage. A high response level was achieved using a low viscosity SPLAT formulation containing only 10 mM MeJA that was sprayed on the foliage. Further work is needed to determine the duration of the expression of foliar volatiles induced by exposure to MeJA and to devise optimal loading levels for achieving a maximal response from the foliage.

- Field tests are planned to determine if spray treatment with MeSA and MeJA influence recruitment of ACP and its natural enemies to treated v. control potted orange
- Field trials of ACP scent attractants using DPI 3D psyllid traps: Field tests of ACP traps made with a 3D printer by Florida DPI are in progress. A scent lure formulation based on the volatiles emitted by orange jasmine flush and are being tested at different concentrations of this formulation in 3D printer traps placed in orange jasmine hedges infested with ACP.
- Testing synthetic ligands of olfactory binding proteins: A new rapid screening assay is being developed to measure ACP attraction to novel synthetic ligands of olfactory binding proteins isolated from the antennae of ACP. The goal of this project is to discover highly effective scent lure attractants, either on their own or in combination with terpene compounds that are emitted naturally in the aroma of host plant flush.
- The sustainability/economics/modeling team continued to develop analytical infrastructure based on the generic needs to understand system dynamics and potential impacts of technology adoption.
- Keynote papers by Ryan McAllister of CSIRO on the importance of social science in invasive species responses and by Paul Mitchell on the economic impacts of HLB on Florida citrus were given at IRCHL in March.
- Short versions of these papers are available online in the Journal of Citrus Pathology: http://escholarship.org/uc/iocv_journalcitruspathology
- The modeling team has been working with the CPDPC in California helping to disentangle the possible effects of inter-seasonal variation and ACP control treatments on the observed dynamics of ACP, particularly in the outbreak that occurred in Kern Co. in 2015-16. This work has been important in understanding the cost-effectiveness of the grower-funded urban treatment program in suppressing ACP in non-commercial situations.
- The outreach team conducts monthly teleconference calls to select and discuss projects that showcase research programs addressing the HLB problem. With funding from other agencies the subject matter has been expanded beyond the nuPsyllid program, with the goal of educating growers about new technologies (both genetic engineering and non-GE) for managing HLB. The Science for Citrus Health web site http://ucanr.edu/sites/scienceforcitrushealth/ was made public this quarter. It is divided into four sections: 1) Early Detection Techniques, 2) Strategies for Established Orchards, 3) Strategies that Require Replants, and 4) nuPsyllid. The team has posted the following research snapshots and additional research snapshots are planned for the next quarter. Some topics are found in two sections. Postcards announcing the web site were provided to citrus growers at meetings in Ventura, Santa Barbara, and Exeter CA during this quarter.
- Early Detection:
  Carolyn Slupsky; Metabolite changes in the tree can help detect HLB
  Ali Pourezza; Starch accumulation sensor for early detection of HLB
  Cristina Davis; Using volatile changes in citrus for early detection of HLB
- Established Orchards:

Bryony Bonning; A new, Bt toxin-based strategy for suppression of the Asian citrus psyllid vector
Michelle Cilia; Managing psyllid gut cells to block transmission of CLas
Lukasz Stelinski, Nabal Killiny; Using interference RNA to manage ACP
Bryce Falk; Using insect viruses to combat the Asian citrus psyllid

- Replants:
  Bryony Bonning; A new, Bt toxin-based strategy for suppression of the Asian citrus psyllid vector
  Jim Thomson; Founder lines used to improve HLB tolerance
- NuPsyllid:
  Bryce Falk; Using insect viruses to combat the Asian citrus psyllid
  Kirsten Pelz-Stelinski; Altering the Asian citrus psyllid's beneficial bacteria to stop HLB spread

SUMMARY

There are a number of excellent candidate effector targets including several identified in a functional screen. It would be ideal to test these candidates in a psyllid viral vector. The combined data strongly suggest an "invasion model" in which CLas/CLso transforms the endocytic/exocytic host pathways to facilitate internalization, infection, and circulation in the psyllid host and vector. Briefly, a model involving a putative phage gene that acts as an effector, which may operate in conjunction with a unique ACP gene to alter the function of genes associated with clathrin-mediated endocytosis, actin cytoskeletal rearrangements, and vacuolar formation, and exocytosis.

The translocation driver system is ready if the transformation bottleneck can be overcome. Because of the progress with the effector characterization and driver options, it is an important time for the team to continue to:

- select and prioritize effectors;
- obtain antibody reagents for top effector candidates;
- use the bioassay platform for comparative testing of the phenotypes in ACP, maximizing transmission blockage and minimizing fitness loss;
- accelerate development of the DCPLV vector and be prepared to use others that might be immediately useful for effector prioritization;
- analyze the phenotypes of both native and non-native *Wolbachia* introduced into ACP;
- determine if *Wolbachia* transformation is a feasible goal;
- develop ACP transformation capacity at any level of efficiency;
- continue to ready the engineered translocation and cleavage drive constructs;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions;

- optimize trap design for monitoring and other potential control applications;
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB
- provide support and continuity as additional teams are funded that can build these results into existing and pending research programs seeking HLB solutions.

CITRUS HOST INTERVENTION

Soil Microbial Management, Amendments, and HLB

This portion of the report summarizes research projects on soil and root microbial management to aid in HLB control that have been supported on behalf of the citrus industry by the Citrus Research and Development Foundation from 2010 to 2018. What have we learned and where we need to go? This research topic investigated the hypothesis that naturally occurring soil- and root-based bacteria could aid in the management of HLB. Such beneficial bacteria might also occur in commercially available microbial soil amendments and could act as antimicrobials or control agents against HLB bacteria.

The goal of the Wang project (608) was to characterize the effect of the application of beneficial bacteria (MICROBE Program) on the management of HLB. Three bacteria isolates, ATY16, PT6 and PT26A, isolated from Florida soils in previous studies, were tested for their ability to aid in the management of HLB. These isolates were able to promote seed germination and seedling growth of *Arabidopsis* in vitro and to increase grapefruit seedling emergence and growth of stronger root systems in the greenhouse. These three beneficial bacteria also reduced the HLB disease progression and slowed the CLas population growth compared with the non-treated control in a field trial with mild HLB affected citrus trees. In addition, each of the three bacteria has potentially beneficial traits for citrus including phosphate solubilization ability and ability to produce IAA, salicylic acid, nitrogen fixation traits and antibiotics against several plant pathogenic fungi and bacteria. Field trials suggested that after 10 soil drench applications over 19 months, the three bacteria treatments showed a reduced HLB disease severity and lowered CLas bacterial populations in leaf samples of young infected trees compared to the non-treated control trees with mild HLB symptoms when the experiment was initiated. A patent was filed for these beneficial bacteria, but future practical application of the beneficial bacteria will depend on the successful development of soil drencher to apply the microbes. In four field trials, 5 commercially available microbial products: Bioflourish, Ecofriendly, Serenade soil, Aliette, Quantum and an untreated control were tested in Lake Wales and Arcadia. The products were applied by irrigation. No significant effect was observed between the treatments and control in citrus growth, production, HLB symptom development, and CLas populations.

