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

Quarter Ending September 30, 2016

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: 1aI. 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.

New chemicals were submitted for testing by companies previously associated with CRDF. Project managers continue to develop relationships with companies with potential bactericides and focus on chemicals that can be made available to growers in the near-term. In the past quarter no new companies were identified as potential sources of bactericides or chemical libraries.

Ongoing research funded by CRDF and projects funded by other funding agencies appear to be on track. Several discussions on the progress of research projects, as well as presentations on some of these projects, took place at the Citrus Expo in August and the International Citrus Congress in September.

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

Twenty candidate bactericides were advanced through the in vitro assay this quarter, of these candidates, four were advanced to the greenhouse assay. These four materials are in early stages of development and would be considered new active ingredients by the EPA Registration Division if a registration package were to be submitted. One of the tests materials from company D shows promising activity, this material is still in early stages of development and will not be advanced into field trials until it is further developed as an agricultural chemical.

In the next quarter, two biopesticides will be tested in the greenhouse assay as well as a pesticide from Company C, a registered pesticide that has bactericidal activity.

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.

CRDF project managers are working with a company to develop new adjuvants for bactericide delivery. CRDF is working with a researcher to develop a project to compare these new adjuvants with commonly used, commercially available adjuvants. This project will be presented next quarter for Committee and Board approval.

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

A research project being developed to evaluate a new application technology for field applications of bactericides has been developed from a completed CRDF project. This project tested the concept and provided evidence to support moving forward with the next phase.

Subproject Title: 1aIII. 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.

The field trial to evaluate trunk injections as an application method on Hamlins, project 15-048C, was set-up in February 2016. Data from the first six months of data collection were analyzed. Data being collected includes tree growth measurements, disease severity, root and foliar bacterial titer/C_t values, leaf nutrient analysis, yield, fruit drop and fruit quality. Data are not being presented in this report because no significant differences were found between treatments. In the next quarter yield and fruit quality data will be collected. Data collection will be completed in the third quarter of FY 2016-2017 and a full report will be presented in the fourth quarter.

The biopesticide field trial, project 15-049C, was set-up in late February on Hamlins. Data from the first six months of data collection were analyzed. Data being collected includes tree growth measurements, disease severity, foliar bacterial titer/C_t values, leaf nutrient analysis, yield, fruit drop, fruit quality and psyllid counts. Data are not being presented in this report because no significant differences were found between treatments. In the next quarter yield and fruit quality data will be collected. Data collection will be completed in the third quarter of FY 2016-2017 and a full report will be presented in the fourth quarter.

Project 934 was completed this quarter, this project evaluated soil drenches to prevent HLB infection. The data from this trial will be analyzed when all of the data has been received and results will be provided in the report for quarter two.

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 and disease severity data has been collected, fruit drop data are being collected on Hamlins and grapefruit and yield data will be collected in the next quarter on Hamlins and grapefruit. Valencia fruit data will be collected in the third and fourth quarter. PCR samples and disease severity data will be collected in December and. A full analysis of the data will be available in the third or fourth quarter of FY 2016-2017. The analysis will be available to growers on the CRDF website as soon as it is completed. Data are presently being compiled and any useful data summary will be provided to growers as soon as possible.

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

CRDF project managers are working with a company to develop a project evaluating a new application technology for field applications of bactericides. This project will be presented for Committee and Board approval early in the third quarter of FY 2016-2017 if the company is prepared to move forward.

CRDF project managers have met with the bactericide registrants to discuss registrant-managed trials. The continuance of the multi-year HLB field trial funded by CRDF was approved, this trial will evaluate application timings of oxytetracycline and streptomycin on Valencia, Hamlin and grapefruit at the maximum number of applications. Project managers will meet quarterly for updates on this trial.

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

A document was compiled and is available on the CRDF website outlining the results of a grower bactericide use survey. This survey represents the response of 100 growers and more than 50% of the reported acreage in Florida. The document can be found at: http://citrusrdf.org/wp-content/uploads/2012/10/Summary-of-Grower-Bactericide-Use-Survey-8-16.pdf.

A request recertification of the Tree Health Section 18 is being prepared and will be sent to the EPA Emergency Response Team from the Florida Department of Agriculture and Consumer Services in October. This request is for continued use of oxytetracycline and streptomycin in Florida in 2017. The current Section 18 expires on December 31, 2016.

Obstacles Encountered and Breakthroughs:

The greenhouse assay was improved this quarter, the PCR method has been changed to obtain the results in a shorter period of time; this method appears to provide a more accurate representation of the bactericide effect.

Significant Meetings or Conferences:

The CRDF bactericide program was discussed in a session during the educational section of the Citrus Expo in Fort Myers Florida on August 18. Presentations were given by Harold Browning on the results of the grower bactericide use survey, by Stephanie Slinski on the CRDF bactericide trials, and by Bob Shatters of USDA, ARS on replicated field trials of bactericides. The first two presentations can be found on the CRDF website, or at: http://citrusexpo.net/program/

The International Citrus Congress took place September 17-23rd in Foz do Iguazu Brazil. Three project managers from CRDF attended. Citrus researchers attended this conference from citrus growing regions around the world and many topics were covered, including a session entitled “HLB Pathosystem (Plant, Vectors and Bacteria) with talks on the testing of new bactericides.

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Quarter Ending September 30, 2016

1. *Candidatus* Liberibacter asiaticus PATHOGEN INTERVENTION

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

Narrative of Progress by Project Goals:

Obj. 2 - Determine impact of thermal treatment on CLas acquisition by ACP.

Ongoing CRDF-funded research at UF and USDA does not identify how thermal treatment affects availability of CLas 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 CLas acquisition testing on current field trials was suggested as a simple way to accomplish this goal. A project plan was developed by Kirsten Pelz-Stelinski of UF, IFAS, CREC and has been approved by CRDF, and subsequently approved for funding through the USDA MAC HLB program. The one-year research project is underway and has the following update.

