CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-17

Quarter Ending June 30, 2017

1. *Candidatus Liberibacter asiaticus* PATHOGEN INTERVENTION

Project title: 1a. Bactericide Strategies

The goal of this project is to identify bactericides effective against Huanglongbing (HLB). Project managers will identify bactericides from various sources from products in the market to materials in early stages of development that are effective against HLB, and assist with formulation for effective delivery, provide regulatory guidance by engaging regulatory consultants and EPA and assist with commercialization if necessary. This is an ongoing project that will build on the development of an assay pipeline for screening bactericides and the *in vitro* screening of more than eight hundred compounds including material libraries from agriculture, biotech and pharmaceutical companies. Bactericides that have been identified by project managers, as potential short to long-term solutions will continue to be tested in assays and in field trials and steps will be taken to encourage commercialization of these materials to provide a solution to growers for HLB.

Subproject Title: 1a. Bactericide Strategies: Candidate Bactericide Testing

Narrative of Progress against Goals:

Obj. 1 – Form relationships with companies with candidate bactericides for testing in the CRDF assay pipeline. Assemble data on potential bactericides to assist in prioritization.

CRDF is focusing on chemicals that can be available to growers in the near-term, although new active ingredients are tested in the pipeline when appropriate. This quarter two new potential bactericides were discussed with researchers interested in studying the bactericidal effect of these materials. These materials may be tested in CRDF funded assays in the next quarter.

Obj. 2 – Move bactericide candidates through assay pipeline to identify promising materials for field trials.

Five new materials were tested in the in vitro assay this quarter. These materials may be considered minimum-risk or biopesticides, however they are early in the bactericide discovery process.

Subproject Title: 1aII. Bactericide Strategies: Bactericide Delivery

Narrative of Progress against Goals:

Obj. 1 – Coordinate with researchers, companies and other institutions to define formulations and delivery methods for field trials with minimal regulatory requirements.

A project was completed last quarter to test new adjuvants for the introduction of chemicals into citrus. These chemicals are in early stages of development, a time-to-market has not yet been established and will depend on efficacy. Results and next-steps are being discussed by company representatives and CRDF project managers.
A workshop took place last quarter with researchers, growers and industry representatives to discuss

trunk applications of pesticides. This workshop led to development of a project outline to develop application strategies for effective use to trunk application technologies. This project will continue to be developed over the next quarter.

Obj. 2 – Track RMC and CPDC research projects relevant to the formulation and delivery of bactericides against HLB; integrate findings into project planning.

A project ran this quarter to evaluate a new method for delivering bactericides to the canopy. Data collection will continue into the next quarter. This project aims to determine if the method significantly improves bactericide uptake. If this phase of the project is successful, phase two of the project will be developed and presented to the Commercial Product Delivery committee. The second phase of the project will evaluate the benefit to tree health of the increased level of bactericide uptake and will be discussed for finding in the next quarter.

A project was funded last quarter from a University of Florida researcher with the aim to evaluate the hypothesis that thermotherapy increases the uptake of bactericides. This study will evaluate uptake in small greenhouse plants after heat treatment using biochemical analysis. Treatments will take place early next quarter. A complementary field trial set-up last quarter is being evaluated by CRDF to determine if the combination of thermotherapy and bactericides improve the health of the trees in the field.

A research project to evaluate methods of detecting bactericides in plant parts was initiated in July 2016 and is being conducted by a research group at the University of Central Florida. This project uses infrared spectroscopy techniques to detect and quantify both oxytetracycline and streptomycin in a citrus leaf. This goal of this project is to evaluate movement of bactericides in citrus to better understand the dynamics of bactericide movement within the tree to help develop better methods of formulation and delivery. The methods used can successfully detect streptomycin on and in the citrus leaf on a nanoscale, streptomycin has been detected in the phloem tissue of the midrib of leaves treated with streptomycin and the adjuvant Grounded (Helena Chemical), the absorption was greater with the adjuvant. This project continues through the next quarter.

Subproject Title: 1c. Bactericide Strategies: Bactericide Field Testing

Narrative of Progress against Goals:

Obj. 1 – Managing existing field trials including analyzing data, refining treatments and reporting progress to CPDC.

Since March 2016, the bactericides Mycoshield, Fireline and Firewall have been available for use in Florida. CRDF has set-up nearly 70 field trials with growers to evaluate the efficacy of individual grower applications. Data being collected includes disease severity, bacterial titer/C_t values, fruit drop and yield. Initial PCR, disease severity, fruit drop and yield data has been collected on all Hamlins and grapefruit. Valencia harvest data collection will be completed in the next quarter. Data from the majority of the trials, including harvest data. The data analysis has shown that there is much variation in the data. Since these are non-replicated trials, few conclusions can be drawn from the trial results at this time. Data will continue to be analyzed as it is collected. All data is available to growers on the CRDF website under presentations or at http://citrusrdf.org/wp-content/uploads/2012/10/Update-GBT-Tables-4-24-17.pdf. An analysis of the current data will be completed early next quarter and a presentation will be given t the Citrus Expo in Fort Meyers on August 17th.

Registrant trials funded by CRDF continue for a third year. In 2016-2017, these trials focus on the use of alternating applications of oxytetracycline and streptomycin (three applications of each active ingredient) and a mixture of the two active ingredients for use in grapefruit. Application timing include applications at even intervals throughout the year and applications grouped in the spring and fall. This project is on track, objectives for this quarter have been completed.

The biopesticide field trial, project 15-049C, was set-up in late February on Hamlins. Data collection Was completed this quarter and data has been analyzed. The treatments in this trial were Thymeguard (Agro Research International, Ecotrol Plus (Keyplex), OnGuard EO, Xplode (AgXplore) and a research oil product. All products were applied at the recommended rates and with the recommended adjuvants by the registrants. Applications of these products were applied every 60 days (6 applications total)

Two sites were used for this trial, at the initiation of the trial the trees from one site tested PCR negative for HLB, although the trees were visually infected and the second site trees all tested positive for HLB. The field trial design consisted of four replications of blocks of six trees (24 trees per treatment). The evaluations consisted of tree health measurements: canopy volume, trunk cross-sectional area, disease severity (disease index); canker evaluations, polymerase chain reaction to quantify bacterial titer, leaf nutrient analysis, fruit drop counts, fruit quality and yield.

After one year, the results of this trial were not remarkable, but one year may not be adequate for tree recovery. Year two of this trial was approved this quarter and treatments were applied in June.

The following is the report from a project completed in the first quarter of 2016/17:

Title of Project: Soil drenches of products to combat initial HLB infection in young citrus trees

Situation Statement: This was a two-year study to test five soil applied treatments to prevent HLB infection in newly planted citrus trees and also to test the ability of treatments to maintain tree health should they become infected. The materials were a Bayer experimental material (USF 2018A), Aliette®, Aliette® plus USF 2018A and Serenade®. All materials were supplied by Bayer. The impact of treatments on new tree health, foliar nutrition, disease rating, HLB status and root density were evaluated. Fruit quality and yield was not evaluated because the trees did not reach maturity by the end of the trial. The trial was located at a single site in Lake Wales, Florida and began in August 2014.

Objectives of the Project: The objective of this field trial was to test four soil-applied treatments plus a water treated control (five treatment blocks) on health and HLB status of orange trees over two years. The five treatment plots will be 20 trees each was replicated four times.

The five treatments were applied manually as a soil drench to the base of the tree trunk and the surrounding irrigation-wetted root zone in 8 to 12 ounces of water per tree. Applications took place approximately every six weeks.

Table 1. Trial Treatments

	Treatment	Per Acre Rate	Dose/Tree	Comment
1	Water control		12 oz	
2	USF 2018A	3.42 oz	0.024 oz	
3	Aliette®	2.0 lb	0.23 oz	
4	USF 2018A	2.0 lb	0.24 oz	
	+ Potassium Carbonate	0.6 lb	0.07 oz	Increase pH > 5.0
	+ Aliette®	3.42 oz	0.023 oz	
5	Serenade®	2 qt	0.46 oz	

Treatment application dates were the following: 8/15/14, 10/01/14, 11/21/14, 1/21/15*, 3/23/15, 5/07/15, 7/02/15, 8/12/15, 9/22/15, 11/04/15, 12/16/15, 1/29/16, 3/14/16, 4/25/16, 6/08/16, 7/20/16 and 8/30/16.

* Delayed application due to observed phytotoxicity

Narrative:

This study tested the hypothesis that these four soil-applied treatments will prevent the establishment of HLB in young trees, compared to the water control treatment, or at least mitigate the effects of HLB on tree health and yield should they become infected. Data used to test this hypothesis was tree health, disease rating, HLB status, foliar nutrition, and root density.

A block of Valencia on X-639 planted in the late fall of 2013 was chosen for this trial. Good horticultural practices including irrigation, fertility program, leaf nutrition, and recommended young tree psyllid and other pest control measures, were requested of the cooperating grower to be followed. The block had not received soil applied microbial products during the previous two years.

There were five treatments (1-5) of 20 trees each x four replicates (A-D) = 400 total trees.

Trial Set-up:

Table 2. Field Design

Row	1	2	3	4	5	6	7	8	9	10	11	12	13
Replication			2A	4A	3B	1B	5B	4C	2C	3D	5D	1D	
Replication			1A	3A	5A	2B	4B	3C	5C	1C	2D	4D	

Data collection:

Six uniform measurement trees were selected in the middle of each of the 20 tree plots. The two end trees within the 20 tree plots were designated as buffer trees. All 120 measurement trees were initially visually rated using height and width and visually to verify trees were free of HLB. Measurement trees were rated four times per year and leaves were sampled annually by PCR to first verify that nursery trees are free of HLB and thereafter to document any infection. Canker evaluation occurred annually through foliar survey for characterizing canker lesions, as well as evaluating any fruit for presence of canker lesions and canker-imposed fruit drop. Trunk diameter measurements at 10 cm above the graft union was conducted annually.

Results:

In January 2015, chlorosis and leaf drop was reported in the USF 2018A treatment. The January treatment applications were delayed by two weeks because of the phytotoxicity. New flush and no new phytotoxicity symptoms were reported in February. In March 2015, tree recovery was observed although the USF 2018A treatments were stunted compared with the other treatments. The USF 2018A plus Aliette treatment exhibited chlorosis and stunting but no visible leaf drop.

At the trial initiation, a number of trees were found to be HLB positive (Figure 3). The number of positive trees increased over the length of the trial with most trees detected as positive a year and a half after the initiation of the trial. PCR data showed a change from a mean HLB negative for all treatments to a mean PCR positive for all treatments in a one year period (Table 3). The last round of PCR sampling occurred in September 2016 and only Serenade and the control were PCR positive, but sampling during this time of years often leads to inconsistent results. Based on disease severity ratings, all the experimental trees show symptoms and are likely to be infected.

The psyllid pressure appeared to be high in this block. No scouting was conducted for this trial, but when project managers visited the block, psyllids and psyllid damage was apparent on a number of experimental trees.

Disease severity was evaluated twice and at both time-points, Aliette exhibited fewer symptoms than the control (Table 4). Aliette also showed some additional growth over the control based on trunk cross-sectional area and tree height, but did not have an improved canopy volume (Table 5, 6 & 7) or root dry weight (Figure 4).

The canker level in this block was negligible in both evaluation years.

Leaf nutritional values were within the optimal range for all elements tested.

Conclusion:

Based on these data, none of the soil applied treatments prevented HLB, but Aliette had fewer symptoms based on visual disease symptom and tree growth. The USF 2018A treatments caused significant damage to the experimental trees, because of this, these treatments cannot be directly compared to the other treatments. More studies are necessary to determine the extent to which Aliette applications can to contribute to keeping young, HLB infected trees healthy and productive, or to determine if Aliette will have the same effect when used post-infection.