Soil-based antimicrobials as control agents against HLB were evaluated by Wang (780C/ RSA 13-02). The goal of this research was to test whether the rhizosphere soil from healthy appearing survivor trees could repress disease development in HLB inoculated seedlings in a greenhouse

study. Soil samples from around survivor trees were mixed with cow manure to improve soil texture and promote the growth of beneficial microbes. CLas populations in HLB-infected plants were monitored throughout a year. Eighteen plants appeared to be free of CLas after growing in suppressive soil for 12 months. The results suggested possible involvement of beneficial microbes in the health of survivor trees. To understand the potential involvement of beneficial bacteria in suppressing CLas, we isolated many bacteria groups from the soil and tested their effect on promoting plant growth at the presence of CLas. 35 bacteria containing potentially beneficial traits were identified. Survivor trees in the field and greenhouse tests suggest that some soil microbes delay the HLB symptom development.

Currently, ongoing research by Wang (#15-042) seeks to confirm whether the endophytic microbes from survivor trees can diminish effects of HLB, improve plant defenses and affect their attractiveness to psyllids. By comparing healthy and HLB infected root/soil samples, we found several bacteria phyla, such as Proteobacteria, Acidobacteria, and Bacteroidetes that were enriched in the healthy root-associated microbiome. Burkholderia sp. were isolated and inoculated into citrus plants using the soil drench method. Populations survived for 7 months, and expressions of beneficial traits were characterized. In addition, roots and shoots from survivor trees were grafted to healthy and HLB diseased trees in the greenhouse to check for the effect of endophyte changes in the grafted trees. Both healthy and HLB diseased trees were planted in soil from surviving trees in the greenhouse. Effects of application of potentially beneficial bacterial isolates on plant defenses and attractiveness to psyllids were also tested.

CRDF designed three separate field trials (928C) to test the hypothesis that commercially available soil-applied microbial products can mitigate the effects of HLB on citrus tree health and yield. There were six soil amendment treatments: BioFlourish, EcoFriendly, Serenade, Quantum, and Aliette plus an untreated control (Untrt Cont) applied to Valencia/Swingle trees at a Ridge site with 19-yr-old trees, at an East Coast site with 6-yr-old trees, and at a Southwest Florida site with 11-yr-old trees. Treatments were applied by contracted crop consultants over three seasons (2014–2016) at recommended label rates, and a subset of trees within each treatment was also mulched annually with mature cow manure. At the Ridge site after 3 years of treatments, the Serenade treated trees had a higher visible disease index (DI) than the Untrt Cont trees, but the other treatments had intermediate DIs that did not differ from each other. All treatments had similar average cycle threshold (CT) values from PCR that were less than 30 (HLB positive), and there were no treatment effects on canopy volume, fruit yield, average fruit weight, or juice quality. At the East Coast site, all trees were HLB positive, and no treatment CT values differed from the Untrt Cont trees. Again, there were no treatment effects on canopy volume, fruit yield, DI, average fruit weight or juice quality. At the SW Florida site, all treatment trees had average CT values that were greater than 30 (HLB negative). Aliette treated trees had higher CT values than EcoFriendly treated trees, while the other treatments had intermediate CTs that did not differ from each other. BioFlourish and Aliette treated trees had larger canopy volumes than the Untrt Cont and Serenade treated trees, but there were no treatment effects on DI or fruit yield. Here with little HLB, the mulch treatment increased yield, but there were no significant effects of mulch anywhere else. Overall, there were no persistent treatment effects

on root density in the soil or on leaf mineral nutrition as all nutrient values were within optimum ranges. Thus, there were no positive effects of these soil microbial amendments on tree health and yield of HLB-affected trees.

The bacterial survival in soil and effects of treatments on the soil bacterial community were determined (# 928.4). The results suggested that the application of these soil amendment products did not affect the citrus rhizosphere bacterial community because the additional bacteria products did not survive in the soil.

Plant Improvement and Development of HLB Resistant Rootstocks and Scions

Citrus genetic plant improvement for the development, evaluation, and delivery to the industry, of better than industry standard rootstocks and scions, is an important part of the CRDF research portfolio. In the current environment, identification of varieties that remain economically viable in the face of HLB is an urgent short term goal pursued concurrently with the longer term goal of finding economically viable HLB resistant varieties.

Plant breeding programs in Florida at both the University of Florida (UF) and the United States Department of Agriculture (USDA) continue to follow objectives to improve citrus. Over time, hundreds of rootstock and scion field trials have been established across the state to evaluate the performance of experimental germplasm in comparison to industry standards in the diverse environments. Evaluation and possible selection of varieties with economic viability from these pre-HLB legacy trials is an ongoing part of the core breeding programs (Bowman 15-002 and 15-003, Gmitter 15-010, Grosser 736). Field trial support continues for new projects evaluating populations generated with HLB resistance as a primary of objective or more advanced materials selected for some degree of HLB tolerance (Bowman 15-002 and 15-003, Gmitter 15-010, Grosser 736, Stover 15-025, and 15 026, Rogers 15-030c).

Other short term projects target citrus physiology using scion varieties exhibiting HLB tolerance as interstocks to potentially transmit tolerance via the graft union to more susceptible rootstocks or scions (Gmitter 15-010).

Longer term breeding projects involve germplasm screening to identify potential parents for economically important traits such as yield and fruit quality as well as sources of resistance to diseases such as HLB and citrus canker.
Several germplasm screens have identified HLB tolerance in several *Citrus*, *Poncirus* and other citrus relatives compared to commercial varieties (Gmitter 724, LaPointe 853, Stover 15-025). Hundreds of hybridizations have subsequently been made to generate populations which can be screened for HLB tolerance and commercial performance (Gmitter 15-010, Stover 15-025). Populations that are segregating for tolerance/resistance genes, as well as other genes conferring commercially important traits, can also serve as tools for genetic mapping studies to identify and locate genes of interest (Gmitter 724,Stover 15-025). Other studies are utilizing similar populations to differentiate the types and quantities of volatile chemicals produced by

different populations of citrus when exposed to HLB as potential tools for identifying tolerant varieties earlier in the breeding process.

In addition to conventional projects looking for native sources of disease resistance, many projects are using a combination of conventional and biotechnology techniques as means of generating HLB resistant varieties. New genes conferring resistance to HLB and other diseases may be introduced into citrus through several methods including Agrobacterium mediated transformation, biolistic particle delivery (gene-gun) and new techniques such as genome editing.

Supporting research in genetic solutions to HLB also includes funding long term exploratory research as well as the development of tools which may accelerate research and delivery of solutions. Several projects have worked on tools to improve laboratory techniques, the success rate of generating transgenic plants and confirmation of transgene presence (Orbovic 15-033c, Zale 15-045c). Researchers have identified genes which improve transformation efficiency and precision of gene engineering (Li 747, Li16-001, Zale 15-045c). The long juvenility period in citrus increases the number of years needed to determine whether an experimental variety will produce commercially acceptable fruit. Several projects have identified a citrus early flowering gene (FT) and successfully produced transgenic citrus plants which flower much earlier than the expected juvenility period (Moore 573 and Grosser 547).

Other projects are evaluating unique sources of HLB resistance including synthetic antibodies targeting a *C*Las membrane protein (McNellis11-125 424). The Nodt transgenic 'Duncan' grapefruit plants have been characterized in the laboratory will be evaluated in the field for HLB tolerance and general horticultural performance. Another project targeting systemic acquired resistance genes such as NPR1 has generated plants which show tolerance to HLB and separately potential citrus canker tolerance (Mou 754, Mou 15-020).