The objective of this project is to evaluate the effect of thermal therapy treatment on *Candidatus* Liberibacter asiaticus (Las) transmission by the Asian citrus psyllid (ACP). Since the initiation of this project, a citrus grove, located at the Citrus Research and Education Center, has been identified for conducting bioassays. Trees in this grove are 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.

Whereas, this was a one-year funding project, CRDF extended the duration to allow the PI to continue the evaluation of transmission. The work is now complete and the publications pending from the work have not yet submitted.

However, as a result of the project extension by CRDF, the team was able to complete additional replicates of the thermal treatment followed by controlled ACP acquisition experiments. The repeat experiments also allowed the CLas acquisition to be measured following two sequential thermal treatments. While the data summary and interpretation have not yet been finalized in a final report, the findings include:

- Suppression of Clas in the treated trees by PCR measurements compared to untreated controls. This was relatively small, with retention of ability to measure Clas following treatment, a phenomenon more fully investigated in the studies reported below in the other objectives.
- Rates of acquisition of Clas from ACP following thermal treatment were reduced, and the time series of exposures by ACP demonstrated a rebound, where rates of acquisition returned to those measured in the untreated controls.
- Multiple thermal treatments showed the temporary reduction in acquisition, but there did not appear to be a significant cumulative effect.

The published results of this completed work will be made available when received by CRDF.

Obj. 3 - Refine requirements and environmental conditions for most effective thermal treatment.

The USDA, APHIS MAC group was charged to manage the federal funding to put HLB solutions in the hands of growers. This group quickly identified thermal therapy as a "shovel-ready" project area and encouraged development of project ideas and mechanisms to attract and encourage solvers to come forward with plans for scale-up, and to propose how this funding could facilitate rapid scale-up.

USDA, APHIS 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 were in place at the end of this quarter. 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 will have machines ready for testing or will have them field-ready within the next couple of months. 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 CLas and/or HLB and tree response, a before and after protocol was developed to document tree and environmental conditions surrounding thermal treatments and a data plan for follow-up so that individual trials will be evaluated similarly and treatments can be compared. This protocol has been publicized on the CRDF web page so growers can do some self-assessments of their own thermal therapy trials and has been implemented on a small scale with grower and research trials. The protocol will become 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 is engaged in follows:

<u>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>.

Most trees being 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 conducted by those involved in scale-up is ongoing by the CRDF evaluation team. Six enterprises are operating field thermotherapy machines in Florida. At least two other companies are supported by USDA, APHIS, MAC to deliver additional thermal therapy to Florida for field trials. Those with capability are operating at multiple locations in Florida, and the evaluation team is in the field conducting the evaluations.
At this reporting period, 14 trials are being evaluated, with varying intensity and with different machinery delivering a range of temperature/duration combinations. Since the trials continue to be set up as opportunities arise, we are providing the current data sets associated with trials currently being conducted. None of these trials are completed, but the results to date provide a glimpse of the variation of measures and tree responses. Significant additional data analyses will be available following the 2015-16 fruit harvest, providing yield, quality and other metrics.

All of these trials have been subjected to the protocol for evaluation as outlined per the approved work plan. The CRDF evaluation team is working with commercial scale-up thermal treatment applicators, helping to lay out field trials, collecting pre-treatment PCR bacterial measures, and other parameters. According to the protocol, periodic data collection following treatments will assess the tree health response as well as the specific impact on CLas bacteria. In cases where there are no untreated controls, the test will likely be reduced to the "short version evaluation". Having 14 locations under evaluation is ahead of the plans, and we anticipate being able to conduct additional evaluations as others get dropped to fulfill the work plan and budget.

Additional trial evaluations have been established as thermal therapy providers are ready for evaluation of their machinery and treatments. Additional treatment sites have been established to evaluate the new generation machines from Dr. Ehsani (UF, IFAS), Premier Energy, and Daniel Scott.

Status at end of 27 months of the scale-up program.

While this project does not control the tempo of innovation or the timetables for the various solvers who are commercializing thermal therapy for HLB-infected trees in Florida, there is significant progress being demonstrated on several fronts that is driving the evaluation component of this project. Several participants have revised designs in response to early evaluation results, and have deployed next generation machines.

The 14 sites reported on in this period are all ongoing thermotherapy projects where tree responses to different thermotherapy conditions are being monitored. Some treated trees displayed previous short-term responses that have since disappeared. The different sites are of various aged trees and varieties. Most projects have recent post-treatment leaf samples awaiting PCR analyses. All data and observations should be considered preliminary, as monitoring tree status and data analysis are continuing.

Notes for October, 2016 report:

While summaries of data to date are provided below, please note the following scheduling plans:

- Fall/Winter PCR testing has proven to be the most reliable, with less random variation that during other times of year. Thus, during the next reporting period, additional PCR tests will be conducted.
- Fruit drop, fruit harvest and fruit quality data will be forthcoming as citrus harvest begins in November and continues into spring, 2017. This will provide measures that matter most to citrus growers as they evaluate the value of thermal therapy.
- During the reporting period, meetings were held and discussions advanced for the expansion of evaluation of thermal therapy to the Texas citrus growing region. A meeting in August with potential grower, research and industry organizations in South Texas indicated their interest and willingness to participate in tests. Since the Texas citrus production region has widely varying climatic and horticultural requisites compared to Florida, it is expected that some logistical mechanical and biological variances will occur in attempting to deploy thermal therapy on trees with only a few years of infection there. The researchers involved in Florida have followed up with the Texas partners to develop a plan to explore testing of thermal therapy in Texas.