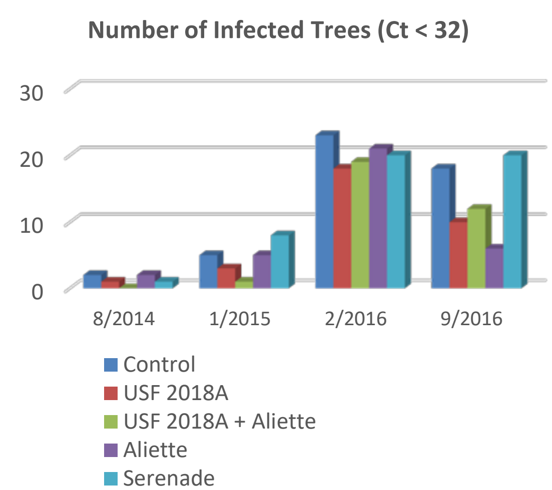

Figure 3. Number of infected trees

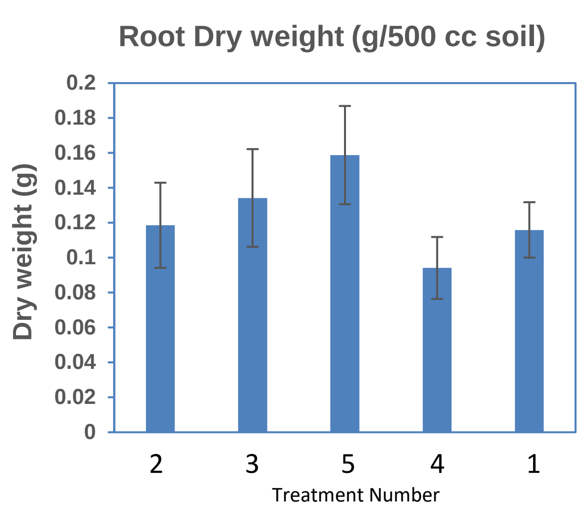

Figure 4. Root Dry weight and standard error

Table 3. Average PCR (Ct) Values

Variable	Jan. 2015		Feb. 2016		Sept. 2016	
Aliette	35.79 ± 6.99*	A**	25.46 ± 5.07	A	32.49 ± 7.63	B
USF 2018A	37.65 ± 5.96	A	26.71 ± 6.5	A	33.7 ± 6.55	AB
USF 2018A + Aliette	38.83 ± 3.21	A	25.61 ± 5.26	A	35.59 ± 6.78	A
Serenade	34.16 ± 7.28	A	25.05 ± 5.84	A	27.08 ± 4.14	C
Control	37.15 ± 5.72	A	23.62 ± 3.01	A	28.68 ± 6.85	C
N=	120		120		120	
Model P=	0.06		0.07		<.0001	
Treatment P=	0.0664		0.3222		<.0001	

Table 4. Average Disease Severity (0-40) Score

Variable	DI April 2016		DI Oct. 2016	
Aliette	2.9 ± 0.57	B	19 ± 9.12	B
USF 2018A	3.23 ± 0.61	AB	25.5 ± 8.01	A
USF 2018A +Aliette	3.29 ± 0.64	AB	24.08 ± 5.77	A
Serenade	3.13 ± 0.68	AB	25.58 ± 5.56	A
Control	3.44 ± 0.52	A	25.17 ± 7.2	A
N=	120		120	
Model P=	<.0001		<.0001	
Treatment P=	0.0106		0.001	

Table 5. Average Canopy Volume (m^3)

Variable	Oct. 2016	
Aliette	13.58 ± 4.77	A
USF 2018A	8.42 ± 3.15	B
USF 2018A + Aliette	7.76 ± 0.61	B
Serenade	12.49 ± 4.79	A
Control	12.4 ± 5.55	A
N=	120	
Model P=	0.00	
Treatment P=	<.0001	

Table 6. Average Trunk Cross-Sectional Area (cm^2)

Variable	Aug. 2014		Dec. 2015		Feb. 2016		Sept. 2016	
Aliette	1.35 ± 0.22	ns	4.21 ± 0.58	A	16.12 ± 5.14	A	22.67 ± 6.91	A
USF 2018A	1.1 ± 0.3	ns	2.32 ± 0.69	C	7.91 ± 2.98	C	13.64 ± 4.01	D
USF2018A+Aliette	1.28 ± 0.33	ns	2.61 ± 0.68	C	8.35 ± 2.11	C	13.94 ± 3.08	CD
Serenade	1.24 ± 0.33	ns	3.61 ± 1.03	AB	13.11 ± 4.5	AB	19.68 ± 6.22	AB
Control	1.13 ± 0.36	ns	3.35 ± 0.79	B	12.9 ± 5.25	B	18.09 ± 6.58	BC
N=	120		120		120		120	
Model P=	0.23		<.0001		<.0001		<.0001	
Treatment P=			<.0001		<.0001		<.0001	

Table 7. Average Tree Height (cm)

Variable	Aug. 2014		Dec. 2015		Feb. 2016		Sept. 2016	
Aliette	104.67 ± 8.34	ns	113.88 ± 10.69	ns	154.94 ± 19.29	A	158.64 ± 22	ns
USF 2018A	101.07 ± 8.99	ns	105.2 ± 9.14	ns	129.12 ± 13.31	C	143.09 ± 23.43	ns
USF 2018A+Aliette	102.55 ± 9.87	ns	108.27 ± 12.42	ns	134.62 ± 22.18	C	138.11 ± 21.24	ns
Serenade	103.72 ± 5.88	ns	114.19 ± 9.37	ns	151.34 ± 23.12	AB	156 ± 29.43	ns
Control	101.39 ± 9.32	ns	107.95 ± 10.05	ns	135.57 ± 19.81	BC	145.73 ± 25.06	ns
N=	120		120		120		120	
Model P=	0.63		0.09		0.00		0.12	
Treatment P=					<.0001			

* Standard deviation
**Tukey comparison lines for least squares (LS) means of treatment
LS-means with the same letter are not significantly different.
ns = the model is not significant, therefor no mean separations were calculated

Obj. 2 – Develop new field trials to test promising bactericidal therapies.

No field trials on new bactericide therapies is presently in development.

Obj. 3 – Provide communication of progress towards project goals and results to CPDC, CRDF and growers.

The Tree Health Section 18 was recertified by the US EPA for use through December 31, 2017. This is for continued use of oxytetracycline and streptomycin in Florida in 2017. An interim report will be compiled in the next quarter, this is a requirement for the section 18. A resistance monitoring program is also required by the EPA. A protocol was developed by CRDF, FFVA and FDACS with the assistance of George Sundin, a researcher at the University of Michigan. This protocol was approved by EPA, researchers were recruited to conduct the work and grower blocks were identified for the sampling. The first round of samples were collected and processed this quarter and a report will be completed early next quarter to be used for the required interim report to the EPA.

Significant Meetings or Conferences:

- CRDF Project managers attended the Florida Citrus Mutual Annual Conference. The education session for this conference focused on bactericide use in citrus.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-17

Quarter Ending June 30, 2017 – Final Summary

1. *Candidatus Liberibacter asiaticus* PATHOGEN INTERVENTION

Project Title: 1b. Thermal Therapy to Reduce Las Titer in Infected Trees

Project goal(s) for this project were:

1. Track ongoing research on thermal therapy and its role in HLB and tree health
2. Determine impact of thermal treatment on *Candidatus* Liberibacter asiaticus (Las) acquisition by ACP.
3. Evaluate HLB infected citrus trees before and after thermal therapy treatments to encourage scale-up of individual tree, over-the row and root supplemental heat and evaluation of their performance in reducing disease and improving health of treated trees.
4. Continue outreach efforts to inform growers of optimized thermal treatments including CRDF sponsored field days to include thermal therapy researchers and active steaming commercial companies.

Narrative of Progress by Project Goals:

<u>2. Determine impact of thermal treatment on Las acquisition by ACP</u>. CRDF-funded research at UF and USDA previously has not identified how thermal treatment affects availability of Las to be acquired 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 Las 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 one-year research project has been summarized in previous quarterly reports is now completed.

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). Trees in the CREC grove were 4-year-old Hamlin oranges. Of the 203 trees tested using quantitative PCR (qPCR), 50 trees have been identified with cycle threshold (Ct) values below 36, indicating the presence of Las.

In May 2015, bioassays were completed to quantify the rate of Las 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 Las infection. Thirty newly-emerged adult ACP were enclosed on individual branches of Las-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 of Las acquisition by psyllids will be quantified from these samples using nested quantitative polymerase chain reaction analysis (qPCR).

After initial acquisition bioassays were conducted, thermal therapy treatments were postponed until late July to ensure there were no residual imidacloprid effects. Thermal therapy was applied to Las-infected trees in the experimental plot during the second week of July. Using a steam-generating machine, trees were heated to 55ºC for 30s. In approximately 4 weeks, the rate of Las acquisition by immature and adult ACP was compared among infected trees receiving thermal therapy, untreated infected, and uninfected, untreated trees.

Following steam treatment of Las-infected trees in July, test trees were monitored for defoliation and re-emergence of flush. After new flush was evident (approximately 5 weeks after treatment), adult and immature psyllids were bagged on treated trees during the Las acquisition access periods and insect and leaf samples were collected. Samples were stored at -20ºC for subsequent nucleic acid preparation. Analysis of these samples via quantitative real-time polymerase chain reaction (qPCR) is ongoing. Changes in plant Las titer pre- and post-treatment were completed in November to evaluate acquisition efficiency following steam applications. The next acquisition assay also was in November.

Adults and nymphs were enclosed in mesh sleeves on trees for acquisition feeding approximately 5 weeks following the July treatments. Following acquisition feeding, insect and leaf samples were collected (45 d post-treatment) from trees and taken to the lab for subsequent nucleic acid extraction and analysis. Acquisition feeding assays were repeated approximately two months later, with samples collections beginning 114 d post-treatment. Nymphs were collected from plants after adult emergence, until no psyllids remained in the mesh sleeves. The titer of Las in trees receiving steam treatment did not significantly differ from untreated trees on days 0, 45, or 114 post-treatment (p = 0.99, 0.11, and 0.81, respectively; Tukey's Honestly Significant Difference (HSD) test); however, Las titers in treated and untreated trees were lower at 45 d post treatment as compared to days 0 and 114 post-treatment. This is likely due to naturally-occurring seasonal decreases in Las titers. Las titers were significantly higher in steam-treated trees than untreated trees on day 0 as compared to day 45. Las 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 Las 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 Las under controlled conditions. Two-year-old Valencia trees were inoculated with Las by enclosing plants with Las-infected psyllids for two weeks. untreated or thermal-treated, acquired significantly more Las than insects collected from uninfected trees for sampling period 3 ($F_{2,15}=6.32$, $P=0.008$, $P=0.045$, respectively). No other significant differences were observed in nymph acquisition assays. Nymph acquisition from Las-positive trees did not differ significantly between trees that were untreated or treated with thermal therapy. Nymph acquisition was significantly correlated with Las titer in leaves ($R2 = 0.35$; Pearson’s correlation co-efficient = 0.59, $F = 5.315$, $P = 0.044$)
These data suggest that treating the above-ground portion of citrus trees at 55ºC for 30 seconds is not adequate to replicate the efficacy of previously observed in whole plant thermotherapy in potted trials (Hoffman et al. 2012).

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

Obstacles: None for this period. All activities followed prescribed plans.