A gene causing susceptibility to citrus canker was identified in citrus and genome editing techniques used to disrupt the expression of the CsLOB1 gene. As a result, several Duncan grapefruit plants with canker resistance were generated (Wang 922). Research to improve the precision of the gene-editing techniques to generate plants which do not contain the foreign DNA for regulatory purposes in addition to generating canker resistant plants of economically important varieties is ongoing.

The plant improvement research into HLB, citrus canker and other traits of interest is facilitated by CRDF funding for service and support projects such as the core citrus transformation laboratory for juvenile citrus (Orbovic-15-033c) and the mature transformation laboratory (Zale 15-035c). These transformation laboratories provide transformation services for researchers as well as continue to work on improving transformation techniques and transformation efficiency. A key step in HLB resistance research is efficiently exposing citrus plants of interest to *C*Las infected Asian Citrus Psyllids (ACP) to inoculate the plants with the bacteria to test for tolerance or susceptibility. A USDA laboratory in has developed an effective method for rearing

and maintaining *C*Las infected ACP and an effective high-throughput assay for infecting plants (Hall 15-016c).

Once varieties have been selected for superior performance in field trials and a decision to release them to the industry is made, various steps to must be taken to provide disease free propagation plant material to the industry through DPI. Recently, a shortage of available seeds for rootstock propagation has resulted in an increase of propagation through cuttings and tissue culture of newly released rootstocks. It is important to determine ideal conditions for propagation techniques which require different chemical treatments than seed propagation. It has been determined that although greenhouse conditions may vary to some degree and therefore require slight changes in light, temperature and hormone levels for propagation, there does not a appear to be difficulty in propagation that is rootstock or scion specific (Beeson 940c).

Supplemental Citrus Nutrition and HLB

From the first time Huanglongbing (HLB, Citrus greening) disease was recognized in citrus, it has been characterized as "nutrient deficiency-like" disorder. Thus, there has been considerable interest in the role that nutritional programs, both foliar and soil, may play in sustaining Florida citrus tree health in the presence of HLB. This communication summarizes research projects on citrus nutrition/HLB that have been supported on behalf of the citrus industry by the Citrus Research and Development Foundation from 2010 to 2018. What have we learned and where we need to go?

Analyses comparing HLB symptomatic (blotchy mottle from starch accumulation) and asymptomatic leaves from healthy trees have shown that HLB increased leaf K while Mg, Ca, and B decreased. The micronutrients Zn and Mn, whose deficiency symptoms are commonly seen on HLB-infected trees, were not actually deficient in HLB-infected samples when the dry mass of the samples was corrected for the large amounts of starch accumulation caused by HLB. Since root decline and phloem disruption impact uptake and delivery of nutrients, any stress to trees will accentuate symptom development and tree decline. Clearly, supplemental nutrient applications can compensate for some HLB symptoms and declining root systems and potentially allow trees to maintain profitability. It was logical to assume that foliar applications of nutrients could reduce the effects of HLB to overcome the root uptake limitations and to promote nutritional therapy. This facilitated the development of the therapeutic concept of "spoon-feeding" nutrients (and irrigation) in small frequent doses. There are differences in absorption efficiency of both macro- and micro-nutrients by roots and leaves so the mode of application (foliar vs. soil) continues to be controversial depending on soil pH or leaf permeability.

The National Academy of Science (NAS) planning effort commissioned by the Florida citrus industry in 2009, made reference to citrus nutrition as a component of cultural practices for new approaches to sustaining citrus in the presence of HLB. The NAS Evaluation Team reported that supplemental levels of micronutrients have been credited with eliminating mineral

deficiency symptoms associated with HLB. Such trees will not be cured of HLB infection, however, and may continue to serve as sources of inoculum.

Beginning with the organized response to HLB in 2008, research projects have included consideration of how cultural practices affect disease development and spread and have addressed nutritional effects on HLB originally funded by FCPRAC. Many of these projects (for example, the evaluation of intensively managed fertigation in high-density plantings, #191 Schumann), fit well into the discussions and recommendations provided by the NAS team. Other nutrition research projects have expanded beyond the scope of the NAS priorities to include maintaining productivity, decreasing fruit drop, increasing root health and HLB effects on fruit quality.

The following is a brief summary of recent and continuing projects focused on nutrition/HLB funded through CRDF. Like other areas of research, institutional investments (e.g., UF, IFAS and USDA, ARS) complement the nutrition projects funded by CRDF. Individual grower-sponsored trials and experiments are prevalent across the state, and fertilizer suppliers are investing resources in evaluating nutritional responses in HLB trees.

Adequate nutrients need to be supplied at appropriate rates to avoid deficiency levels in leaves. Since roots are adapted to absorb nutrients and leaves are not, if roots are sufficiently healthy, root uptake of most nutrients is more efficient than foliar uptake. Small frequent doses of irrigation water and nutrients appears to be a pathway to aid recovery of declined root systems. High doses of some minor nutrients including B (#329 Schumann) have also been shown to be of benefit (#15-013 Grosser). Rouse and Morgan (#179) found that applications of 3 times the annual recommended amounts of selected micronutrients increased trees growth and yield, however, applications of 6 times recommended rates of minor elements on an annual basis could increase tree vegetative growth at the expense of fruit yield. In irrigation management studies, Morgan showed that reduced water uptake by HLB affected trees was proportional to reductions in tree leaf area. This supports spoon feeding irrigation water to HLB compromised trees to maximize water uptake and growth. In this regard, there have been a number of studies reporting good success with maintaining tree health using “constant” nutrition of from fertigation (#191 Schumann) and from slow release materials (# 548 Grosser).

Stress from high pH in the soil and bicarbonates in irrigation water (#731 Graham) can decrease leaf nutrient status (#838 Morgan) and increase the susceptibility of HLB infected trees to other infectious disease such as *Phytophthora* (# 545 Graham). Long-term previous recommendations for soil pH have been a target pH of 6-6.5, and recent studies (#731 Graham) have focused on a target pH of 5.5-6. Such low pH levels should be viewed with caution, however, as they can solubilize toxic levels of Cu and can leach out necessary Ca, Mg and K.

Current Status of Understanding the Role of Supplemental Nutritional Programs
There is high tree-to-tree variability in yield that is both site and season dependent which is worsened by HLB infection and disease decline. Additionally, the rate of decline of HLB-infected groves is highly dependent on the age of infection and overall condition of the grove, including

genotype, environmental interactions, soil, pH, salinity, root disease and the overall level of abiotic and biotic stress already imposed on a specific site. Schumann's current project (#15-023 through 2018) seeks to understand the variability in response to enhanced nutritional programs and to define optimal nutritional therapies to maintain root growth and target leaf levels of nutrients. It is likely that, in the presence of HLB, the range of nutritional requirements for optimal yield is narrowed and/or shifted by HLB infection and the general susceptibility to stress, possibly including the stress of psyllid feeding (#447 Stansley). Rouse's project (#179) incorporated measurements of phloem health in HLB symptomatic versus asymptomatic trees with/without supplemental nutritional treatments and psyllid management. Stansly (#477) evaluated psyllid management and nutritional treatments on growth and productivity in field plots, and it was clear that aggressive psyllid management should continue even after all trees are HLB positive.