Specific Thermal Therapy Field Trial Results:

<u>Conserve Trial 1</u>

On 2-26-16, 15 sets of paired uniform of trees of Valencia on Swingle rootstock were selected for evaluation. Fifteen trees were non-steamed control trees and 15 trees were steamed at 131 F for 30 sec. On 6-3-16 and 9-16-16, Canopy Volumes (CV, m^3), tree height (TH, m), trunk cross sectional area (TCSA, cm^2) and Disease index (DI, 0-40) were evaluated. There were no treatment effects on these variables. The latest PCR values sampled on 4-11-16 have been include for reference.

Control				TCSA			
DATE	N	CV (m^3)	T H (m)	(cm^2)	DI (0-40)	CT	CN/DNA
_6_3_16	15	4.7	1.9	33.6	23.8	26.3	3109
_9_16_16	15	5.3	1.9	33.7	20.5	4_11_16	4_11_16
Treatment=131 F30s							
DATE	N	CV (m^3)	T H (m)	TCSA	DI	CT	CN/DNA
_6_3_16	15	4.9	2.0	32.3	23.9	25.6	2595
_9_16_16	15	5.3	2.0	33.3	20.6	4_11_16	4_11_16

Conserve Trial 2

On 3-2-16, 15 sets of paired uniform of trees of Hamlin on Swingle rootstock were selected for evaluation. Fifteen trees were non-steamed control trees and 15 trees were steamed at 131 F for 30 sec. On 6-3-16 and 9-16-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated. There were no treatment effects on these variables. The latest PCR values sampled on 4-11-16 have been include for reference.

Control				TCSA			
DATE	N	CV (m^3)	Tr Ht (m)	(cm^2)	DI (0-40)	PCR CT	CN/DNA
CV_6_3_16	15	4.5	1.9	27.3	23.1	27.2	3293
CV_9_16_16	15	5.4	2.0	29.1	18.5	4_11_16	4_11_16
Treatment=131F 30s							
DATE	N	CV (m^3)	Tr Ht (m)	TCSA	DI	PCR CT	CN/DNA
CV_6_3_16	15	4.5	2.0	26.4	23.1	27.2	1154
CV_9_16_16	15	5.3	2.0	28.8	18.9	4_11_16	4_11_16

Conserve Trial 3

On 2-26-16, 10 sets of 3 uniform trees of Valencia on Swingle rootstock were selected for evaluation. Ten trees were non-steamed control trees, 10 trees were steamed at 131 F for 30 sec., and 10 steamed at 120 F for 40 sec. On 6-3-16 and 9-16-16, canopy volumes, tree height, trunk cross sectional area and Disease index were evaluated. There were no treatment effects on these variables; TCSA data are not shown. The latest PCR values sampled on 2-24-16 have been include for reference. Both the treated sets of trees had a significantly lower copy number per DNA than the untreated control trees on 2-24-16.

Canopy volume (CV, m^3) _6_2_16

DMRT	CV (m^3)	N	Treatment
A	7.1	10	131F30s
A	7.0	10	120F40s
A	6.9	10	Control

Canopy volume (CV, m^3) _9_16_16

DMRT	CV (m^3)	N	Treatment
A	8.0	10	120F40s
A	8.0	10	131F30s
A	7.9	10	Control

Tree height (TH, m)_6_3_16				Tree height (TH, m)_9_16_16			
DMRT	TH (m)	N	Treatment	DMRT	TH (m)	N	Treatmer
A	2.2	10	131F30s	A	2.2	10	Control
A	2.1	10	120F40s	A	2.2	10	131F30s
A	2.1	10	Control	A	2.1	10	120F40s

Disease index (DI, 0-40)_6_3_16				DI_9_16_16			
DMRT	DI	N	Treatment	DMRT	DI	N	Treatment
A	22.4	10	131F30s	A	17	10	120F40s
A	22.1	10	Control	A	16.6	10	Control
A	21.7	10	120F40s	A	16.5	10	131F30s

PCR CT_2_24_16				CN / DNA_2_24_16			
DMRT	CT	N	Treatment	DMRT	CN/DNA	N	Treatment
A	29.463	10	131F30s	A	44341	10	Control
A	26.354	10	120F40s	B	8387	10	120F40s
A	25.288	10	Control	B	4793	10	131F30s

Cutrale Trial 1. Discontinued.

Cutrale Trial 2

There were 10 sets of paired uniform trees of 2 year-old Valencia on Swingle rootstock were selected for evaluation. Ten trees were non-steamed control trees and 10 trees were steamed at 127 F for 90 sec. On 6-20-16 (baseline) and 10-18-16, canopy volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. There were no treatment effects on these variables except treatment trees had a higher DI on 10-18-16.

Untreated Control				TCSA			
DATE	N	CV (m^3)	TH (m)	(cm^2)	DI (0-10)	PCR CT	CN/DNA
_6_20_16	10	2.0	137.3	13.6	0.7	31.2	10196
_10_18_16	10	2.4	152.4	17.1	4.6 b		
Treatment=127F_90sec							
DATE	N	CV	TH	TCSA	DI	PCR CT	CN/DNA
_6_20_16	10	2.1	144.4	14.9	0.7	34.3	2132
_10_18_16	10	2.6	158.5	18.8	5.3 a		

Cutrale Trial 3

There were 10 sets of paired uniform trees of 4 year-old Hamlin on Swingle rootstock selected for evaluation. Ten trees were non-steamed control trees and 10 trees were steamed at 127 F for 90 sec. On 6-20-16 (baseline) and 10-18-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. There were no treatment effects on these variables except treatment trees had a lower DI on 10-18-16.