<u>3. Refine requirements and environmental conditions for most effective thermal treatment</u>.
The USDA, APHIS MAC group was charged to manage the federal funding to put HLB solutions in the hands of growers. Based on preliminary results, this group quickly identified thermal therapy as a “shovel-ready” project area and encouraged development of project ideas. Further, they encouraged solvers to come forward with plans and proposals for rapid scale-up.

USDA, APHIS thus responded with consideration of a mechanism that has been used by their agency previously in seeking solutions to challenges, and plans were established to solicit solvers for thermal therapy scale-up. Two Mac projects were approved to facilitate scale-up and both have been in place for 2 years. Evaluation of thermal therapy conducted by those involved in scale-up is being initiated by the CRDF evaluation team. Six enterprises are either field testing machines in Florida or have had machines in the field. Those with capability are operating at multiple locations in Florida, and the evaluation team is in the field conducting the evaluations.

CRDF CPDC moved forward with plans to coordinate evaluation efforts of thermal therapy. Building on the methods used to evaluate effects of other treatments (antimicrobials, soil amendments, etc.) on Las 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 described above. An overview of current field activity that the CRDF evaluation team has been engaged in follows.

<u>3. Encourage scale-up of individual tree, over-the row and root supplemental heat and evaluation of their performance in reducing disease and improving health of treated trees</u>.
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 machinery have been used delivering a range of temperature/duration combinations. Current data sets associated with these trials have been summarized in previous quarterly reports over the past two years. 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 non-remarkable as thermotherapy treatments only achieved short-term improvements in tree appearance and titer reductions that lasted 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 Las bacteria. Having 14 locations under evaluation allowed us to drop some sites as we fulfill 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.

Status at end of 24 months of the scale-up program. While this project did not control the tempo of innovation or the timetables for the various solvers who expressed a willingness to commercialize thermal therapy for HLB-infected trees in Florida. Based on disappointing results, several participants revised designs in response to early evaluations and deployed next generation machines. Overall participation has slowed as most potential solvers have not participated.

There were 14 sites reported on in the last period and most of the treated trees that displayed previous short-term responses to thermotherapy have now become not different from non-treated control trees. All trials had post-treatment leaf samples that have been analyzed by PCR in 2017. CRDF has thus discontinued monitoring tree status and data analysis. This report summarizes repeated evaluations on two of the sites for which we have continuous data over 1 to 2 years.

<u>Davis Site</u>

Valencia/Swingle trees 10 years old. All 24 trees were steam treated on 4/9/15 at 120 F for 30 s. Canopy growth, fruit drop, and visible disease index (DI) have been monitored monthly since April 2015. On 6-22-16, 12 trees were retreated with steam at 120 F for 30 s (2x 120 F 30 s) while 12 trees were left as a TT/control. By 2-3-17, there were no treatment effects on Cycle threshold (CT) for Las from qPCR or copy number per DNA. On 4-5-17, Canopy Volume (CV), tree height (TH), trunk cross sectional area (TCSA) and Disease index (DI) were not affected by treatments. Thus, 10 months after the second steam treatment, there were no treatment effects (summarized from the March 2017 quarterly report in Table 1).

Table 1. Davis Site.

Treatment	CV (m3) 4_5_17	TH (cm) 4_5_17	TCSA (cm2) 4_5_17	DI 4_5_17	CT 2_13_17	CN/DNA 2_13_17
TT/Control	20.7	257.0	124.8	22.8	27.8	3363
2x 120F_30s	21.1	248.8	125.8	21.1	29.2	2166

Figure 1 is a summary of Disease index (DI) and canopy volume over the two-year period from 2015 to 2017. On 6-22-16, 12 trees were retreated (retrt) with steam at 120 F for 30 s (2x 120 F 30 s) while 12 trees were left as a TT/control. Average visible DI went down after all trees that were steam treated in 2015 and half the trees were retreated on 6-22-16 (Fig 1 top). The retreated trees had a lower DI on 12-12-16 but here were no significant treatment effects on DI by 4-5-17. Canopy volumes (CV) increased with decreasing DI in 2015 but then decreased from 11-15 to 4-16 (Fig 1 bottom). There were no treatment effects on CV after retreatment. Likewise, Figure 2 shows that CT values changed little over time and there were no treatment effects on CT throughout the 2 years (Fig 2 top). The retreatment of half the trees on 6-22-16 did however, numerically lower the copy number (CN) per DNA by 6-24-16 (Fig 2 bottom) but this difference disappeared by 2-13-17. Thus, the only significant treatment effect was the temporary decrease in DI on 12-12-16 which did not persist.

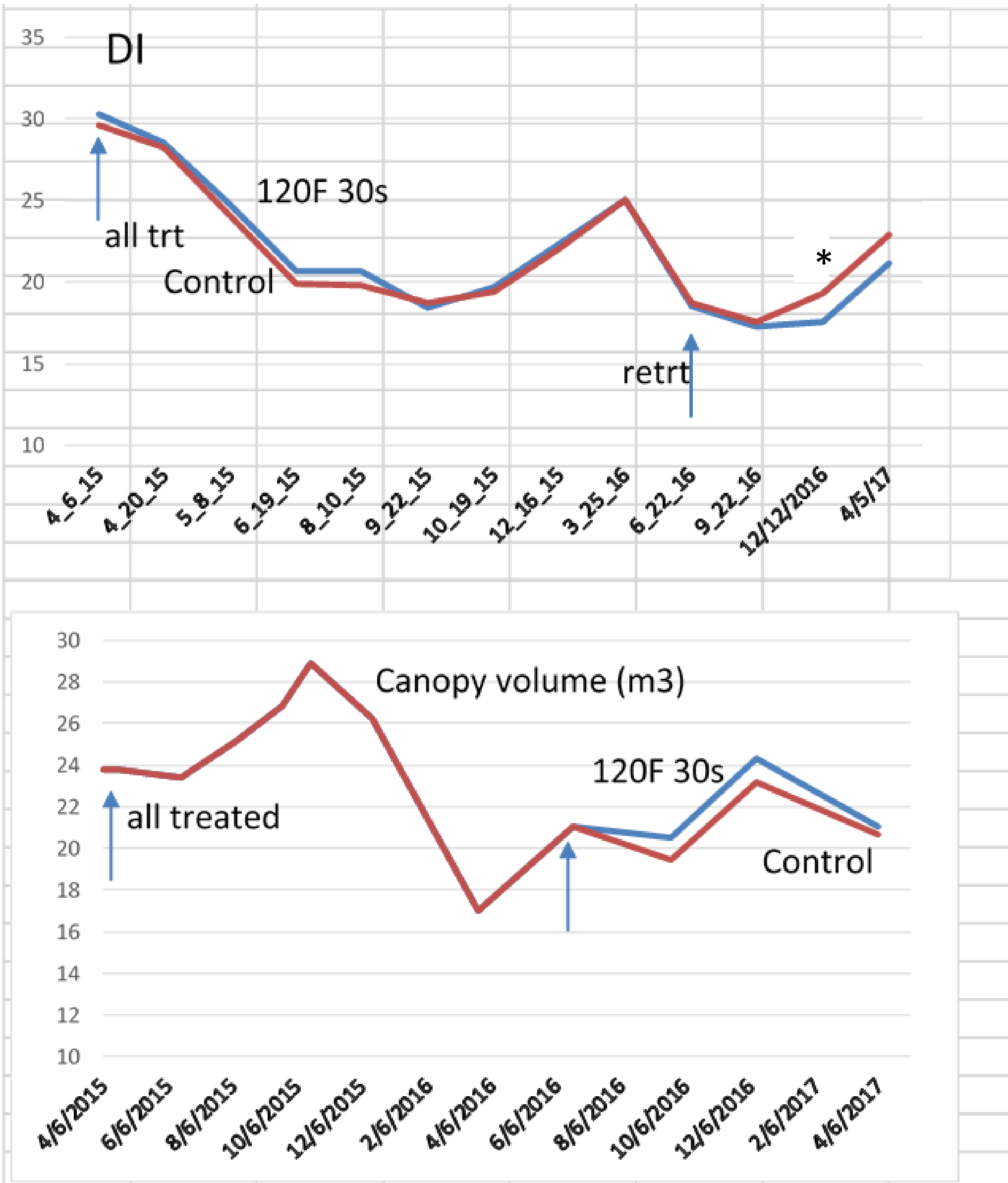

Figure 1. Davis disease index (DI) and Canopy volume from 4-6-15 to 4-5-17.

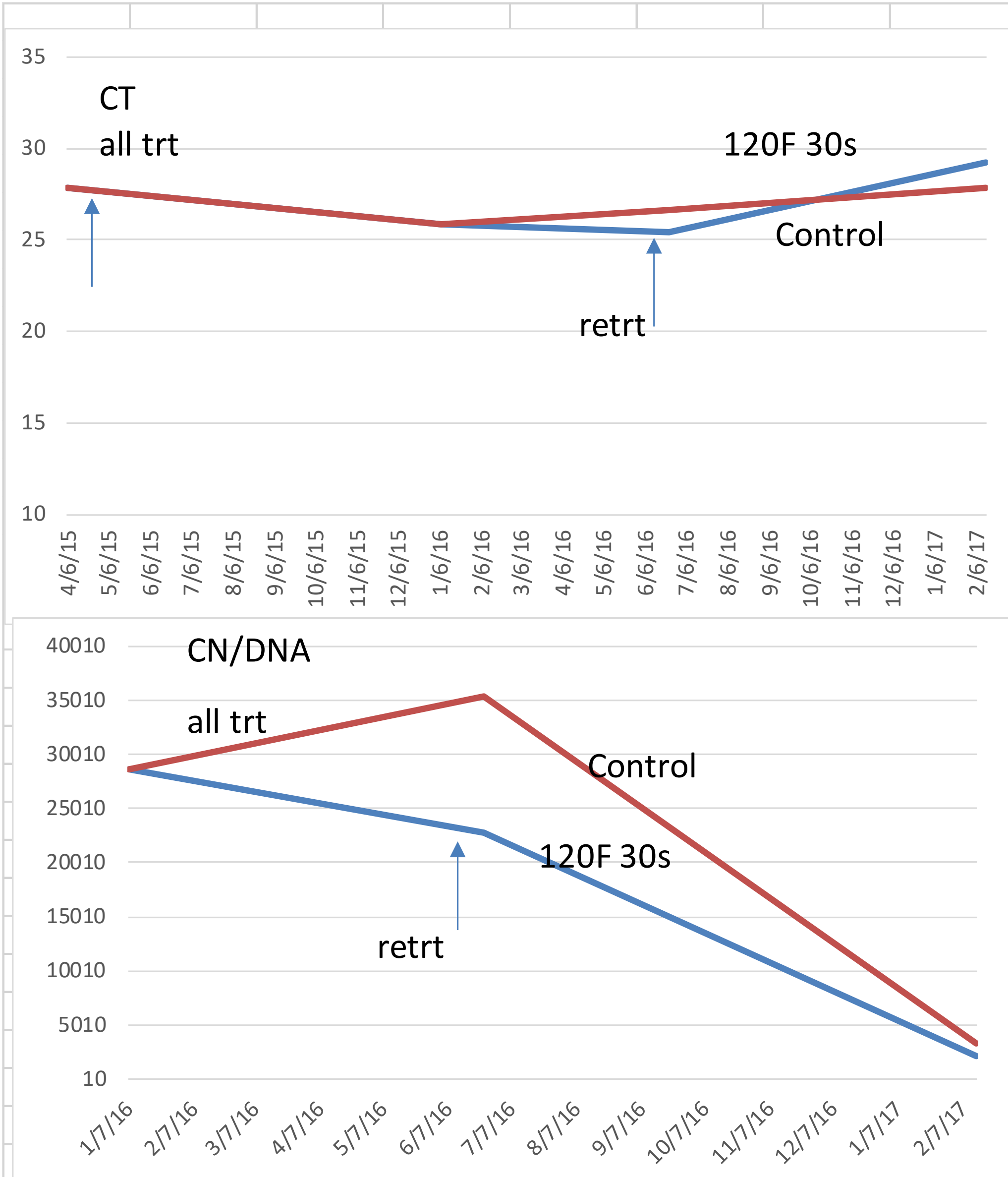

Figure 2. Average Cycle threshold (CT) and Copy number per DNA (CN/DNA) of N = 12 trees at the Davis site from 4-6-15 to 2-7-17.