Tree to tree variations make randomized, controlled nutritional trials difficult. Boman's (#903) supplemental foliar K in grapefruit studies failed to show significant effects even though there was an indication that foliar K supplements increased grapefruit size. It is difficult to adequately test nutritional trials over multiple years when dealing with tree-to-tree variations from HLB. A more practical alternative may be to develop a system to aggregate data from profitable grower experience that is based on large acreage commercial operations, data mining, and meta-analysis. Initial attempts at this were not successful, but CRDF is currently entertaining ways to synthesize nutritional management programs that appear to be superior in maintaining tree health and yield.

Currently, there is no better HLB management practice than to manage frequent irrigation and balanced nutrition applications to avoid stress coupled with good psyllid control. During July and August 2017, CRDF formed a committee made up of growers, researchers and CRDF staff to interview successful growers to determine if there were best management practices in this HLB era. The committee visited four anonymous growers, 2 on the Central Ridge and 2 in SW Florida. Discussions dealt with a wide breadth of varieties, rootstocks, tree ages and soil types. There was a free exchange of information and the committee is confident that they we gathered a good representation of what successful Florida citrus growers are doing. Clearly, there were common practices among these growers that included block-specific management of irrigation, nutrition and pest control. Spoon-feeding was a common denominator regardless of whether fertilizer programs were by fertigation, granular, foliar or controlled release formulations. All growers felt that they were better managers now than they were before HLB. In general, growers were willing to modify scheduling, rates and formulations as required, even as frequent as every month, to avoid any nutrient stress.

CRDF will continue to consider justifiable research proposals to address solutions to HLB and to work with the industry via CRDF committees to encourage growers to share data associated with their successful as well as less successful field trials. With hundreds of such trials spread across the state, there is much we can learn through interpreting the common features that lead to success. These are underutilized resources that could lead to a better understanding of the optimum role of supplemental nutrition in HLB citrus management.

Plant Growth Regulators to Mitigate HLB Symptoms

This communication summarizes research projects on the use of plant growth regulators (PGRs) to aid in HLB management and control that have been supported on behalf of the citrus industry by the Citrus Research and Development Foundation from 2009 to 2018. What have we learned and where we need to go? This research topic investigated hypotheses that PGRs could be of use in killing diseased trees, as aids in growth flush management for psyllid control, and to reduce HLB symptoms especially preharvest fruit drop.

Trees infected with HLB are a reservoir for spread of the disease by the Asian citrus psyllid (ACP) vector. Growers often want to kill infected trees in-place after scouting and then wait until one time a year to physically remove the trees. Project 200 (Singh) investigated the possibility of eliminating HLB infected trees without physical removal through application of herbicides. Although a successful trunk cutting, herbicide spray applicator device was constructed and mounted on a small tractor, little industry interest evolved in its use because growers became more interested in saving trees through nutritional management.

Burns (204) sought to determine effects of summer and fall hedging timing of 'Hamlin' and 'Valencia' sweet orange on new flush growth in conjunction with the use of the PGR prohexadione-calcium (Apogee) to control regrowth to aid in psyllid management. Hedging practices can be modified to affect flushing patterns in a way that benefits ACP control strategies. However, since environmental effects on flushing are poorly understood in citrus and the hedging effect cannot be consistently reproduced, it is likely not to be adapted by growers. ACP feeding on PGR-treated, infected plants acquired CLas at a higher rate than did ACP held on untreated, CLas-infected plants. The effect of PGRs on ACP did not appear to be due to growth retardation or specific toxicity of the materials, suggesting a yet undefined effect on plant physiology that altered host quality.

Albrigo (776C, 777C, 778C and 779C) tested if frequent Low Rate Application of 2-4,D and Cytokinin affected plant symptom reduction in HLB affected trees. Preharvest fruit drop rates were variable from year to year. Tests did not support the idea that 2, 4-D could consistently reduce preharvest drop associated with HLB disease when applied in late January or before harvest. Tests did support the idea that more declined HLB trees will usually drop more of their fruit than declining trees before harvest especially in severely declined trees.

Grubber (894) tested the question "Are there declines in hydraulic conductivity and drought tolerance associated with HLB"? Supplemental support to expand plant growth regulator trials to determine the efficacy of plant growth regulators (PGRs) as a tool to mitigate declines in citrus tree root and canopy growth resulting from HLB. Results were not conclusive and did not support any effects of 2,4-D in reducing citrus fruit drop.

Field trials (942C) were designed by CRDF staff established at four locations, 2 sites at 2 central Florida locations to determine if a single on-label application of 2,4-D (Citrus Fix) spray late December (2014 and 2015) could mitigate 'Valencia' preharvest fruit drop from HLB affected trees. The grove floor under 32 similar trees at each site was raked prior to treatment and 16

trees were sprayed with Citrus Fix. Approximately every two weeks thereafter all trees were checked for fruit drop counts until harvest in March 2015 or April 2016. Total overall fruit drop was quite high (from 25% to 76% drop) and variable from tree to tree and from location to location. One trial was harvested before final counts could be made. Combining the percentage of fruit drop results from 7 remaining trials at four sites over the 2 years, the percentage of fruit drop was significantly reduced from 5% to 28% by the single 2, 4-D treatment but in only 3 of the 7 trials. This work resulted in a publication entitled, “A Single Application of 2,4-D Can Decrease Preharvest Fruit Drop in HLB-Affected ‘Valencia’ Orange Trees” in the 2015 Proc. Fla. State Hort. Soc. 128: 70-72.

Strigolactones (SL) are plant growth regulating hormones that may be of use to combat HLB in Florida and Etxeberria (899) is engaged in evaluation. Strigolactones applications by foliar spray or soil drench in greenhouse HLB trees resulted in increases in growth flushes, increased vascular phloem (by microscopy) and increased root growth over non-treated HLB trees. Thus, Strigolactones may regulate shoot architecture of HLB trees resulting in restored tree health. Presently SL are relatively expensive, however, yet costs have declined substantially (reduced in half) during the experimental period. Although effective in improving health to HLB-affected trees in the greenhouse, the cost remains prohibited at the moment making it unlikely to be of commercial use in ameliorating HLB symptoms of trees in the field.