Treatment=Control				TCSA			
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**
_6_20_16	10	7.4	233.4	33.1	15.8	23.2	117557
10_18_16	10	6.8	229.4	36.2	15 a		
Treatment=127 F for 90 sec							
DATE	**N**	**CV**	**TH**	**TCSA**	**DI**	**PCR CT**	**CN/DNA**
_6_20_16	10	7.1	231.6	31.8	15.8	23.8	178795
10_18_16	10	7.0	229.6	36.5	14.3 b		

Blue Goose Trial 1

There were 10 pairs of uniform trees selected for evaluation, 10 steamed and 10 non-steamed control trees. On 6-16-16 (baseline) and 10-14-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR on 8-1-16. Paired variables on each date followed by different letters differed significantly at $p<0.05$. Canopy volume (CV), tree height (TH) and trunk cross-sectional area (TCSA) of the treatment trees were larger than the untreated control trees on both dates but Disease Index (DI) and PCR values did not differ.

Untreated Control				TCSA					
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**	**PCR CT**	**CN/DNA**
_6_16_16	10	7.9 b	2.2 b	55.6 ns	17.9 ns	33.9	1968	34.1	1263
_10_14_16	10	7.6 b	2.2 b	57.4 b	17.7			**8_1_16**	**8_1_16**
Treatment=128F_30s									
DATE	**N**	**CV**	**TH**	**TCSA**	**DI**	**PCR CT**	**CN/DNA**	**PCR CT**	**CN/DNA**
_6_16_16	10	10 a	2.4 a	61.1	17.5	37.2	1163	36.9	448
_10_14_16	10	9.2 a	2.4 a	68.2 a	16.8			**8_1_16**	**8_1_16**

Blue Goose Trial 2

There were 10 pairs of uniform trees selected for evaluation, 10 steamed and 10 non-steamed control trees. On 6-16-16 (baseline) and 10-16-16, canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR on 6-15 and 8-1-16. There were no significant treatment effects on any of the measured variables.

Nontreated Control				TCSA					
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**	**PCR CT**	**CN/DNA**
_6_16_16	10	8.1	2.1	53.2	22.6	30.8	3640	28.7	5252
10_14_16	10	7.6	2.1	55.9	23.2	**6_15_16**	**6_15_16**	**8_1_16**	**8_1_16**
Treatment=128F_30s									
DATE	**N**	**CV**	**TH**	**TCSA**	**DI**	**PCR CT**	**CN/DNA**	**PCR CT**	**CN/DNA**
_6_16_16	10	8.5	2.1	55.0	22.2	28.1	4647	27.3	7192
10_14_16	10	8.0	2.1	56.8	21.7	**6_15_16**	**6_15_16**	**8_1_16**	**8_1_16**

Blue Goose Trial 3

There were 10 pairs of uniform trees selected for evaluation, 10 steamed and 10 non-steamed control trees. On 6-16-16 (baseline) and 10-14-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated. here were no treatment effects on these variables except TH of treated trees was lower on both dates and TCSA of treated trees was less on 10-14-16. Leaves were sampled for PCR on 6-16-16 and again on 8-1-16. On 6-16-16, the treated trees had a higher CT and lower CN/DNA (less HLB bacteria) than the untreated control trees but this difference disappeared by 8-1-16.

Untreated control				**TCSA**			
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**
_6_16_16	10	10.0	2.3 a	57.7 ns	22.1	26.3 b	7138 a
10_14_16	10	9.3	2.3 a	64.7 a	23.3	27.4 ns	5842 ns
						8/1/2016	**8/1/2016**
Treatment=128F_30s				**TCSA**			
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**
_6_16_16	10	9.0	2.1 b	51.9	21.4	31.4 a	2685 b
10_14_16	10	8.8	2.1 b	56.6 b	22.8	30.7	4510
						8/1/2016	**8/1/2016**

Scott Trial 3

Ray Ruby GF on Sour orange trees, 5 years old. Leaves for PCR were collected from 35 trees on 6/30/15 and 20 trees were steam treated on 7/20/15 at either at 130 F for 15 sec or at 130 F for 30 sec. There were 15 non-treated control trees, rounding out the 3 treatments in this trial. On 6-14-16 and 10-11-16, Canopy Volumes, tree height, trunk cross sectional area, Disease index, % leaf bleaching, % leaf drop and fruit drop were evaluated. There was no visible leaf bleaching. There were no treatment effects on these variables; TCSA did not differ (data not shown).

Canopy Volume CV, (m^3) 6 14 16					**CV (m3)_10_11_16**			
DMRT	**CV**	**N**	**Treatment**		**DMRT**	**CV**	**N**	**Treatment**
A	11.6	12	130F_30s		A	12.1	12	130F_30s
A	10.2	15	untreated		A	11.6	15	untreated
A	10.0	8	130F_15s		A	10.6	8	130F_15s

Tree Height (Tr Ht, m) 6_14_16					**Tree Height (m) 10_11_16**			
DMRT	**Tr Ht**	**N**	**Treatment**		**DMRT**	**Tr Ht**	**N**	**Treatment**
A	2.22	12	130F_30s		A	2.28	15	untreated
A	2.15	15	untreated		A	2.23	12	130F_30s
A	2.08	8	130F_15s		A	2.22	8	130F_15s

Disease Index (DI)_6_14_16					**Disease Index (DI)_10_11_1**			
DMRT	**DI**	**N**	**Treatment**		**DMRT**	**DI**	**N**	**Treatment**
A	21.9	15	untreated		A	19.9	15	untreated
A	21.5	12	130F_30s		A	19.8	12	130F_30s
A	20.8	8	130F_15s		A	19.3	8	130F_15s

Scott Trial 4

Ray Ruby Grapefruit on Sour orange trees, 6 years old. 12 trees each were steam treated on 9/8/15 at either 128 F for 120 seconds, 132 F for 1 second (turned off immediately when temperature in canopy reached 132 degrees, or 132 F for 10 seconds. 12 trees were left untreated as a control. On 6-21-16 and 10-4-16, Canopy Volumes, tree height, trunk cross sectional area, Disease index, % leaf bleaching, were evaluated. There was no visible leaf bleaching. On 10-4-16, treated trees all had smaller canopies than the untreated control trees. On 6-21-16, trees treated at 132 F for 1 sec had the lowest DI but by 10-4-16, trees treated at 128 F for 20 sec. had the highest DI and the untreated control trees had the lowest DI. TCSA did not differ (data not shown).