<u>Lee Jones Site</u>

Based on initial PCR evaluations of HLB status, 22 uniform trees that were HLB positive and 22 uniform HLB negative were selected for evaluation of thermotherapy. On 3-22-16, one tree in each pair was steam treated at 128 F for 30 s while the other paired tree was left as an untreated control. By 10-12-16, Disease index (DI) was higher (* = significant @ P<0.05) and canopy volume (CV) was lower in previously HLB positive trees than in negative trees but neither were affected by the thermotherapy treatment. Trees were sampled for PCR and harvested in January 2017. Average cycle threshold (CT) revealed that all trees were HLB negative (CT < 31) on 1-13-17 so the previous HLB positive trees had become negative post treatment. Copy number (CN) per DNA was no longer affected by previous HLB status or by treatment. Fruit yield, however, was lower in previously HLB positive trees than in HLB negative trees and was also further reduced by the treatment.

Table 2. Jones Site.

HLB	Treatment	DI 10_12_16	CV (m3) 10_12_16	CT 1_13_17	CN/DNA 1_13_17	Yield (kg) 1_19_17
neg	128F_30s	13.1	10.5	25.1	4438	6.1
pos	128F_30s	14.0	4.5	25.1	3193	2.9
neg	Control	11.5	10.4	27.4	5215	16.1
pos	Control	14.7	3.8	24.9	6636	7.6
	HLB	*	*	ns	ns	*
	Treatment	ns	ns	ns	ns	*

Figure 3 shows the overall decrease in DI over the 8-month period. On 6-1-16, treatment trees had a higher average DI (more visible symptoms) than control trees but this difference disappeared thereafter. Canopy volume (CV) increased over time as tree grew but CV was not affected by treatment on any sampling date. Cycle threshold (CT) values generally decreased over time as the disease progressed but there was no effect of treatment on CT values over time (Figure 4). The CN/DNA decreased on both the treatment and control as there was no treatment effect on CN/DNA throughout. Thus, there was no evidence of any lasting positive effects of thermotherapy on HLB.

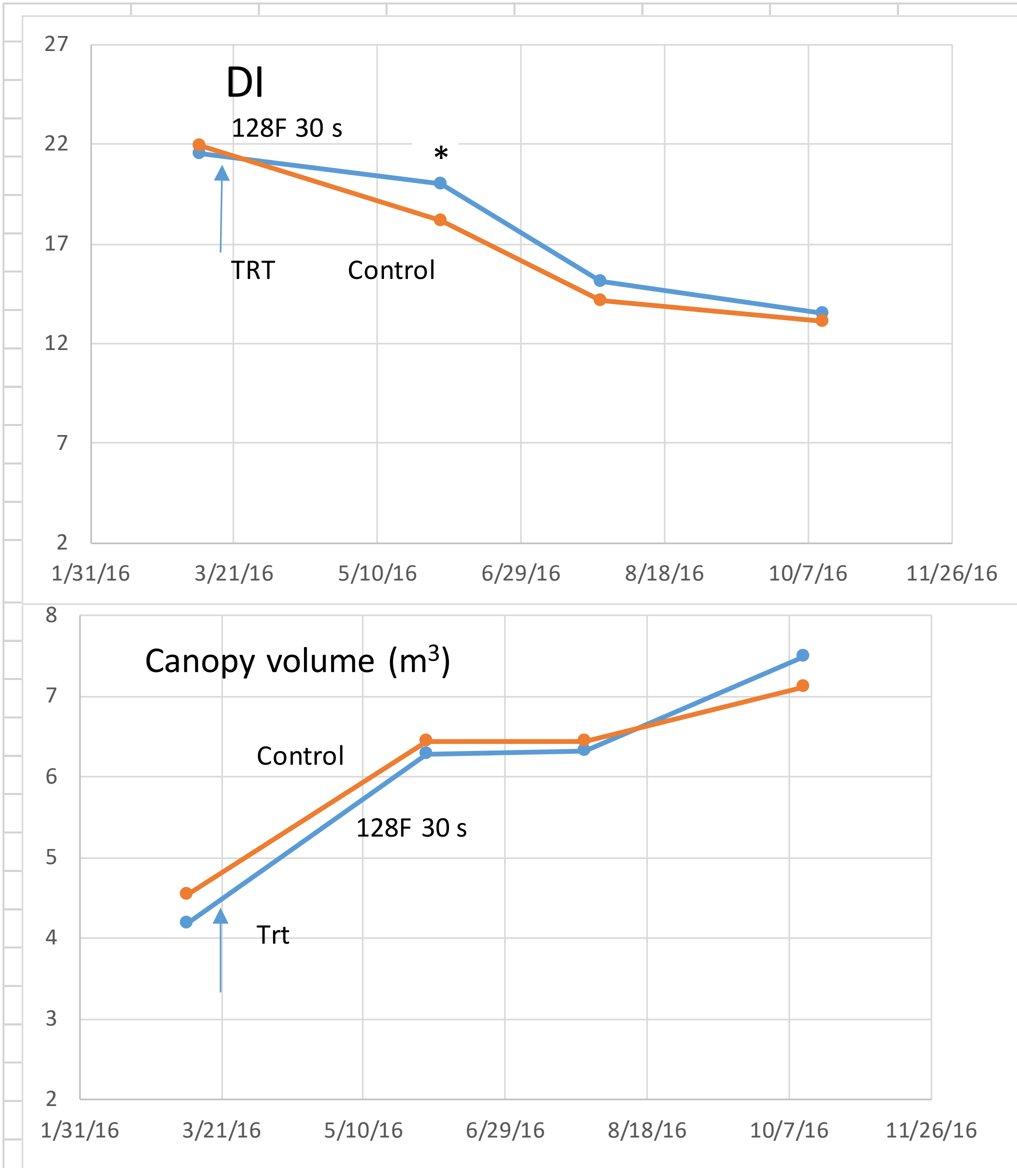

Figure 3. Average Disease index (DI) and canopy volume during 8 months in 2016 at the Lee Jones Site.

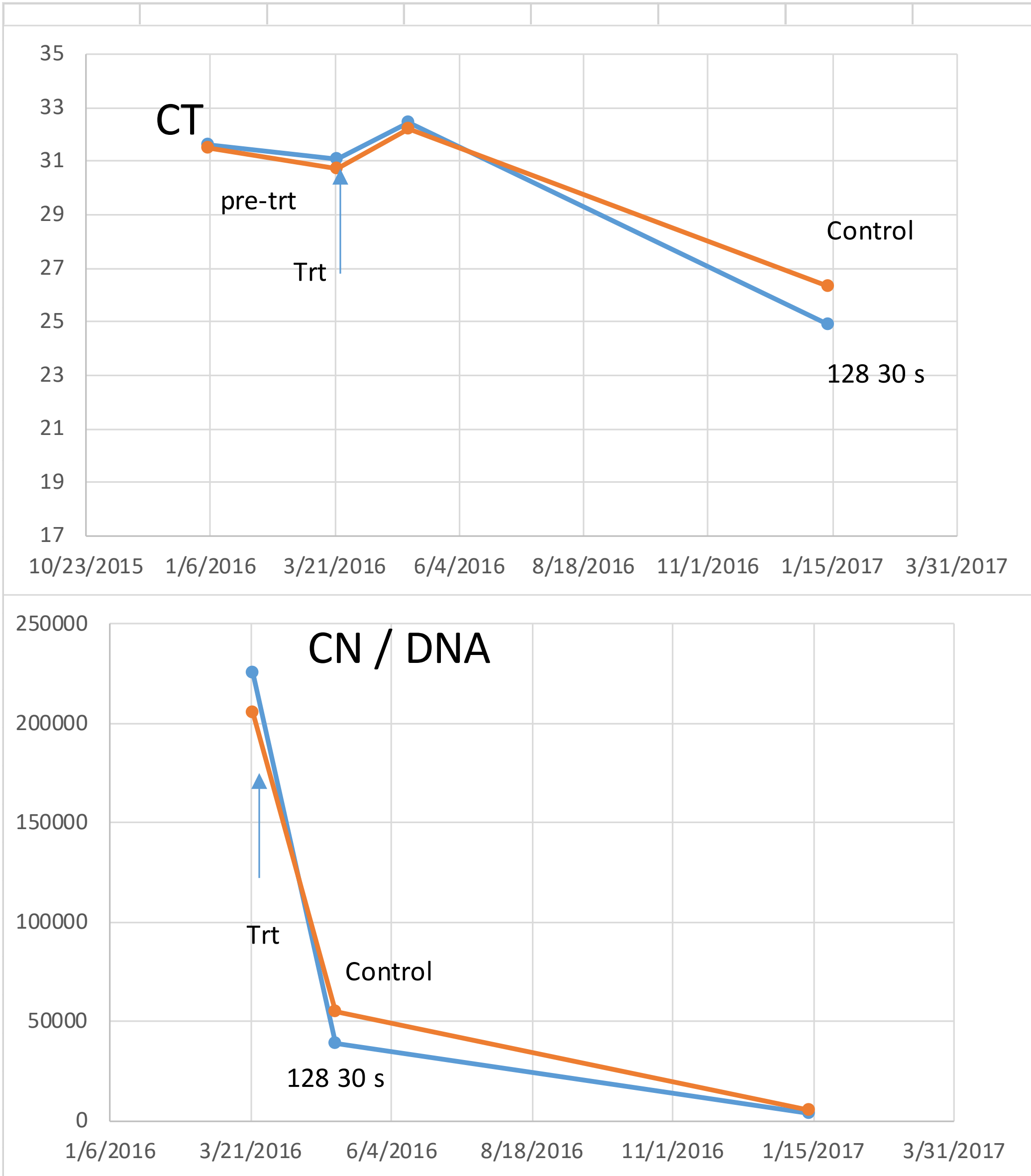

Figure 4. Average Cycle threshold (CT) and copy number (CN) per DNA of n= 12 trees at the Lee Jones Site in 2016-17.

CRDF Commercial Product Delivery Project Progress Report FY 2016-17

Quarter Ending June 30, 2017

2. Asian Citrus Psyllid VECTOR INTERVENTION

Project Title: 2a. Asian Citrus Psyllid Management and Citrus Health Management Areas (CHMAs)

During the final quarter of 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 in the quarterly report for RNAi/Psyllid shield (2a). 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 (#711 Bonning and #15-021 Pelz-Stelinski). 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 is planned for July 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.

Narrative of Progress by Project Goals:

1. <u>Pursue actions that will support expanded tools for ACP management</u>

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 (Swadeshmukul 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.

2. Engage registrants and regulatory entities in need for label modifications

Registrants of Aldicard continue to pursue the opportunity to bring this treatment back to Florida citrus. State labels for use of Aldicard on selected row crops has been completed, and now the company is considering taking steps towards use in citrus.