FY 2016-17 Active CRDF Project List

Project #	PI	Institution/Organization	Project Title	Contract Total	Start Date	End Date
424	McNellis, Timothy	Pennsylvania State University	Functional disruption of the NodT outer membrane protein of Candidatus Liberibacter asiaticus for rootstock-mediated resistance to citrus greening using a phloem-directed, single-chain antibody	$ 55,000.00	7/1/2011	12/30/2016
447	Stansly, Phil	University of Florida	Role of Nutritional and Insecticidal Treatments in Mitigation of HLB in New Citrus Plantings	$ 324,430.00	2/15/2012	2/14/2016
582	Pelz-Stelinski, Kirsten	University of Florida	Factors influencing transmission of the huanglongbing (greening) pathogen by the Asian citrus psyllid and methods for interrupting the transmission process	$ 577,703.00	5/1/2012	2/29/2016
590	Rogers, Michael	University of Florida	Enhancing psyllid control through a better understanding of the effects of pesticide applications on psyllid feeding and mortality	$ 889,701.00	5/1/2012	4/30/2015
593	Schumann, Arnold	University of Florida	Advanced Production Systems (ACPS) for efficient, sustainable citrus groves	$ 414,039.00	7/1/2012	6/30/2015
598	Schumann, Arnold	University of Florida	Bringing young citrus trees infected with Candidatus Liberibacter asiaticus into production using intensive horticultural management strategies.	$ 152,952.00	7/1/2012	6/30/2015
601	Stansly, Phil	University of Florida	Effective and Sustainable Insecticidal Control of Citrus Leafminer, Phyllocnistis citrella (Stainton) Lepidoptera: Gracillariidae) to Slow Spread of Citrus Canker Disease.	$ 205,539.72	6/1/2012	5/31/2015
616	Rogers, Michael	University of Florida	Ultra High Performance Liquid Chromatography – Pesticide Residue Analysis	$ 172,500.00	5/1/2012	4/30/2015
711	Bonning, Bryony	Iowa State University	Identification of Bacillus thuringiensis endo-toxins active against Adult Asian Citrus Psyllid	$ 500,000.00	5/1/2013	4/30/2017
715	Dewdney, Megan	University of Florida	The leaf litter cycle of citrus black spot and improvements to current management practices	$ 577,352.00	5/1/2013	12/31/2016
716	Dewdney, Megan	University of Florida	Improved fungicide control measures for pre- and post-harvest management of citrus black spot	$ 289,300.00	4/1/2013	6/30/2016
717	Duan, Yongping	USDA	Control Citrus HLB by Blocking the Function of two Critical Effectors Encoded by Candidatus Liberibacter asiaticus	$ 410,000.00	5/1/2013	4/30/2016
724	Gmitter, Fred	University of Florida	Accelerating Citrus Gene Discovery for HLB Tolerance/Resistance	$ 399,500.00	5/1/2013	4/30/2016
726	Gonzalez, Carlos	Texas AgriLife Research	A Bacterial Virus Based Method for Biocontrol of Citrus Canker 54% of total	$ 468,016.00	5/1/2013	10/31/2016
730	Graham, Jim	University of Florida	Monitoring streptomycin resistance in Xanthomonas citri in support of FireWall registration for canker	$ 141,129.00	5/1/2013	4/30/2016
731	Graham, Jim	University of Florida	Calcium carbonate may reduce root health and exacerbate HLB expression	$ 198,214.00	4/1/2013	3/31/2016
732	Graham, Jim	University of Florida	Understanding and reducing early root loss in HLB affected trees	$ 359,931.00	4/1/2013	3/30/2016
736	Grosser, Jude	University of Florida	Expedited Indian River Evaluation of Tetrazyg Rootstocks Surviving the HLB-Gauntlet	$ 96,538.00	4/1/2013	3/31/2016
749	Li, Yi	University of Conneticut	Development of Technologies Important for Creation and Commercialization of Transgenic HLB Resistant Citrus	$ 229,683.00	5/1/2013	6/30/2016
750	Ma, Wenbo	University of California - Riverside	Identification of key components in HLB using effectors as probes	$ 299,781.00	4/1/2013	3/31/2016
752	Moore, Gloria	University of Florida	Cell Penetrating Peptides for Citrus Genetic Improvement and Disease Resistance	$ 136,251.00	5/1/2013	4/30/2016

0	PI	Institution/Organization	Project Title	Contract Total	Start Date	End Date
754	Mou, Zhonglin	University of Florida	Application of a natural inducer of systemic acquired resistance and engineering non-host resistance in citrus for controlling citrus canker	$ 366,032.00	5/1/2013	10/31/2016
759	Santra, Swadeshmukul	University of Central Florida	Fixed-Quat: A novel alternative to Cu fungicide/bactericide for preventing citrus canker	$ 260,050.00	4/1/2013	7/31/2016
767	Triplett, Eric	University of Florida	Rapid identification of antibiotics useful in the control of citrus greening disease	$ 406,637.00	4/1/2013	6/30/2016
782	Triplett, Eric	University of Florida	Evaluation of Candidate Antimicrobial Compounds or Combination of Compounds using Liberibacter crescens adday, for Efficacy in Reducing Titer in Bacterium Candidatus Liberibacter asiaticus as Control Agents Against HLB Individually and in Combination	$ 26,040.00	7/1/2013	6/30/2015
803	Duan, Yongping	USDA	Characterization and manipulation of the prophages/phages of 'Candidatus Liberibacter asiaticus' for the control of citrus huanglongbing	$ 574,219.00	5/1/2014	4/30/2017
805	Long, Sharon	Stanford University	Functional genomics of Liberibacter in a model system	$ 540,197.00	4/1/2014	9/30/2016
809	Albrigo, Gene	University of Florida	Citrus preharvest drop related to HLB disease-Nature and control	$ 240,000.00	4/1/2014	3/31/2017
834	Duan, Yongping	USDA	Optimizing Heat Treatment in the Fields and Understanding the Molecular Mechanism Behind the Success of Thermotherapy for the Control of Citrus HLB	$ 385,900.00	4/1/2014	3/31/2016
838	Morgan, Kelly	University of Florida	Effect of selected concentrations of calcium bicarbonate on expression of HLB in the greenhouse and grove	$ 349,491.00	5/1/2014	4/30/2017
850	Albrigo, Gene	University of Florida	Scheduling ACP spring spray selection based on the Citrus Flowering Model	$ 90,000.00	4/1/2014	3/31/2017
853	LaPointe, Stephen	USDA	Why is Poncirus trifoliata resistant to colonization by Asian citrus psyllid?	$ 187,681.00	5/1/2014	8/30/2016
858	Santra, Swadeshmukul	University of Central Florida	New non-phytotoxic composite polymer film barrier as ACP repellent for controlling HLB infection	$ 350,000.00	4/1/2014	3/31/2017
873	McCollum, Greg	USDA	Application of new technologies to expedite cleaning of new accessions for use in Florida	$ 180,000.00	9/1/2014	8/31/2016
899	Etxeberria, Ed	University of Florida	Strigolactones type growth regulators to combat HLB in Florida	$ 347,426.00	5/1/2014	4/30/2017
903	Boman, Brian	University of Florida	Establishing citrus nutrition trials for young & mature trees in the Indian River Region to promote plant growth, mitigate HLB, decrease fruit drop, and improve postharvest fruit storage properties	$ 360,000.00	7/1/2014	6/30/2017
907	Johnson, Evan	University of Florida	Zinkicide: A novel therapeutic zinc particulate based formulation for preventing citrus canker and HLB.	$ 203,744.00	6/1/2014	3/31/2016
910	Powell, Chuck	University of Florida	An integrated approach for establishment of new citrus plantings faced with the HLB threat	$ 369,714.00	4/1/2014	3/31/2017
916	Wang, Nian	University of Florida	Screening and application of antibacterial producing microbes to control citrus Huanglongbing	$ 431,180.00	7/1/2014	6/30/2017
919	Dewdney, Megan	University of Florida	A method to monitor for Guignardia citricarpa (Gc) ascospores in Florida groves.	$ 42,650.00	5/1/2014	10/31/2015
921	Schneider, William L.	USDA	Determining the role of a novel virus in Citrus blight.	$ 400,000.00	10/1/2014	9/30/2016
922	Wang, Nian	University of Florida	Control citrus canker by manipulating the EBE (effector binding element) of CsLOB1 which is the citrus susceptibility gene for citrus canker disease	$ 436,045.00	4/1/2014	3/31/2017