Canopy Volume (m^3) 6 21 16			
DMRT	**Can Vol**	**N**	**Treatment**
A	21.6	12	untreated
A	20.8	12	132F_10s
A	18.4	12	132F_1s
A	18.1	12	128F_20s

Canopy Volume (m^3) 10 4 16			
DMRT	**Can Vol**	**N**	**Treatment**
A	24.3	12	untreated
B	18.4	12	132F_10s
B	18.2	12	132F_1s
B	17.6	12	128F_20s

Tree Height (m) 6 21 16			
DMRT	**Tr Ht**	**N**	**Treatment**
A	2.77	12	untreated
A	2.63	12	132F_10s
A	2.59	12	128F_20s
A	2.58	12	132F_1s

Tree Height (m) 10 4 16			
DMRT	**Tr Ht**	**N**	**Treatment**
A	2.84	12	untreated
A	2.62	12	128F_20s
A	2.58	12	132F_1s
A	2.58	12	132F_10s

Disease Index (DI, 0-40) 6 21 16			
DMRT	**DI**	**N**	**Treatment**
A	18.6	12	untreated
A	18.4	12	128F_20s
A	18.1	12	132F_10s
B	16.5	12	132F_1s

Disease Index (DI) 10 4 16				
DMRT		**DI**	**N**	**Treatment**
	A	23.8	12	128F_20s
B	A	22.4	12	132F_1s
B	A	22.3	12	132F_10s
B		20.9	12	untreated

Davis

Valencia/Swingle trees 10 years old. 24 trees were sampled for PCR on 4/6/15 and leaves have been resampled for PCR in Jan 2016. All 24 trees were steam treated on 4/9/15 at 120 F for 30 seconds. Tree trunk and canopy growth, fruit drop, and visible disease index (DI) has been monitored monthly since April 2015. On 6-22-16, Canopy Volumes, tree height, trunk cross sectional area, Disease index and PCR were evaluated and half the trees were retreated with steam at 120 F for 30 seconds. Canopy measurements were repeated on 9-22-16 but there were no significant effects of this treatment.

Untreated Control				TCSA		
Dates	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**CN/DNA**
CV_6_22_16	12	20.5	2.3	118.8	18.8	35319
CV_9_22_16	12	19.4	2.3	123.9	17.6	**6_24_16**
120F_30sec				**TCSA**		
Dates	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**CN/DNA**
CV_6_22_16	12	21.5	2.3	118.9	18.5	22790
CV_9_22_16	12	20.5	2.3	124.1	17.3	**6_24_16**

Shinn

Valencia /Swingle, 3 years old, double set. Pretreatment leaves were sampled for PCR on 8/7/2015. Eighteen trees were treated 8/7/2015 at 122-127 F (avg 125) for 30 sec. and 18 trees were untreated as controls. On 5-31-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated. This year's earlier PCR data from 1-25-16 are listed for reference. There are no differences between treatment and control values.

		5_31_2016			1_25_16	
Treatment	**N**	**DI (0-40)**	**CV (m^3)**	**Tr Ht (m)**	**CT**	**CN/DNA**
Control	18	17.9	2.4	1.5	26.2	3725.6
125F_30sec	18	18.3	2.1	1.4	25.0	3136.5

Lykes

Hamlin / X639 trees, 4 years old. 24 trees were steam treated on 10/6/15 at 55 C (131 F) for 30 seconds and 24 trees were left as untreated control trees. Leaves were sampled for CT from PCR on 1-26-16. Canopy volume (CV, m^3), tree height (m), trunk cross sectional area (TCSA, cm^2) and disease index (0-40) measured on Jun 17 and Oct 10, 2016. Treated trees had smaller TCSA but had a lower DI (looked better) on Oct 10, 2016.

		6_17_16	**10_10_16**	**1_26_16**	**1_26_16**
Treatment	**N**	**CV (m^3)**	**CV (m^3)**	**CT**	**CN/DNA**
Control	24	12.2	15.4	25.1	1610
131F_30sec	24	11.4 ns	14.5 ns	24.5 ns	3662 ns
		Tr Ht (m)	**Tr Ht (m)**		
Control	24	2.4	2.8		
131F_30sec	24	2.4 ns	2.7 ns		
		TCSA (cm^2)	**TCSA (cm^2)**		
Control	24	70 a	75 a		
131F_30sec	24	58 b	63 b		
		DI (ns)	**DI**		
Control	24	17.3	14.7 a		
131F_30sec	24	17.7	13.6 b		

Lee Jones

Based on initial PCR evaluations, 22 uniform trees that were HLB positive and 22 uniform HLB negative were selected for evaluation. On 3-22-16, one tree in each pair was steam treated at 128 F for 30 sec while the other paired tree was left as an untreated control. On 3-9-16, baseline Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated (reported in last report). On 6-1-16, 7-27-16 and 10-12-16, canopy volumes, tree height, trunk cross sectional area and Disease index were evaluated. There were no significant of the treatment on these variables. The most recent PCR data (5-5-16) are included for reference.