Current review of use of pyrethroids by EPA also is an issue of importance to continuing use of diverse classes of pesticides. Florida growers and support organizations are participating in discussions and information gathering in support of the continued need for diverse tools for ACP management.

Reregistration of pesticides in the neonicotinoid group has commenced, with Imidachloprid being the first active ingredient being reviewed. Due to widespread use of this class of insecticides in agriclulture, landscape management and other arenas across the United States.

3. Continue participation in pesticide stewardship activities

Dr Lukasz Stelinski (#15-038C) is bringing the current phase of resistance monitoring to closure as the quarter ends, and a final report 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.

4. Continue to support CHMA implementation of ACP and other HLB management tools

The current project focused on providing recommendations and support to CHMAs in Florida (#15-035C Michael Rogers) 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 Q4 of 2016-17 by the PIs (#16-027C Steve Futch). 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.

5. Communicate progress and results of project to CPDC, CRDF and growers

Significant Meetings and Conferences:

No Major meetings were held during this quarter.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-2017

Quarter Ending June 30, 2017 – Final Summary

2. ASIAN CITRUS PSYLLID VECTOR INTERVENTION

Project Title: 2b. RNAi Molecules/Psyllid Shield

Narrative of Progress against Goals:

Obj. 1 – Complete planning for and initiate field trials to begin in spring 2017

The principal goals of the 3-year field trials are as follows:

- Determine the efficacy of selected RNAi constructs mediated by CTVvv in controlling ACP on field grown trees
- Determine the effectiveness of CTVvv as a delivery method of RNAi throughout the year
- Determine the effect of CTVvv RNAi on the acquisition and transmission of CLas by ACP
- Determine the effect of CTVvv-RNAi on the spread of HLB

In addition, the field trial will familiarize the regulatory agencies with the technologies and help establish the field testing conditions for trials with RNAi. This will enable the industry to help develop the testing protocols and permit conditions for testing in conjunction with the agencies instead of having the conditions established completely by the agencies or by others.

Based on the results of this field trial, a decision will be made regarding a Phase 2 area-wide "Psyllid Shield"
field trial.

During the quarter the following activities were performed:

- Valencia scion were grafted with bud chips from donor trees that carried the CTVvv RNAi constructs. These trees will be planted in July.
- Two physically separate rearing facilities were established at SGC facilities. These insectaries will serve as the primary rearing facilities for this trial.
- Production target was set at 4000 to 5000 ACP per trial.
- To accomplish the above production target, comparison studies were conducted with differing light sources, light intensity, ACP density, host plant density, and host plan flush in order to establish guidelines to optimize cage conditions for adult ACP production.
- Using the above guidelines, 250-300 ACPs per cage with 16 cages were successfully produced, which is in line with the overall production target.
- During the quarter, field trial management has been training on and learning all aspects of the Southern Gardens diagnostic lab activities, with emphasis on ELISA and rtPCT.
- Sleeve prototypes were also developed for use in the field studies.

Obj. 2 – Continue outreach to other companies engaged in RNAi research and product development for potential collaborations

This is an ongoing effort. Nothing new to report this quarter on communications with either Forrest Innovations or AUM LIfeTech. Project management is continuing to look for other companies that are conducting RNAi R&D and product development to contact for possible relevance to HLB.

Obj. 3 – Continue to monitor ongoing RNAi research, including nuPsyllid project, for insights that may be applied to ACP intervention through Psyllid Shield.

Dr. Turpen collected comments on the expected outcomes from the nuPsyllid project as it comes to a conclusion in August, 2017, as well as recommendations on how the progress will be continued. A draft of this white paper was submitted for committee/board comment in March. He is currently preparing a final report for the nuPsyllid project. This report will be completed by September 2017.

Obj. 4 – Continue to explore potential candidates for long term commercialization of RNAi solutions for ACP intervention

This is an ongoing effort, and there is no new information to report on this front.

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 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.

Significant Meetings or Conferences:

None.

Obstacles/Breakthroughs:

None

Other Information:

This project has effectively combined the results of RNAi research into psyllid control with Psyllid Shield modeling to create the information needed to develop the recommendation to proceed with a two-phased field trial approach. It is expected that enough data would be available by the end of year 2 to make some educated guesses as to the effectiveness of the RNAi constructs to begin planning for larger scale trials. The larger scale field trials would be designed to further validate the technology and to collect the data necessary for a full Section 3 registration.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16

Quarter Ending June 30, 2017 - Project 928C - Final Report

3. Citrus Host Intervention

Project Title: 3a. Naturally Occurring Microbial Product Interactions with HLB

Project goal(s)

1. Track ongoing research on soil microbes and their role in HLB and tree health
2. Conduct field trials to test commercially available naturally occurring microbes
3. Provide communication on project goals, progress and results to CPDC, CRDF and growers

Significant Meetings of Conferences:
A presentation entitled "Soil Microbial Product Interactions with HLB in Valencia/Swingle Trees Over Three Seasons at Three Contrasting Sites in Florida" was presented at the 5th International Research Conference on Huanglongbing March 14-17, 2017 in Orlando. A similar presentation was given at the 130th annual Florida State Horticulture meetings in Tampa in June 4-6, 2017.

From 2014-17, results from these 3 field trial sites have been regularly communicated to the Florida citrus industry by quarterly progress report to the Committees and Board of CRDF which are posted to the CRDF website, and through presentations at grower meeting. To summarize, although several soil amendment treatments increased canopy growth and yield at the low HLB site in SW FL, there were no treatment effects at the other two HLB sites. Thus, these results support recent studies have shown that manipulation of soil bacteria with commercial microbial soil amendments has little effect on HLB disease control. Grower field days were held on Dec 6, 2016 at the east coast (IR) site and on Mar 22, 2017 at the SW Fl (Duda) site to summarize treatment effects on canopy volume, disease index, leaf nutrition and fruit yield after 2-3 years of treatments. The text below details final results from the trials and represents the final project report for CRDF 928 c. This is a manuscript in press as a publication in the 2017 Proceedings of the Fl. State Hort. Society.

SOIL MICROBIAL PRODUCT INTERACTIONS WITH HLB IN VALENCIA/SWINGLE TREES OVER THREE SEASONS AT THREE CONTRASTING SITES IN FLORIDA

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Abstract. **Three separate field trials were designed to test the hypothesis that 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 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 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 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.

Huanglongbing (HLB; *syn*. citrus greening) is presumably caused by the bacterium *Candidatus* Liberibacter asiaticus (*C*Las), a fastidious gram-negative, obligate parasite, phloem-limited α-proteobacterium (Garnier and Bove, 1983; Jagoueix et al., 1994). *C*Las is known to infect citrus roots which contributes to root loss and tree decline (Johnson et al., 2014). *C*Las may interact indirectly with populations of soil bacteria by increasing carbon supply to the rhizosphere from root death (Wang et al., 2017) or by altering soil nutrient cycles (Trivedi et al., 2011). Thus, HLB infection could result in shifts in the composition and functional potential of rhizosphere microbial communities (Trivedi et al., 2012). Microbiological soil amendments can act as disease antagonists, and organic mulch can stimulate resident antagonistic bacteria to suppress disease activity (Graham, 2005). Thus, the addition of beneficial bacteria to the soil beneath HLB trees might benefit HLB disease management. Field studies were designed to compare the ability of commercially available microbial soil amendment products to mitigate the effects of HLB on tree health and yield. We tested the hypothesis that five soil amendment products (BioFlourish, EcoFriendly, Serenade, Quantum, and Aliette), plus an untreated control, could affect HLB status, canopy volume, foliar nutrition, root density, fruit yield and juice quality. All materials were applied in multiple applications per year as recommended over the 3 year study at three Florida sites.

Materials and Methods

The soil amendment treatments were begun in May/June 2014 and were continued until Mar 2017. The three trial sites were well-managed commercial Valencia/Swingle groves located on the Ridge, East Coast, or Southwest Florida. The three sites were typical for their location and all received good horticulture care including irrigation, fertilizer and psyllid management. At the Ridge Site near Balm, FL, trees were 19 years old in 2017, trees were 6 years old at the East Coast Indian River area site, and 13 years old at the SW FL Felda site. Treatments were applied at the Ridge site by Pacific Ag Research, Thonotosassa FL, at the East Coast site by Glades Crop Care, Inc., Jupiter, FL; and at the SW FL site by KAC Agricultural Research, Inc., Deland, FL. Materials were applied as labeled at all three sites. BioFlourish (Triangle Chemical) was applied 3 x per year, EcoFriendly Citrus Soil Amendment (Eco-Friendly Products, LLC) 8 x per year, Quantum Soil (AE Microbiology) 12 x per year, Serenade Soil (Bayer CropScience) 3 x per year, and Aliette (Bayer CropScience) 3 x per year. Materials were applied to the soil under tree canopies at recommended label rates with calibrated hand spray guns at the Ridge and East Coast sites and through a custom irrigation spray jet system at the SW FL site. Each soil amendment treatment had a subset of three trees that received an annual application of 50 lbs of mature cow manure in April as a surface organic mulch.

Each of the three trials consisted of the six treatments (5 soil amendment products plus an untreated control) of 20 uniform trees each with 4 replicated blocks = 24 plots of 20 trees each = 480 trees at each site. There were sub-plots of 3 mulched trees within each of the 24 plots = 72 trees mulched at each site. Tree responses were measured on six unmulched sentinel trees in each treatment, replicated 4 times = 24 trees per treatment plus one mulched sentinel tree within each treatment replicated 4 times = 4 mulched trees per treatment. Thus, there are 28 sentinel trees times 6 treatments = 168 total measurement trees at each site.

Tree measurements included canopy volume (CV), visible disease index (DI) rating, and leaf samples for PCR detection of *C*Las and leaf nutritional analysis. Canopy volumes were measured using dimensional analysis of prolate spheroid using measurements of canopy width and height (Albrigo et al., 1975). The DI rating was done by subdividing each canopy into eight equal sections by two imaginary perpendicular planes (vertical and horizontal at mid canopy height) passing through the axis of the tree trunk. The resulting eight sections (4 on each side of the canopy) were scored individually on a 0–5 scale indicative of the proportion of limbs expressing HLB disease symptoms within each section (Gottwald et al., 1989). Leaf HLB status was determined from the cycle threshold (CT) value from QPCR using the protocol developed by US Sugar Corp. (http://www.flcitrusmutual.com/content/docs/issues/canker/sg_samplingform.pdf)

At harvest in the Spring of 2014, 2015 and 2016, total fruit weight per tree was evaluated from all 168 trees at each site and sub-samples taken for juice quality analysis using standard Florida Test House procedures. For juice quality, sub-samples of about 50 fruit were sampled and combined into one sample from the six unmulched sentinel trees and about 50 fruit were sampled from the mulched sentinel tree in each treatment. Thus, there was a single fruit quality composite sample from the unmulched trees and one additional mulched tree sample from each treatment replicated four times at each site. Since the mulch treatment did not affect any of the measured juice quality variables, treatment effects on juice quality were analyzed by pooling the ± mulch treatments replicated four times in each treatment for n = 8.

At all three sites, leaf nutrient concentrations were determined annually (2014-2016) in August using 6 mo-old leaves sampled and combined into one sample from the six unmulched sentinel trees in each treatment and one sample from the mulched sentinel tree in each treatment. Tissue concentrations (% dr wt) were determined by a commercial laboratory (Waters Agricultural Lab, Camilla, Ga.). Leaf nutrient concentrations were also expressed on a leaf area basis to check for any starch effects on leaf nutrient responses (Cimo et al., 2013) but any treatment effects were unchanged. Root density per tree was sampled annually in December from all 168 trees at each site. There were eight soil cores (1 x 6 in. deep) spaced between the tree trunk and canopy dripline under each tree which were pooled for root analyses. Roots were separated from soil with a 2-mm sieve and expressed as g dry wt per 500 cc of soil. The bacteria in soil amendments were determined using methods from Trivedi et al. (2011) and found to be similar to the product labels.