0	PI	Institution/Organization	Project Title	Contract Total	Start Date	End Date
925	Dutt, Manjul	University of Florida	Diaprepes control using a plant based insecticidal transgene approach	$ 80,000.00	7/1/2014	12/30/2016
15-002	Bowman, Kimberly	USDA	Development of Supersour and Other Promising Rootstocks for Florida.	$ 959,860.00	7/1/2015	6/30/2018
15-003	Bowman, Kimberly	USDA	Metabolomic profiling to accelerate development of HLB tolerant rootstocks	$ 539,104.00	7/1/2015	6/30/2018
15-005	Dewdney, Megan	University of Florida	Asexual inoculum production of Guignardia citricarpa, the causal agent of citrus black spot	$ 255,227.00	7/1/2015	6/30/2018
15-008	Etxeberria, Ed	University of Florida	Determination of CLas signal in HLB-affected citrus trees	$ 53,572.00	7/1/2015	12/31/2016
15-009	Gabriel, Dean	University of Florida	Exploiting the Las phage for potential control of HLB	$ 419,500.00	8/1/2015	7/31/2017
15-010	Gmitter, Fred	University of Florida	Development and Commercialization of Improved New Disease Resistant Scions and Rootstocks - the Key For a Sustainable and Profitable Florida Citrus Industry	$ 1,797,148.00	11/1/2015	10/31/2018
15-013	Grosser, Jude	University of Florida	Understanding and Manipulating the Interaction of Rootstocks and Constant Nutrition to Enhance the Establishment, Longevity and Profitability of Citrus Plantings in HLB-Endemic Areas.	$ 340,778.00	10/1/2015	9/30/2018
15-016C	Hall, David	USDA	High-Throughput Inoculation of Transgenic Citrus for HLB Resistance	$ 375,000.00	8/1/2015	7/31/2018
15-017	Killiny, Nabil	University of Florida	Disrupt LuxR solo quorum sensing that mediates plant virulence and insect transmission of Candidatus Liberibacter asiaticus to control the disease	$ 157,144.00	8/1/2015	7/31/2017
15-020	Mou, Zhonglin	University of Florida	Create citrus varieties resistant to Huanglongbing (HLB) through transgenic and nontransgenic approaches	$ 112,688.00	7/1/2015	6/30/2018
15-021	Pelz-Stelinski, Kirsten	University of Florida	Regulation of Las transmission and microbial colonization by the Asian citrus psyllid immune system	$ 185,732.00	9/1/2015	8/31/2017
15-022	Reuber, T. Lynne	Two Blades Foundation	Engineering citrus for canker resistance	$ 367,598.00	7/1/2015	6/30/2018
15-023	Schumann, Arnold	University of Florida	Citrus nutrition studies for improved survival of HLB-affected trees	$ 281,804.00	7/1/2015	6/30/2018
15-024	Stelinski, Lukasz	University of Florida	Predicting When, Why, and Where Asian citrus psyllids move to increase effectiveness of insecticide sprays.	$ 161,116.00	8/1/2015	7/31/2017
15-025	Stover, Ed	USDA	HLB Resistance and Tolerance in Citrus Scion Breeding	$ 441,935.00	10/1/2015	9/30/2018
15-026	Stover, Ed	USDA	Implementing Transgenic Tools to Produce Commercial Scion Cultivars Resistant to HLB and Canker	$ 540,758.00	8/15/2015	8/14/2018
15-027	Triplett, Eric	University of Florida	Developing a culture medium for Liberibacter asiaticus through comparative multi 'omics analysis with its closest cultured relative, L. crescens	$ 325,912.00	7/1/2015	6/30/2017
15-028	Wang, Nian	University of Florida	Control citrus Huanglongbing (HLB) by counteracting the SA hydroxylase of Candidatus Liberibacter asiaticus	$ 430,697.00	7/1/2015	6/30/2018
15-030C	Rogers, Michael	University of Florida	Continuing Field Trial Support for CRDF CPDC	$ 322,129.00	7/1/2015	6/30/2017
15-031C	Etxeberria, Ed	University of Florida	Development of a laser-based system to deliver antimicrobials to citrus trees: Greenhouse testing.	$ 197,585.00	7/1/2015	6/30/2016
15-032C	Irey, Mike	US Sugar Corp/Southern Gardens	Continued Support for the Southern Gardens Diagnostic Laboratory	$ 294,677.00	7/1/2015	6/30/2017

0	PI	Institution/Organization	Project Title	Contract Total	Start Date	End Date
15-033C	Orbovic, Vladimir	University of Florida	Support role of the Citrus Core Transformation Facility remains crucial for research leading to production of Citrus plants that may be tolerant or resistant to diseases.	$ 270,000.00	7/1/2015	6/30/2018
15-034C	Roberts, Pam	University of Florida	Continuation of diagnostic service for growers for detection of Huanglongbing in citrus and psyllids to aid in management decisions	$ 287,688.00	7/1/2015	6/30/2017
15-035C	Rogers, Michael	University of Florida	Continuing support of Citrus Health Management Areas (CHMA's)	$ 155,017.00	7/1/2015	6/30/2017
15-036C	Rogers, Michael	University of Florida	Correlating pesticide residue analysis with psyllid feeding to improve protection of young trees	$ 451,603.00	7/1/2015	6/30/2018
15-037C	Santra, Swadeshmukul	University of Central Florida	T-SOL™ antimicrobial for the management of citrus canker and HLB	$ 240,224.00	7/1/2015	6/30/2017
15-038C	Stelinski, Lukasz	University of Florida	Insecticide resistance monitoring and management in Florida citrus to maintain sustainable control of Asian citrus psyllid within Citrus Health Management Areas	$ 129,491.00	7/1/2015	6/30/2017
15-039C	Stover, Ed	USDA	Secure site for testing transgenic and conventional citrus for HLB and psyllid resistance	$ 260,980.00	1/1/2016	12/31/2018
15-040C	Triplett, Eric	University of Florida	Rapid turn-around evaluation of up to 1200 promising antimicrobial compounds (or combinations), using the L.crescens assay	$ 26,040.00	7/1/2015	6/30/2016
15-042	Wang, Nian	University of Florida	Control citrus Huanglongbing using endophytic microbes from survivor trees	$ 467,000.00	7/1/2015	6/30/2018
15-043C	Wang, Nian	University of Florida	Rapid turn-around evaluation of up to 25 antimicrobial compounds for efficacy in reducing titers of the bacterium Candidatus Liberibacter on diseased 6-year old trees Hamlin on Swingle.	$ 55,000.00	7/1/2015	6/30/2016
15-045C	Zale, Janice	University of Florida	Continued Funding for the Mature Citrus Facility to Produce Disease Tolerant, Transgenic Citrus	$ 928,550.00	7/1/2015	6/30/2018
15-048C	Minter, Tom	Florida Pesticide Research. Inc.	Field Trials of Bactericide Application Methods.	$ 74,250.00	10/1/2015	3/31/2017
15-049C	Booker, Brad	Florida Ag Research	Evaluation of minimal-risk and biopesticide products as a protectant and therapy for HLB	$ 14,000.00	11/1/2015	3/31/2017
15-050C	Behlau, Franklin	Fundecitrus	Effect of windbreaks, copper bactericides and citrus leaf miner control on temporal and spatial progress of citrus canker.	$ 44,000.00	11/1/2015	10/31/2016
16-001	Li, Yi	University of Conneticut	Enhancing Genetic Transformation Efficiency of Mature Citrus	$ 314,168.00	7/1/2016	6/30/2019
16-009	Triplett, Eric	University of Florida	Developing second generation antimicrobial treatments for citrus greening disease.	$ 248,744.00	7/1/2016	6/30/2018
16-010C	Dewdney, Megan	University of Florida	Enhancement of postbloom fruit drop control measures	$ 268,643.00	3/1/2016	2/28/2018
16-011C	Adair, Robert C.	Florida Research Center for Agricultural Sustainability	Increasing the yield and decreasing the bearing age of citrus trees in new plantings by using metalized reflective mulch while determining ACP populations.	$ 89,439.00	7/1/2016	6/30/2017
16-015C	Irey, Mike	US Sugar Corp/Southern Gardens	Enhanced Fruit Quality Assessment from Field Trials. RSA	$ 10,000.00	3/1/2016	6/30/2017
16-016C	Irey, Mike	US Sugar Corp/Southern Gardens	Use of RNAi delivered by the Citrus Tristeza Virus Ciral Vector to control the Asian Citrus Psyllid	$ 554,485.00		
16-017C	Tetard, Laurene	University of Central Florida	Quantitative Detection and Mapping of Bactericides in Citrus	$ 51,553.00	7/1/2016	6/30/2017
726L	Gonzalez, Carlos	Texas AgriLife Research	A Bacterial Virus Based Method for Biocontrol of Liberibacter	$ 386,902.00	5/1/2013	10/31/2016