Untreated control				**TCSA**			
DATE	**N**	**CV (m^3)**	**TH (m)**	**(cm^2)**	**DI (0-40)**	**PCR CT**	**CN/DNA**
_6_1_16	22	6.4 ns	2.3 ns	22.2 ns	18.2 b	32.2 ns	54707 ns
_7_27_16	22	6.4 ns	2.3 ns	24.5 ns	14.1 ns	**5_5_16**	**5_5_16**
_10_12_16	22	7.1 ns	2.4 ns	26.0 ns	13.1 ns		
Treatment=128F_30s							
DATE	**N**	**CV**	**TH**	**TCSA**	**DI**	**CT**	**CN/DNA**
_6_1_16	22	6.3	2.4	22.6	20.1 a	32.4	38628
_7_27_16	22	6.3	2.4	25.6	15.1	**5_5_16**	**5_5_16**
_10_12_16	22	7.5	2.5	27.5	13.6		

Obstacles: There are few obstacles related to the field testing described above. However, this evaluation protocol has not been applied to two of the three MAC projects that are funding delivery of field-ready equipment to apply thermal treatment. The Ag-Harvesters machinery has not been delivered for field use, and therefore cannot be included in these evaluations. The same is true for the project funded through the BAA that proposes to test trunk application of heat for CLas suppression. A part of this project was to evaluate additional thermal treatment equipment as it became available for testing in Florida, so we continue to be prepared to expand to participate in these evaluations when appropriate.

Obj. 4 - Outreach on Performance of Thermal Therapy Treatment for HLB:

During this period, presentations and written progress of the evaluation of thermal therapy continued. This included presentation by Dr. Reza Ehsani, UF, IFAS and by Mr. Bill Barber, Lykes Brothers Citrus, and Mr. Larry Davis, Davis Cattle and Citrus on design and performance of thermal therapy equipment. A research update was provided by Dr. Ehsani at the August Citrus Expo in Fort Myers, and he also displayed the equipment in the trade show. At the same meeting, the two growers presented updates on their company efforts to build and deploy thermal therapy equipment to the audience of around 300 growers.

Premier Energy, the Georgia-based company that has deployed nearly 20 thermal therapy machines in Florida, provided a written summary of their evaluation efforts to the industry during this quarter as well. Informal and formal field days also were conducted involving grower cooperators, grower providers of thermal therapy and research teams working to fine-tune equipment design.

Finally, a tour was provided during this past quarter for USDA, APHIS leaders to see demonstration of several strategies for management of HLB in Florida. The two-day tour included an on-site demonstration of the equipment developed by Dr. Ehsani of UF, IFAS, as well as a static display of the technology being developed through MAC funding to deliver heat to citrus tree trunks. These demonstrations occurred at the USDA, ARS Horticultural Research Laboratory in Fort Pierce and in nearby commercial groves.

CRDF Commercial Product Delivery Project Progress Report FY 2016-17

Quarter Ending September 30, 2016

2. Asian Citrus Psyllid VECTOR INTERVENTION

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

Narrative of Progress by Project Goals:

Obj. 1 - Pursue actions that will support expanded tools for ACP management

The continuing CRDF ACP portfolio has a number of active projects. Among them are projects that may contribute to addressing the increased ACP pressure this year. Work on movement of ACP in and out of groves, attraction and suppression strategies and overwintering locations continues.

With leadership from the USDA HLB Multi-Agency Coordinating Group (MAC), a subsample of the US ACP research team convened by phone during the quarter to develop a group work plan to integrate attract/repel results from individual projects into a deployable tool. The approved MAC project seeks to validate individual components and to conduct field trials of these integrated tools to monitor and/or affect ACP field populations. Florida leadership on this project group will allow CRDF to keep updated on progress.

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

FFVA has been active in pursuit of recertification of the neonicotinoid insecticide Belay (Clothianidin) in concert with Valent and the industry. Submission of the recertification request occurred during this quarter, proposing the same use details as the prior Section 18 period. The goal is to have approval for continued use as the current Section 18 expires at end of November.

Obj. 3 - Continue participation in pesticide stewardship activities

15-038C Resistance Monitoring: Dr. Stelinski continues to monitor at locations around the state for resistance development. Discussion among the researchers and growers have highlighted the importance of rotation of active ingredients. This topic is becoming more contested as growers are attempting to reduce ACP suppression costs and resort to lower cost spray materials, relying on more applications of the same active ingredients.

15-036C Distribution and behavior of pesticides targeting ACP: Correlating pesticide residue analysis with psyllid feeding to improve protection of young trees is providing results that will improve our understanding of the movement and retention of pesticides on/in targets and inform adjustments to spray recommendations, both timing and choice of materials. Dr. Rogers is completing another round of evaluations of the movement and residues of systemic materials in use to suppress ACP.

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

Brandon Page and the IFAS team are continuing to contribute to CHMA meetings, and the focus is emphasis on APC suppression in areas that are seeing much higher populations during this season. These meetings addressed the increase in ACP populations in mid-summer cycles of CHRP scouting, and focused on proper rotation of active ingredients and timing of sprays which complement the scheduled CHMA applications

Removal of abandoned citrus progressed during the quarter, with all MAC funded block removal completed by end of the quarter. Callie Walker with FDACS has evolved plans for using State Legislative funding to eliminate further acreage of abandoned groves, building on what has been accomplished through the two years of MAC-funded pilot programs. The state funding program will follow what has been done through contracted removal of abandoned acreage, and will add incorporation of ACP suppression to be conducted by the contractors prior to removing the acreage. With higher than normal ACP populations in the field, this suppression ACP spray prior to tree removal will reduce the movement of adult psyllids from trees being removed.

During the past quarter, CRDF, FFVA/TPR and registrants have been working to develop a plan for the use of herbicide products to quickly and effectively kill standing abandoned citrus trees, effectively eliminating them as reservoirs of psyllids and CLas. Regulatory considerations are among the topics being worked out, along with field use, technical recommendations, and best use practices to avoid non-target impacts. CRDF has reached out to UF, IFAS Extension to engage them in outreach once a tool is available.

Significant Meetings and Conferences:

Expo August 18-19, 2016, Fort Meyers. Over 1,000 growers participated in this two-day program which featured many CRDF topics and funded projects. Dr. Phil Stansly presented an overview of the evaluation of an ACP program, incorporating all of the available tools and strategies to minimize HLB movement via ACP.