Data were analyzed using a completely randomized block design with four blocks using proc GLM in SAS (2016, Cary, NC, 9.4TS1M4 in Enterprise Guide 7.13HF4). There were two main factors: the five soil amendment products plus the Untrt Control replicated on seven sentinel trees per treatment and the mulch versus no-mulch factor where one of the seven treatment replicates within each block was mulched. Thus, there were 24 trees in the unmulched treatment, and four trees in the mulched treatment. Treatment means were separated with Tukey's HSD test with a significance threshold of $P = 0.05$.

Results and Discussion

During the first 2 years of the study (2014–2015) there were very few significant treatment effects on any of the monitored variables. Disease index (DI) ratings varied over time and any small treatment effects were transient. There were a few treatment effects on leaf nutrients but all major and minor leaf nutrients remained within optimum ranges (Obreza and Morgan, 2008) throughout the 3-year study (data not shown). On a few occasions, an element was affected by a treatment, but this difference disappeared in subsequent leaf analyses. Consequently, this report will focus on only the latest results from tree measurements, fruit yield, and juice quality from all three sites in 2017.

Ridge Site, Balm, FL site. After 3 years of treatments, there were no effects of the mulch treatment so the unmulched and mulched treatments were combined for n = 28 trees per treatment. On 30 Jan 17, there were no treatment effects on average tree canopy volume (CV), but all of the treatment trees had numerically greater CV than the Untr Cont trees (Table 1). Fruit yields (on 13 Apr 17) were relatively low for mature trees, from 1.6 to 1.9 (90 lb) boxes per tree, and no yields from treatment trees differed from the Untrt Cont trees. On 2 Mar 17, the Serenade treatment had a higher visible disease index (DI) rating than the Untrt Cont trees but the other treatments had similar intermediate DIs. Average CT values (on 13 Jan 17) indicated that all trees were HLB positive (CT < 30) and no treatment trees differed from the Untrt Cont trees. Juice quality was analyzed from the 13 Apr 17 harvested fruit samples using standard state test house methods, and none of the measured variables were significantly affected by treatment (Table 2).

East Coast, Indian River site. There were no effects of the mulch treatment so the plus and minus treatments were combined for n = 28 trees per treatment. In Feb-Mar 2017, there were no treatment effects on tree canopy volume (9–11 m^3), yield (1.5–1.8 boxes per tree) or DI (18-20, Table 3). All trees were HLB positive having a CT less than 30. Quantum treated trees had a higher CT than EcoFriendly trees, but again, none of the treatment CTs differed from the Untrt Cont trees. Although these trees were also HLB positive, yields from these much smaller 6-yr-old trees were similar to the larger 17-yr-old trees at the Ridge site. Juice quality was analyzed from the 21 Feb 17 harvested fruit samples, and none of the measured variables were significantly affected by treatment (Table 4).

SW FL Felda site. Valencia/Swingle trees were 11 years old. On 3 May 17, BioFlourish and Aliette treated trees had the largest canopy volumes, whereas the Untrt Cont and Serenade trees were the smallest (Table 5). Even though there were no significant treatment effects on fruit yield on 5 Apr 17, the ranking of yields corresponded to the ranking of canopy volume. Yields of the larger trees tended to be greater than smaller trees. Interestingly, when the yield data were pooled across treatments, the mulch treatment (n = 24) had significantly higher average yields, 4.2 boxes/tree, than the unmulched trees, 3.6 boxes/tree (n = 144). There were no significant treatment or mulch effects on DI. Leaves were sampled for PCR on 18 Jan 17. All average CT values would be considered HLB negative (CT >30), but the Aliette treated trees had a lower HLB titer (higher CT) than the EcoFriendly trees. None of the treatment CT values differed significantly, however, from the Untrt Cont. These are important results because after 3 years of treatments, they show some indication of treatment effects on canopy growth and positive mulch effects on fruit yield at least in non-HLB trees.

Juice quality was analyzed from the 5 Apr 17 harvested fruit samples and most of the measured variables were not significantly affected by treatment (Table 6). There was no significant treatment effect on juice brix but juice from Aliette treated fruit had a higher percentage acid than the Untrt Cont fruit. This resulted in the Aliette treatment having a lower brix/acid ratio than juice from the Serenade, EcoFriendly, and the Untrt Cont treatments.

The bacterial survival in soil and effects of treatments on the soil bacterial community were determined (Trivedi et al., 2012). 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 (N. Wang, unpublished).

Root density. There were no soil amendment treatment effects on root density per tree at any of the three sites in Dec 2014, 2015, or 2016 (data not shown). There were no significant effects of the mulch treatment on root density per tree in Dec 2014 and 2015 but mulch tended to increase root density in 2015 (data not shown). In Dec 2016, the mulch treatment increased root density over the unmulched treatments at the Ridge and SW FL sites (when the other soil amendment treatments were ignored) but not at the East Coast site (with the youngest, smallest trees) which tended to have less root density than the other two sites (Table 7).

Although there was an increase in yield from the mulch treatment at the low HLB, SW FL site, none of the small increases in fruit yield from the microbial soil amendment treatments were statistically significant. There were only small increases from 0.1 to 0.5 boxes per tree from Untrt Cont to treated trees from the best products. There were increases of only 0.3 and 0.1 boxes in HLB trees (on the Ridge and East Coast, respectively) and 0.5 box increases on well-managed non-HLB trees in SW FL. In an effort to gain statistical power, known differences in sites and tree age were ignored and data were combined across the three sites so there were n = 84 (28x3) trees per treatment and n = 24 (8x3) juice samples per treatment. In these analyses, Bioflourish trees had larger canopy volumes than Untrt control trees but there still were no treatment effects on yield, DI or lb solids/box.

Conclusions

All trees were HLB positive at the Ridge and East Coast sites, whereas average CTs at the SW FL site revealed that very little HLB was present. Overall, the small treatment effects on CT values were not remarkable. Canopy volume of the 6-yr-old trees at the East Coast site were the smallest, but the 19-yr-old old HLB-positive trees on the Ridge were smaller than the 13-yr-old non-HLB trees at the SW FL site. Canopy volume of the BioFlourish treated trees tended to be larger (though non-significantly so) than the other treatments on the Ridge and East Coast sites, and BioFlourish trees were significantly larger than the other treatments at the SW FL site where there was little HLB. By the third year, the mulch treatment increased root density in the Ridge and SW FL sites but not at the East Coast site. There were greater yields in the mulched than the unmulched trees at the low HLB SW FL site. Overall, there were no persistent treatment effects on leaf mineral nutrition status and none of the small increases in fruit yield from the microbial treatments were statistically significant. Although several treatments increased canopy growth and yield at the low HLB SW FL site, there were no treatment effects at the other two HLB sites. Thus, these results support recent studies have shown that manipulation of soil bacteria with commercial microbial soil amendments has little effect on HLB disease control (Wang et al., 2017).

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Table 1. Ridge site. Soil amendment treatment effects on average Canopy volume (CV), fruit yield, disease index (DI) rating, and cycle threshold (CT) from leaf PCR.

Treatment	CV (m^3) 30 Jan 17	Yield (boxes) 13 Apr 17	DI 2 Mar 17	CT 13 Jan 17
Aliette	27.9	1.8	11.2 AB	25.0
BioFlourish	35.2	1.9	13.6 AB	25.5
EcoFriendly	28.1	1.6	11.6 AB	24.9
Quantum	28.6	1.7	13.5 AB	24.8
Serenade	32.2	1.8	15.5 A	24.4
Untrt Cont	26.7	1.6	9.6 B	27.8

n = 28 sample trees per treatment. Column values followed by different letters differ significantly at $P < 0.05$.

Table 2. Ridge site. Soil amendment treatment effects on average fruit weight (Avg frt wt from the 13 Apr 17 harvest) and juice quality characteristics.

Treatment	Avg frt wt (lb)	% juice	Brix	% acid	Ratio	lb solids/ box	Color
Aliette	0.35	62.67	11.06	0.88	12.71	6.24	38.18
BioFlourish	0.34	62.66	10.82	0.91	11.99	6.10	38.84
EcoFriendly	0.35	63.28	10.96	0.91	12.14	6.24	38.29
Quantum	0.34	62.69	11.24	0.88	13.10	6.35	38.19
Serenade	0.38	62.55	11.23	0.86	13.36	6.32	38.19
Untrt Cont	0.34	62.99	10.85	0.93	11.82	6.16	38.25

n = 8 samples per treatment. No significant differences.

Table 3. East Coast site. Soil amendment treatment effects on average Canopy volume (CV), fruit yield, disease index (DI) rating, and cycle threshold (CT) from leaf PCR.

Treatment	CV (m^3) 1 Feb 17	Yield (boxes) 21 Feb 17	DI 8 Mar 17	CT 13 Jan 17
Aliette	10.4	1.8	20.3	24.9 AB
BioFlourish	11.6	1.6	19.1	24.3 AB
EcoFriendly	10.8	1.6	18.7	23.6 B
Quantum	10.3	1.8	18.9	28.3 A
Serenade	10.0	1.6	18.5	24.9 AB
Untrt Cont	9.2	1.5	18.1	26.4 AB

n = 28 sample trees per treatment. Column values followed by different letters differ significantly at $P < 0.05$.

Table 4. East Coast site. Soil amendment treatment effects on average fruit weight (Avg frt wt from the 21 Feb 17 harvest) and juice quality characteristics.

Treatment	Avg frt wt (lb)	% juice	Brix	% acid	Ratio	lb solids/ box	Juice color
Aliette	0.38	0.62	10.06	0.94	10.9	5.57	37.5
BioFlourish	0.34	0.61	9.91	0.95	10.5	5.46	37.2
EcoFriendly	0.36	0.62	9.89	0.93	10.7	5.49	37.5
Quantum	0.34	0.61	9.93	1.04	9.6	5.46	36.9
Serenade	0.36	0.62	9.94	0.94	10.6	5.50	37.0
Untrt Cont	0.37	0.60	9.86	0.94	10.7	5.35	37.1

n = 8 samples per treatment. No significant differences.

Table 5. SW Fl site. Soil amendment treatment effects on average Canopy volume (CV), fruit yield, disease index (DI) rating and cycle threshold (CT) from leaf PCR.

Treatment	CV (m^3) 3 May 17	Yield (boxes) 5 Apr 17	Mulch	Yield (boxes) 5 Apr 17	DI 3 May 17	CT 18 Jan 17
Aliette	45.1 AB	4.2	Mulched	4.2 A	21.3	36.4 A
BioFluorish	47.4 A	4.1			21.4	35.3 AB
EcoFriendly	39.0 B	3.9	Unmulched	3.6 B	22.5	31.2 B
Quantum	40.4 B	4.0			20.9	33 AB
Serenade	31.1 C	3.5			23.8	31.3 AB
Untrt Cont	32.3 C	3.6			22.5	31.6 AB

n = 28 sample trees per treatment except for Mulched where n =24 trees and Unmulched where n = 144 trees per treatment. Column values followed by different letters differ significantly at $P < 0.05$.

Table 6. SW FL site. Soil amendment treatment effects on average fruit weight (Avg frt wt from the 8 Apr 17 harvest) and juice quality characteristics.