Project #	PI	Institution/Organization	Project Title	Contract Total	Start Date	End Date
776C	Albrigo, Gene	University of Florida	Frequent Low Rate Application of 2-4,D and Cytokinin to study plant symptom reduction in HLB affected trees	$ 132,660.00	1/1/2014	6/30/2016
801nu	Browning, Harold	Citrus Research and Development Founfation, Inc.	Rear and Release Psyllids as Biological Control Agents - An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) disease.		9/1/2012	8/31/2017
926.1C	LaPointe, Stephen	USDA	Sub on Large-scale mating disruption of citrus leafminer validation and product launch	$ 80,000.00	2/1/2014	1/31/2016
927C	Rogers, Michael	University of Florida	Field Trial Support for CRDF CPDC	$ 331,466.00	3/1/2014	6/30/2015
928.1C	Sutherland, Dudley	Glades Crop Care, Inc	Field Trial of Naturally Occuring Microbes	$ 100,788.00	4/11/2014	3/31/2017
928.2C	Booker, Brad	Florida Ag Research	Field Trials of Soil Microbials to combat HLB - Ridge Site crop Consultant	$ 81,146.00	4/1/2014	3/31/2017
928.3C	Yonce, Henry	KAC Agricultural Research, Inc.	Field Trials of Soil Microbials to combat HLB - Southwest FL Site crop Consultant	$ 187,296.00	4/1/2014	3/31/2017
928.4C	Wang, Nian	University of Florida	Field Trials of Naturally occuring microbes to combat HLB	$ 99,156.00	4/1/2014	3/31/2017
929.2C	Rucks, Phil	Phillip Rucks Citrus Nursery, Inc.	Field Trial of HLB Tolerant RootstockPRCN Contract #2659 deposit on orange trees paid March 2014; delivery in Spring 2015. Trees are tagged, inspection by appointment only.	$ 124,740.00	3/25/2014	5/31/2016
931C	Gonzalez, Claudio	University of Florida	In Vitro testing of chemicals on tree leaves collected from HLB-infected trees to determine their efficacy against HLB	$ 203,917.50	2/1/2014	7/31/2015
932.1C	Keesling, James	University of Florida	Mathematical Modeling to evaluate Psyllid Shield Concept	$ 113,523.00	6/1/2014	5/31/2016
934.1C	Wang, Nian	University of Florida	Soil Drenches of products to combat initial HLB infection in young citrus trees	$ 11,028.00	6/1/2014	5/31/2016
934C	Curtis, John	Better Crops, LLC	Soil Drenches of products to combat initial HLB infection in young citrus trees	$ 15,000.00	6/1/2014	9/30/2016
935C	Wang, Nian	University of Florida	Assays - continuation testing of Powell RSA 1 - antimicrobials	$ 88,000.00	7/1/2014	6/30/2015
936C	Richardson, Taw	AgroSource, Inc.	Firewall Section 18 Grapefruit Canker (and HLB) Field use Evaluation	$ 155,500.00	7/1/2014	6/30/2016
937C	Richardson, Taw	AgroSource, Inc.	Firewall Canker Efficiency on Round Oranges	$ 366,680.00	7/1/2014	6/30/2016
938C	Richardson, Taw	AgroSource, Inc.	Large Scale Lab/Greenhouse/Field Trial Evaluation - HLB	$ 1,877,041.00	7/1/2014	6/30/2016
940C	Beeson, Richard C.	University of Florida	Propagation of Rootstock Tree Production in Greenhouses by Seed, Stem Cuttings and Tissue Culture to Accelerate Budded Tree Production for Outplanting	$ 333,774.00	10/1/2014	9/30/2017
941C	Pelz-Stelinski, Kirsten	University of Florida	Influence of Thermal Therapy on Transmission of Candidatus Liberibacter asiaticus	$ 105,782.00	2/1/2015	7/31/2016
942.2C	Yonce, Henry	KAC Agricultural Research, Inc.	Field Trials of Plant Growth Regulators	$ 15,000.00	12/19/2014	6/30/2015
943C	Rogers, Michael	University of Florida	Support for scale-up of Thermal Therapy Treatment: Evaluation before and after thermotherapy heat treatments to combat HLB	$ 82,456.00	3/1/2015	2/28/2016
944C	Pelz-Stelinski, Kirsten	University of Florida	RSA - Small plant assay for testing the efficacy of antimicrobial materials against HLB	$ 125,797.33	3/1/2015	7/31/2016
946C	Nufarm	Nufarm Americas, Inc.	Mycoshield Magnitude of Residue Study for Citrus Crop Group.	$ 291,370.00	3/12/2015	9/30/2016

2016-17 Citrus Industry Research Priorities and Gaps Analysis

The By-Laws of CRDF define the purpose of the Industry Research Coordinating Committee and assign the Committee responsibility for recommending the research priorities for the Florida citrus industry to the Board that are outside the scope of work of the Research Management Committee. This committee will focus efforts on threats or opportunities for all elements of the industry that fall within its scope. This committee will perform a GAP analysis and, working with all elements of the Florida citrus industry, establish research priorities that fall within its scope. Previous efforts to conduct analysis of ongoing research projects and compare those to industry needs were carried out by the Florida Citrus Industry Research Coordinating Council (FCIRCC). This committee annually conducted a gaps analysis and shared the results with the industry. In 2011, CRDF assumed the responsibilities previously associated with the FCIRCC, and a report representing the transitional efforts of IRCC to complete the analysis for 2011-12 was presented in August 2012, and a second report in 2014. This report represents the 2016-17 priorities, research inventory and gaps analysis.

The annual effort conducted by CRDF, IRCC informs the citrus industry and research leadership of areas of research prioritized by the Florida citrus industry as important as well as the current inventory of projects being conducted by various institutions. The ensuing Gaps Analysis leads to identification of priority areas that require attention, and for each gap, an action plan can then be developed.