In August, 2016, an informal grower forum Sebring was organized by production managers to discuss ACP population resurgence during 2016. The meeting was attended by about 30 production managers, and also included citrus entomologists from UF and USDA, ARS who are conducting field research on ACP. Discussion focused on reasons for higher population pressure in 2016 and a review of best practices to fight this high ACP pressure. Growers provided specific data from their operations that included ACP populations from scouting, as well as their IPM plans in place in 2016. Researchers answered questions and summarized the needs going forward to continue pressure on ACP populations. The need for higher participation in CHMA programmed applications was stressed.

The International Citrus Congress held in Brazil in September included oral and poster presentations across the breadth of topics affecting citrus, and technologies available to address solutions. HLB and ACP were topics addressed in about 1/3 of the program. Three keynote addresses focused on ACP topics, including: advances in RNAi (Bryce Falk, UC Davis); multiple approaches in citrus to integrate biological control (Jose Parra, UNESP); and engineered citrus to repel ACP populations (Leandro Pena, Fundecitrus). There also were a number of oral presentations and posters on the psyllid biology and

ecology, suppression tactics for integration into citrus management, and the use of biotechnology to address solutions to ACP. These represented authors from citrus countries around the world. Similarly, the International Congress of Entomology was held in Orlando in late September, one week after the Citrus Congress. This broader meeting draws more than 2,000 participants, and the program has numerous presentations on Asian citrus psyllid, including an in-depth symposium on ACP biology and management.

Obstacles Encountered and Breakthroughs:

It is apparent that during 2016, many citrus growers are seeking ways to remove costs from their production budgets to balance reduced fruit harvests. Difficult decision on where best to invest the production budget on a balance of nutrition, irrigation, ACP suppression, application of bactericides, and other cultural operations has caused some growers to reduce their investment in ACP suppression. Fewer growers are participating in cooperative sprays, in addition to fewer applications of less expensive materials that in general have shorter residual activities. The additional complication is that more groves are transitioning to "unmanaged". The net result with regards to ACP can be summarized:

- Fewer groves are being treated
- Growers are applying fewer applications per season on average
- Less expensive products which may have shorter residual are being chosen at a higher level
- Summer rainfall patterns are favoring abundant flushes, which also encourages ACP oviposition and population development.

These factors in combination lead to the likelihood of higher ACP populations during this time, and enhance the value of concerted efforts to keep ACP populations in check.

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

Quarter Ending September 30, 2016

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

A contractual agreement between CRDF and Southern Gardens was completed, effective September 1, 2016, in which CRDF will fund a portion of the direct expenses related to the trial, and Southern Gardens/Southern Gardens Nursery will provide in-kind funding to cover some direct and all indirect costs of the trial. A first quarterly report from Southern Gardens will be provided to CRDF in January 2017 covering October to December activities.

This agreement is based on a May, 2016 approval by the CPDC and CRDF Board, of a proposal submitted by Southern Gardens to conduct a three-year field trial with a targeted start in the Spring of 2017. The purpose is to evaluate in a field trial selected dsRNAs that were found effective in a greenhouse environment against Asian Citrus Psyllids when expressed in plants using the Citrus Tristeza Viral Vector (CTVvv). This CRDF funded research (618C), under Dr. William Dawson, ended in September, 2015 and is currently being funded by a USDA Specialty Crop Grant, under which Dr. Dawson will continue to identify the most effective target sequences, test multiple sequences, and optimize delivery methods. Dr. Dawson is also working with Southern Gardens to provide plants for the field trial. Based on results of this trial, a decision will be made regarding a Phase 2 area-wide field trial.

Goals of the field trial are as follows:

- Determine if selected target sequences are effective in controlling ACP
    - Cage studies on field grown trees
    - Natural infestations on field grown trees
- Determine the effectiveness of the CTVvv as a delivery method of RNAi
    - Is CTVvv + RNAi effective year round or are there periodicity issues
    - Does CTVvv + RNAi work equally well on young and old leaves
- Determine the effect of CTVvv + RNAi on CLas
    - Acquisition of CLas by ACP
    - Transmission of CLas by ACP
- Determine the effect CTVvv + RNAi on the spread of HLB

A secondary goal of the trial is to familiarize the regulatory agencies with the technologies and to help establish the field testing conditions for trials with RNAi. By establishing the trials, the industry can 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.

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

During the quarter, efforts continued to identify and explore opportunities for collaboration with companies investing in RNAi research and development for use in agriculture. Communications are continuing with Forrest Innovations, which is developing RNAi products to help shore up the defense mechanisms within citrus trees to modulate the effects of the HLB pathogen. The company is currently focused on its mosquito control product due to the Zika outbreak, so progress has been slow.

During the quarter, the prospects of pursuing RNAi discussions with Syngenta were explored as part of a broader discussion of cooperation. It was determined that it was premature at this point to pursue such discussions.

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

This is an ongoing effort. As the nuPsyllid project progresses, there are an increasing number of insights that may be useful in implementing the Psyllid Shield concept of wide area deployment of "altered" psyllids with reduced capability to spread HLB.

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

Commercial partners will be needed for follow-on work to the phase one field trial described above This includes support for an area wide "Psyllid Shield" field trial, as well as supporting regulatory, product development and other work needed to bring products to market.

Past discussions with University of Florida Office of Technology Licensing (Dr. Byatt) and outreach to potential partners suggests the most likely commercial partners will come from within the citrus industry due to the relatively limited size of the market and opportunities for return on investment.

CRDF continues to facilitate, accelerate and incentivize corporate action and is prepared to provide regulatory, commercial delivery and other support, as appropriate, to candidate partners.

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 2016-17

Quarter Ending September 30, 2016

3. CITRUS HOST INTERVENTION

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

Narrative of Progress by Project Goals:

Obj. 2 - Conduct field trials to test commercially available naturally occurring microbes.