Treatment	Avg frt wt (lb)	% juice	Brix	lb solids/ box	Color	% acid	Ratio
Aliette	0.51	0.61	12.39	6.76	37.90	0.84 A	14.78 B
BioFluorish	0.49	0.61	12.54	6.87	37.98	0.82 AB	15.29 AB
EcoFriendly	0.48	0.61	12.24	6.71	38.00	0.76 BC	16.11 A
Quantum	0.48	0.61	12.24	6.76	38.19	0.78 BC	15.82 AB
Serenade	0.47	0.61	12.41	6.79	37.86	0.77 BC	16.23 A
Untrt Cont	0.51	0.61	12.07	6.64	38.25	0.75 C	16.08 A

n = 8 samples per treatment. Column values followed by different letters differ significantly at $P < 0.05$.

Table 7. Mulch treatment effects on average root density (RD) in soil samples taken at the Ridge, East Coast or SW Fl sites in Dec 2016.

		RD (g/500 cc of soil)		
Treatment	n	Ridge	East Coast	SW Fl
Unmulched	144	0.84 B	0.56	0.93 B
Mulched	28	0.98 A	0.65	1.34 A

Column values followed by different letters differ significantly at $P < 0.05$.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-17

Quarter Ending June 30, 2017

3. HOST PLANT INTERVENTION

Project Title: 3c. Deployment of Disease Resistant or Tolerant Citrus Rootstocks and Scions

Narrative of Progress against Goals:

Obj. 1 – Track ongoing research projects evaluating emerging scion and rootstock genotypes for tolerance or resistance to HLB, citrus canker, and other diseases.

a) Project 940c: Propagation of Rootstock Tree Production in Greenhouses by Seed, Stem Cuttings, and Tissue Culture to Accelerate Budded Tree Production for Out planting

The comprehensive report for this project and a propagation manual for rootstocks are being written.

b) Grower Field Trials

Dr. Hatcher has been working with a grower to plan a scion field experiment to assess HLB tolerance in volunteer grapefruit identified by a grower. Scions and the scion/rootstock combination entry lists and field map have been finalized. The volunteer trees have been tissue sampled twice and samples analyzed by PCR for the presence of *Candidatus* Liberibacter (Clas) as an indicator of HLB infection and to get a baseline for infection. The results (data not shown) showed the presence of Clas in the commercial composite red and white grapefruit. Very few samples of the volunteer (mature) trees had detectable Clas titer. The trees were sampled again after three months and samples were sent to the lab for PCR analysis.

The grower has finished constructing a screened house where trees will be propagated and maintained until planting.

c) Transgenic field trials

Researchers involved with projects 754 and 15-020 are collaborating with others to develop a pre-proposal for a field trial with side by side comparisons of transgenic scions to assess field performance and HLB tolerance at the USDA-ARS secure permitted site in Ft. Pierce, Fl. The project is in the planning stages to propagate plants, acquire necessary permits and outline work plans for data collection.

Obj. 2 – Cooperate in in-depth evaluation and planning exercises related to Florida (and the US) citrus breeding to better focus on HLB solutions and rapid evaluation and deployment of rootstocks and scions.

Dr. Hatcher has done completed a preliminary analysis of the list of field trials submitted to CRDF by IFAS and USDA researchers. The exercise involved assigning data collection priority based on the age of the trial, statistical design, and replication and in the material evaluated. The next step is to have discussions with the researchers and shift priorities as necessary. Part of this process includes evaluating trials during site visits to ascertain to exclude or add trials due to tree health which could shift priorities.

Obj. 3 – Develop and implement plans for expanded management of tolerant and resistant citrus

Obj. 4 – Facilitate identification of best performing candidate rootstocks that appear to have HLB tolerance or resistance from Florida (and other) breeding programs

There are ongoing discussions between CRDF and IFAS to develop and execute a memorandum of understanding outlining each organization's expectations and responsibilities to facilitate data field trial data collection as well as resource allocation for other support such as central data repository and grower education field days.

Obj. 5 – Implement and evaluate Phase I and II grower field trials of most promising candidate HLB tolerant rootstocks using standard varieties as scions.

Phase I field trials: Rootstock Trial Project 927c

Field evaluations of field trials are ongoing using standardized CRDF protocols for evaluation and data collection of HLB disease incidence and horticultural traits. During the second quarter of 2017 horticultural data tree height (cm), canopy volume (m^3) and trunk cross-sectional area (cm^2) were collected and analyzed for rootstock differences within each site. HLB disease index (DI) was rated on a maximum scale of 0 to 5 on two sides of the crown, with 0 denoting no visual symptoms and 5 severe tree decline on more than 80% of the canopy. The maximum possible score for DI in these trials is 10.

Data Analysis and Results

All sites are planted in a completely randomized design (CRD) with 5 replications per rootstock.

Data were analyzed using a mixed model analysis procedure GLIMMIX using SAS® software (SAS Institute Inc, 2002 -2012) with the appropriate comparisons to test for differences among rootstock means when it is appropriate.

All the rootstock data collected is currently analyzed within each site and not compared across all sites. It will be important to compare rootstock performance across sites as the trials mature, especially when yield and fruit quality data become available. Current results suggest it is too early to make such a comparison, although one can be made retrospectively later.
Results for the two ridge sites (BHG and Peace River) are presented for all rootstocks for informational purposes. However, UFR-16 was planted late at both locations and cannot be fairly compared to the other rootstocks at this time. Although there two planting dates of UFR-3 at the ridge sites, inclusion or exclusion from data sets did not affect the results.

Results for previously unreported (new) data are presented by location.

CRDF DUDA Rootstock Trial, Felda, FL (Southwest)

The trial is planted in a completely randomized design (CRD) with five replications of each rootstock budded with '1-14-19 Valencia' for straight comparison of rootstock performance. All trees were planted on March 18, 19, 2015. The rootstocks were US-812, US-942, UFR-2, UFR-3, UFR-4, UFR-16 and Swingle (as a standard). Eight sentinel trees were randomly assigned to each plot at planting for data collection.

Yield Data

During the fall 0f 2016 staff begun fruit stripping at Duda but quickly determined that there was a lot of fruit on the trees and stopped after one replication was completed. The trial was harvested by plot at maturity in April 2017. Approximately 20lbs of fruit was subsampled for juice quality analysis at the University of Florida's pilot plant. The standard State test was conducted for juice quality and maturity testing. One plot sample did not pass the maturity test. Data was analyzed using the GLM procedure of SAS® software (SAS Institute Inc, 2002 -2012). There were statistically significant differences ($p < 0.05$) among the rootstocks for pounds of juice per box, pounds solids per box, boxes of fruit per acre, total brix, titratable acid, and pounds solids per acre (Table 1).

Table 1 CRDF Duda site rootstock trial yield and fruit quality data means ± standard error of the mean data collected in spring 2017

Rootstock	Pounds of juice per Box	Titratable acid	Total brix	Brix acid ratio	Pounds solids per box	Juice color	Boxes per acre	Pounds solids per acre
Swingle	48.00 ± 0.4 A	0.66 ± 0.0 A	9.76 ± 0.1 A	14.89 ± 0.3	4.69 ± 0.09 A	37.7 ± 0.3	7.12 ± 0.4 BC	33.9 ± 2.0 AB
UFR_16	43.78 ± 0.5 A	0.56 ± 0.0 AB	8.66 ± 0.1 AB	15.55 ± 0.2	3.80 ± 0.09 AB	36.7 ± 0.8	9.26 ± 0.3 BC	35.2 ± 1.3 AB
UFR_2	45.83 ± 0.0 A	0.60 ± 0.0 AB	8.85 ± 0.1 AB	14.77 ± 0.2	4.05 ± 0.03 A	37.3 ± 0.4	2.24 ± 0.0 C	9 ± 0.7 B
UFR_3	47.42 ± 0.4 A	0.58 ± 0.02AB	9.53 ± 0.2 A	16.62 ± 0.3	4.53 ± 0.09 A	37.2 ± 07	13.63 ± 1.2 AB	60.0 ± 4.3 A
UFR_4	45.06 ± 0.3 A	0.65 ± 0.0 AB	9.39 ± 0.1 AB	14.53 ± 0.2	4.23 ± 0.07 A	37.6 ± 0.9	5.45 ± 0.5 C	22.43 ± 1.4 B
US_812	44.88 ± 0.5 A	0.59 ± 0.0 AB	8.9 ± 0.1 AB	15.21 ± 0.2	4.00 ± 0.09 A	37.2 ± 0.3	14.96 ± 0.3 AB	59.51 ± 1.2 A
US_942	36.75 ± 0.2 B	0.55 ± 0.0 B	8.23 ± 0.1 1B	15.00 ± 0.2	3.03 ± 0.03 B	36.8 ± 0.4	20.38 ± 1.6 A	61.92 ± 5.1 A

Values represent the mean ± standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using the Tukey-Kramer method. Values followed by the same letter do not differ significantly at the 5% level.

The highest yielding rootstocks for pounds of solids per acre were US_942, US_812, and UFR_3. US_942 had significantly less juice and low pounds of solids per box but yielded more fruit than Swingle, which had higher pounds of solids per box, which resulted in low pounds solids per acre for Swingle than for US_942. Although, the other traits that were evaluated were statistically significantly different they are small differences.

Horticultural Trait Data

There were significant differences ($p < 0.05$) among rootstocks for canopy volume (m^3), trunk cross-sectional area (TCSA) (cm^2) and tree height (cm) at this location (Table 2).

Table 2 CRDF Duda site rootstock trial horticultural traits, HLB Disease index (DI) and PCR Cycle Threshold means ± standard error of the mean

Rootstock	Canopy Volume (m^3)	TCSA (cm^2)	Tree Height (cm)	HLB DI[a]
Swingle	5.71 ± 0.22 BC	26.04 ± 1.72 CD	190.05 ± 3.10 BC	2.90 ± 0.21 B
UFR_16	4.74 ± 0.29 C	24.99 ± 1.81 CD	188.38 ± 4.08 BC	3.00 ± 0.24 B
UFR_2	4.95 ± 0.15 C	24.58 ± 1.40 CD	181.73 ± 2.86 C	2.80 ± 0.18 B
UFR_3	3.16 ± 0.20 D	20.99 ± 1.29 D	163.40 ± 4.15 D	4.20 ± 0.27 A
UFR_4	6.82 ± 0.26 A	33.14 ± 1.01 AB	207.63 ± 3.51 A	2.85 ± 0.17 B
US_812	6.32 ± 0.26 AB	29.58 ± 1.96 BC	200.98 ± 2.92 AB	2.30 ± 0.15 B
US_942	6.81 ± 0.26 A	37.13 ± 1.11 A	214.38 ± 4.02 A	2.65 ± 0.15 B

Values represent the mean ± standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using the Tukey-Kramer method. Values followed by the same letter do not differ significantly at the 5% level.

Differences in HLD disease index (HLB DI) and PCR cycle threshold were not statistically significant. Rootstock groupings for each variable can be separated by the best performing rootstocks in order US_942, US_812, UFR_4, Swingle,UFR_16, UFR_2, and (UFR_3).
UFR_3 horticultural performance data indicate smaller trees and significantly higher HLB disease index, but the overall yield in pounds solids per acre (Table 1) is similar to that of US_942.

Peace River CRDF Rootstock Trial, Babson Park, FL (Ridge)

The trial is planted in a completely randomized design (CRD) with five replications of each rootstock budded with '1-14-19 Valencia' for straight comparison of rootstock performance.Valencia trees on seven of eight rootstocks (US-897, US-942, US-812, UFR-2, UFR-4, UFR-3 (short half of the trees), & Carrizo (as a standard) were planted in April 2015. Planting of UFR-3 trees was completed in September 2015. Trees on UFR-16 were planted in August 2016. Eight sentinel trees were randomly assigned to each plot at planting for data collection.