Florida Citrus Research Priorities – Top Gaps and Proposed Actions

This section of the report reflects the final results and recommendations of the Industry Research Coordinating Committee and their efforts to accomplish the following for 2016-17:

- Organize citrus research priority input from all sectors of the industry
- Assemble an inventory of current research projects related to citrus (focus on Florida, but with information from federal as well as Texas and California citrus efforts)
- Review priorities versus level of effort (inventory) and identify gaps
- Discuss each gap and characterize needed actions
- Assist in implementing the actions approved by the CRDF Board of Directors.

The following six gaps were identified by the IRCC during their evaluation of the priorities of the industry and the available inventory of projects. The list of major priorities and their sub-priorities (Appendix I) provides the context for the gaps identified, compared to other priorities identified that currently appear to be addressed adequately, according to the IRCC efforts.

For each gap, there is reference to its placement in the overall priorities, summary of the discussion, and recommended action.

Gap #1: Priority A. Citrus HLB (Greening): Sub-element: All. With the continuing epidemic of HLB expanding in Florida's mature tree inventory, declines in productivity continue. Despite an array of previous and current research projects, the results to date have not successfully addressed the decline in productivity that affects growers, processors, packers and all aspects of the industry. The lack of available tools to collectively impact productivity is the most important research gap.

Discussion: While there are approximately 210 research projects listed in the U.S. Citrus Research Inventory assigned to the topic of HLB, the collective tools that have emerged to date are not adequate to stabilize and restore health to the current tree inventory in Florida. This gap can be described several ways, but includes the need to deliver useful information on which tactics will lead to improved returns to growers though improved productivity, and to take all steps to accelerate this process towards additional tactic delivery. Productivity on a per-acre basis is the standard unit to describe the HLB decline situation at present, but declines in average brix and in fruit size represent other important metrics, and are factors that greatly influence market channels for citrus fruit and processed products.

Given a hiatus in post-production research to characterize the cumulative effects of progressive HLB-induced decline on productivity and complexities of this disease, there is little clarity on the specific disease factors (phloem plugging, root decline, and/or thinner canopy of smaller leaves, etc.) that most affect productivity. Further, we have limited knowledge on how prospective interventions individually and collectively impact productivity by changing symptoms of disease or reversing tree health decline. Lack of specific information in these areas may interfere with selection of best tactics to deploy to achieve the desired improvement in tree health at the grower level, and may impair the prioritization of research to advance additional solutions.

Action: Establish improved productivity as an overarching factor as research project results are evaluated and decisions are made on subsequent research and delivery programs. Prioritize research that is likely to directly affect productivity. Deliver the planned grower play book as soon as possible to guide growers in use of the best available tactics. Include research focused on establishing mechanisms responsible for lower productivity in portfolio review and requests for additional research, linking the declines in productivity with disease as affected by grower practices and disease management strategies.

Gap #2: Priority A. Citrus HLB (Greening): Sub-element 9. Culturing CLas as an important tool for developing solutions to HLB.

Discussion: The inability to culture the causal agent of HLB has limited research progress. Although there are continuing efforts funded by the USDA, limited progress has been made that will provide this research tool to the community.

Action: A concerted effort should be made to learn from culturing efforts to date and foster an organized approach to achieve this goal. There are available resources that can be directed to

assist in this effort. The NAS study should be encouraged to recommend how to accelerate current efforts to culture the bacteria.

Gap #3: Priority B. Post-Bloom Fruit Drop: Sub-element 1. Rainfastness of PFD treatment products for use in preventing PFD losses at bloom.

Discussion: Research here in Florida and in other locations (e.g., Brazil) have demonstrated the critical importance of delivering fungicides to citrus during vulnerable periods to prevent widespread infection by Colletotrichum, leading to PFD. A range of materials are available, and previous research has pinpointed the relationship between bloom phenology, timing of rainfall events, and the incidence of disease. Control recommendations highlight the need to place fungicides on susceptible flower tissues at precise times, often coincident with rainfall. Seeking rainfast products may be as important as expanding the breadth of products available to suppress PFD.

Action: Developing formulations or specific products that were rainfast would greatly expand the ability of growers to control PFD during infection periods. The extended period of effectiveness is one benefit, but also the ability to apply immediately prior to rainfall episodes would strengthen grower ability to protect larger acreage.

Gap #4: Priority G. Citrus Black Spot: Sub-element 1. Life Cycle and Epidemiology as it affects both spread dynamics and treatment development and recommendations. Impact of single infected tree. Better detection of ascospores to confirm life cycle. Cooperation with Brazil in evolution of management tools and epidemiology.

Discussion: Citrus Black Spot has continued to expand in Florida, although at a controlled rate. The pattern of spread experience in Brazil predicts that more aggressive expansion might be expected at some point. Current regulatory and grower initiated practices are in place to slow the spread, but advanced understanding of the disease in Florida might better inform if we are doing enough/too much to interfere with disease spread. Research results from Florida indicate unique characteristics of the pathogen here, and additional research may clarify what this means in terms of spread dynamics and control.

Action: Engage the new group of plant pathologists to join with current researchers (primarily Dr. Megan Dewdney) to expand the research goals to include addressing the issues identified here. Having science-based information to assist regulatory action is vital. It is recommended that international counterparts be engaged in planning and serve in cooperative roles in ensuing research. Brazilian citrus scientists and growers have been addressing CBS for a lengthy period, and we should formalize the relationships to learn from their experiences.

Gap #5: Priority K. Integrated Pest Management: Sub-element 1. Develop rust mite control under intense psyllid control. Limited materials available, potential resistance.

Discussion: The situation with citrus rust mite in Florida has been largely ignored from a research perspective since HLB took center stage. Despite that shift, injury and losses to rust mite continue. HLB interventions in general do not automatically cover rust mite management needs, and in an environment where input costs are escalated, rust mite management has not kept pace. The issues of limited materials available and the potential for resistance development are among the targets for further research.

Action: Establish a rust mite working group to investigate, update, define recommendations in the presence of HLB and current pest/disease treatments. This group of researchers, production managers, and perhaps registrants could define opportunities and needs, and might encourage a group effort leading to a set of rust mite research priorities.

Gap #6: Priority L. Emerging Pests and Diseases: Sub-element 1. Develop a rapid response/proactive approach to future exotic pests and diseases, with particular focus on Citrus Leprosis and Citrus Variegated Chlorosis (CVC).

Discussion: Use of multi-pest survey to address these (and other) diseases has been a component of CHRP, but this may need to be strengthened. There also is a need to increase local awareness of exotic diseases, including the disease biology/epidemiology, as well as practices and treatments that might be applied if disease occurs. We have learned with HLB that proactive information gathering will contribute to a quicker response.

Action: Knowing that exotic citrus pests and diseases are on the move, and include serious diseases such as CVC and Leprosis, Florida citrus should organize a more comprehensive proactive program for exotics. This effort necessarily needs to be multi-agency, and through research should import knowledge from other industries impacted by the target diseases. Research collaboration/participation in areas of occurrence of target exotics is one way to address the needs of Florida. Results should include advanced recommendations for early response to an exotic find, and should offer pre-emptive next steps to respond to a detection in Florida. Numerous mechanisms exist to strengthen preparation for a new disease find, and they should be employed to bring together the appropriate components to address this need.