Horticultural Trait Data

There were significant differences ($p < 0.05$) for horticultural traits reported in this period at the peace river location for canopy volume (m^3), trunk crossectional area (cm^2), and tree height (cm) (Table 3). It should be noted that UFR_16 means are presented for information and should not be directly compared to other rootstocks because it was planted 11 months later at this site. Tree canopy volume, TCSA and tree height varied widely among rootstocks in a trend similar to that observed at the other two sites. With the exception of DI at this site, rootstock performance can be divided into three groups across traits as in the tallest trees, tend to have larger canopy volume and TCSA although it is not likely due cause and effect.

Table 2. CRDF Peace River site rootstock trial horticultural traits and HLB Disease index (DI) means ± standard error of the mean data collected in spring 2017

Rootstock	Canopy Volume (m^3)	TCSA (cm^2)	Tree Height (cm)	HLB DI[a]
Carrizo	2.48 ± 0.18 BC	17.61 ± 1.01 AB	151.80 ± 4.17 AB	1.45 ± 0.15 A
UFR_16	0.74 ± 0.08 D	4.78 ± 0.31 F	113.20 ± 3.06 C	0.65 ± 0.10 B
UFR_2	1.96 ± 0.11 C	11.59 ± 0.55 D	140.73 ± 3.12 B	1.70 ± 0.14 A
UFR_3	1.17 ± 0.16 D	7.85 ± 0.96 E	123.80 ± 4.95 C	1.75 ± 0.20 A
UFR_4	2.84 ± 0.14 AB	16.95 ± 0.65 BC	154.80 ± 3.24 AB	2.05 ± 0.16 A
US_812	3.09 ± 0.13 A	17.95 ± 0.54 AB	162.08 ± 2.79 A	1.65 ± 0.17 A
US_897	2.45 ± 0.13 BC	14.36 ± 0.57 CD	155.25 ± 3.17 AB	1.95 ± 0.16 A
US_942	3.42 ± 0.14 A	20.50 ± 0.61 A	166.40 ± 2.79 A	1.85 ± 0.13 A

Values represent the mean ± standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using the Tukey-Kramer method. Values followed by the same letter do not differ significantly at the 5% level.

BHG CRDF Rootstock Trial, Venus, FL (Ridge)

The trial is planted in a completely randomized design (CRD) with five replications of each rootstock budded with '1-14-19 Valencia' for straight comparison of rootstock performance. Eight sentinel trees were randomly assigned to each plot at planting for data collection. Valencia trees on 5 of 7 rootstocks were planted July 2015. Only trees on 5 rootstocks were initially planted: UFR-2, UFR-4, US-942, US-812 and Sour orange as a standard. Trees on UFR-3 were planted in September 2015 and trees on UFR-16 were planted in June 2016.

Horticultural Trait Data

There were significant differences ($P < 0.05$) in rootstock performance for canopy volume (m^3), TCSA (cm^2), tree height (cm) and HLB disease index (Table 4). UFR_16 was planted ten months later at this site so performance data is provided for information purposes and may not be used in direct comparison with other rootstocks at this time. US_942, US_812, Sour, and UFR_4 had similar canopy volume performance while UFR_2 and UFR_3 smaller canopy volumes. Rootstock TCSA split into four groups (excluding UFR_16). US_942, Sour, and US_812 had the largest TCSA and UFR_3 the lowest. Tree height rootstock performance followed a similar pattern except UFR_4 having comparable tree height to US_942, US_812, and SOUR. HLB DI values split into three groups and indicate a low incidence of HLB.

Table 3. CRDF BHG site rootstock trial horticultural traits, HLB Disease index (DI) and PCR Cycle Threshold means ± standard error of the mean data collected in spring 2017

Rootstock	Canopy Volume (m^3)	TCSA (cm^2)	Tree Height (cm)	HLB DI[a]
Sour	1.60 ± 0.08 AB	12.49 ± 0.55 A	132.93 ± 2.27 AB	0.98 ± 0.18 ABC
UFR_16	0.43 ± 0.03 D	4.64 ± 0.29 E	98.25 ± 1.84 E	0.40 ± 0.10 C
UFR_2	1.07 ± 0.05 C	8.59 ± 0.25 C	121.13 ± 1.58 C	0.55 ± 0.11 BC
UFR_3	0.69 ± 0.04 D	6.71 ± 0.34 D	112.35 ± 1.94 D	0.45 ± 0.10 C
UFR_4	1.37 ± 0.06 B	10.34 ± 0.33 B	130.40 ± 1.95 B	0.80 ±0.19 ABC
US_812	1.76 ± 0.09 A	13.36 ± 0.46 A	138.78 ± 2.42 AB	1.28 ±0.19 A
US_942	1.82 ± 0.09 A	14.04 ± 0.48 A	139.60 ± 2.19 A	1.18 ±0.17 AB

Values represent the mean ± standard error. Means were analyzed with a one-way ANOVA, and letter groupings were obtained using the Tukey-Kramer method. Values followed by the same letter do not differ significantly at the 5% level.

Obj. 6- Communicate progress and results of evaluation of rootstocks to industry

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-17

Quarter Ending June 30, 2017

1. CITRUS HOST INTERVENTION

Project Title: 3d. Genetic technology (MCTF): Deploying Canker-Resistant Genes

Project goal(s) for this project area for the next year:

Make measurable progress toward producing and introducing to Florida citrus growers new transgenic citrus lines based on mature tissue transformation of commercially available cultivars. These citrus lines will have disease resistance to citrus canker and HLB and will flower and bear fruit in a short time period. For FY 2015-2016, measurable progress is defined as:

Narrative of Progress against Goals:

Obj. 1 – Continue *Agrobacterium*-mediated transformation of mature citrus rootstock and scion as a service for internally funded CRDF grant recipients, and expand as a fee-based service for other research institutions and industry.

The mature citrus transformation protocol is dependent upon the vigor induced by budding mature buds onto immature rootstock. Since refreshing the mother trees and repeatedly re-budding experimental material onto immature rootstock, the vigor of the explants in tissue culture has increased, and we have resumed producing transgenic, mature citrus and saw an increase in productivity in the last quarter. During this last quarter, approximately 36 transgenics were produced, 25 have survived micrografting, & we are waiting to see if the remaining 11 survive micrografting.

Obj. 2 – Test alternative selectable markers

Obj. 3 - Increase transformation efficiencies/throughput of the Agrobacterium protocol and develop a biolistics protocol for mature citrus. Conduct molecular analyses to verify transformation and quantify gene expression as necessary. The maize knotted1 (kn1) gene significantly increased stable transformation efficiencies of immature citrus (Hu et al. 2016), but it only increased stable transformation efficiencies of mature citrus to ~5% (Dr. Yi Li, personal communication). If mature citrus transgenics will be commercialized, it is undesirable to add additional genes because maize kn1 interferes with anthesis. We have purified the kn1 protein from E. coli on a small scale and are in the process of scaling up to a larger volume (10 L). The immature & mature labs will test whether the protein increases transformation efficiencies after it has been added to the co-cultivation medium. The E. coli cells will be grown at different temperatures to avoid inclusion body formation.

Obj. 4 – Increase micro-grafting efficiencies/throughput

This remains a team effort lab process that is ongoing.

Significant Meetings or Conferences:

Dr.Zale attended the CRDF, National Academy of Sciences meeting in Irvine, CA on May 22, 2017, at which I gave a short talk along with other scientists & staff from CREC, USDA, UCR & Southern Gardens.

Obstacles Encountered and Breakthroughs:

It was determined that one vector submitted by a client was rearranged in Agrobacterium strain EHA105 or a DNA mixture of 2 colonies was submitted. Attempts to sequence through the T-DNA were unsuccessfully, and all sequencing led to the plasmid backbone. The transgenics produced with the rearranged plasmid were positive for the nptII selectable marker, the GFP reporter, and we could not determine how much of T-DNA was present in these transgenics. New DNA from the client was obtained and will be transformed into strain AGL1, which has a mutation in the RecA gene to diminish rearrangements.

Considering this difficulty, new quality control measures are required in the lab for strain EHA105. All Agrobacterium colonies must be PCR verified for the gene of interest (GOI) before transformation if using this strain. Additional quality control measures are necessary when screening transgenic plants produced with this strain. The lab can no longer conduct PCR for the reporter & selectable marker genes flanking either side of the GOI; PCR must be included for the GOI as well. This quality control measure is a departure from past practices in both the mature or immature labs.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2016-17

Quarter Ending June 30, 2017

4. Other Citrus Diseases

Project title: 4a. Post-Bloom Fruit Drop

Narrative of Progress against Goals:

Obj. 1 – Summarize grower experiences in suppressing PFD during 2016 epidemic year

A CRDF Research Management committee meeting was called on June 29, 2016 to discuss post-bloom fruit drop (PFD) caused by *Colletotricum spp.* in Florida. In this meeting, it was suggested that there is an opportunity to retrospectively investigate PFD management strategies from 2016 to determine if grower treatment programs led to variable results. A survey and protocol for data collection was developed and growers were engaged to participate.

The survey was accompanied by the collection of current field counts of residual fruit calyx buttons and the numbers of set fruit to determine the results of individual grower PFD programs. The CRDF field crew collected this data using the following standardized methods:

1. Twenty trees were evaluated per location.
2. Trees of similar in age, variety and disease severity within the site were evaluated.
3. Fruit and residual fruit calyx buttons were quantified within a 0.5 square meter frame twice on each side of the tree (4x total).

Data was collected from Hamlin, Valencia or Navel blocks at 21 sites. Bloom date at each location was calculated using the Citrus Flowering Monitor program (http://disc.ifas.ufl.edu/bloom/model.jsp). Weather data was collected from the Florida Automated Weather Network (FAWN) weather station in closest proximity to each site. Dates of fungicide applications and the material applied was collected from the grower participants. The CRDF field crew collected average buttons and fruit and a button to fruit ratio was calculated for the data analysis.

Results:
No significant effect of any fungicide treatment was found, but not applying fungicides resulted in significantly more buttons and less fruit. All other results support what is known about the disease; rainfall and temperature conducive to disease development during bloom resulted in less fruit and more buttons.

Discussion:
This retrospective survey was not robust enough to detect an effect of specific treatments or treatment timing on post-bloom fruit drop. Earlier implementation of this survey may have improved the data. Increasing the number of sites surveyed would also have greatly improved the survey. Sites with onsite weather and bloom timing data were originally sought for this survey, but FAWN and Citrus Flowering Monitor data was used for analysis. More sites could have been surveyed if the plan originally called for using FAWN and Citrus Flowering Monitor data.

Obj. 2 – Evaluate PFD management tactics under field conditions

The ongoing project titles “Enhancement of postbloom fruit drop control measures” was initiated in March 2016. This project is evaluating the efficacy and economics of PFD treatments, evaluating the period of efficacy of Luna Sensation during flowering, and determining if the flowering period can be narrowed using plant growth regulators, to eliminate offseason bloom.

Post-application button counts were taken for all trials this quarter. Price gathering for the products was completed and the economic analysis continues. Work on the prediction model continues.

In year one of the plant growth regulator field trials, GA and auxin treatments were evaluated for an effect on synchronizing and compressing flowering on HLB-affected citrus. A reduction in number of flowers was seen with the use of GA when multiple applications were made. Use of auxin did not change the flower number or flowering trend in HLB-affected citrus in past year. Collection of yield data this year will be critical to see the effect of reduced flower number on fruit number.