The overall goal of the project is to screen candidate antimicrobials and deliver best performers through field trials to commercial use. This study is a side-by-side comparison of these 5 soil-applied commercially available products (+water control) as well as organic mulch as recommended by growers. We are testing the hypothesis that soil-applied products will mitigate the effects of HLB on tree health and yield. We expect that differentiation in tree health and disease status will appear in year 2, and after 3 years, we will have valid information on the true impact of these treatments on tree health, disease rating, HLB status, foliar nutrition, root density, yield and fruit quality.

Experimental protocols were developed to provide a sound scientific assessment of HLB effects of 5 commercially available microbial soil amendment products (BioFlourish, Ecofriendly, Serenade, Quantum and Aliette) plus a water treated control (UTC), in multiple applications per year as recommended. A subset of trees within each treatment was mulched with mature cow manure.

Ongoing treatments (quarterly or monthly) were began in May/June 2014 and are being applied with and without an organic mulch at the 3 Valencia/Swingle trial sites, Ridge, East Coast, Southwest Florida. This report summarizes the project after the 2nd year anniversary which occurred May/June 2016 after the second harvest. All required field work at all 3 sites is on schedule and all the data has been submitted on time.

The Field Trial Project Manager, the Field Trial Administrator and Staff are monitoring the project activities. CRDF established data repositories for each project site so that all photos, data and treatment data are provided to CRDF as they are collected. Each of the 3 trials consists of the 6 treatments of 20 trees, 4 reps = 24 plots of 20 trees = 480 trees at each site plus. Sub-plots of 3 trees within each of the 24 plots = 72 trees mulched at each site.

Contracted crop consultants are applying product treatments plus mulch, monitoring canopy volume and Decline Index (DI), photographing sentinel trees and taking leaf samples for PCR and nutrient analysis. At harvest, total fruit weight fruit is evaluated and samples are taken for juice quality analysis. Soil cores were sampled in Dec 2014 and Dec 2015 to determine root density. Soil amendment effects on root densities were not remarkable but were summarized in the June 2016 report. This report (Sept 2016) focuses on leaf nutrient values from samples taken in August, 2016, after 27 months of treatments.

Site Results to Date:

Ridge Site, Balm FL:

Valencia/Swingle trees are 17 years old. After 2 years of treatments, there were no effects of the mulch treatment so + an – mulch treatments were combined for n=28 hereafter. There were no treatment effects on DI but the Bioflourish treatment resulted in larger canopy volumes than the Quantum and untreated control treatments. From the Jun 2016 report, there were no treatment effects on CT values nor on fruit yield from the April 27, 2016 harvest. All trees were HLB positive having a CT less than 31. Fruit yields were relatively low (1-1.2 boxes) for 17-year-old trees with canopy volumes of 29-41 m^3.

Leaves were sampled for nutrition analyses on 8-2-16, brought to the lab, washed, oven dried and sent to the IFAS commercial lab for analyses. Leaf samples from the 6 measurement trees within each of the 4 replicate blocks per treatment were pooled (n=4 for each treatment) plus a separate sample from the mulched trees in each block for a total of N = 8 for each treatment. In general, leaf N was below the optimum range but most leaf nutrients were near or within the optimum range (on a standard leaf dr wt basis). There were no treatment effects on leaf N, P or K but leaves from untreated control and Bioflourish treated trees had lower Cu than the other treatments. There were no treatment effects on all other leaf nutrients measured as all others were at or near the optimum ranges for each element. There was no mulch effect on any measured nutrient.

Optimum: 2.5-2.8 % dw			**.12-.17 % d w**		**1.2-1.7 % dw**		**6-16 ppm**	
N	**% N dw**	**Treatment**	**% P**	**Treatment**	**% K**	**Treatment**	**% Cu**	**Treatment**
8	2.1	Quantum	0.20	Untreated	1.6	Untreated	12 a	Quantum
8	2.1	BioFlour	0.20	BioFlour	1.5	Serenade	10 b	Aliette
8	2.1	Untreated	0.19	EcoFrien	1.5	BioFlour	10 b	Serenade
8	2.0	EcoFrien	0.19	Serenade	1.5	Aliette	10 b	EcoFrien
8	2.0	Serenade	0.19	Aliette	1.5	Quantum	9 b	Untreated
8	1.9	Aliette	0.19	Quantum	1.5	EcoFrien	9 b	BioFlour

Disease Index (DI, 0-40)_8_29_16			
DMRT	**DI (0-40)**	**N**	**Treatment**
A	10.2	28	Quantum
A	10.0	28	BioFlour
A	9.8	28	Serenade
A	9.7	28	EcoFriend
A	9.6	28	Aliette
A	7.5	28	Untrt Cont

DI_8_29_16			
DMRT	**DI (0-40)**	**N**	**Mulch**
A	11.0	24	Yes
A	9.2	144	No

Likewise, the Disease index (DI) was not affected by treatment or by the mulch treatment. All 168 measurement trees were indexed for DI so there were 6 measurement trees + 1 mulched tree = 7 trees per treatment and 4 replicate blocks = 28 trees per treatment. To check for potential treatment effects or HLB starch accumulation effects on leaf tissue density or dry weight per area that could affect nutrient concentration expressed on a dr wt basis, leaf dry weight per area (LDW/A) was calculated and used to express nutrient concentrations on a leaf area basis (mg/m^2). There was no effect of

amendment treatment or mulch treatment on LDW/A). Thus, treatment effects on leaf nutrients expressed on a leaf area basis did not differ from treatment effects on leaf nutrients expressed on a leaf dr wt basis.

LDW/Area (g/m^2)

DMRT	LDW/A	N	Treatment
A	227	8	Aliette
A	223	8	Serenade
A	221	8	EcoFriend
A	219	8	BioFlour
A	216	8	Quantum
A	214	8	Untrt Cont

LDW/Area (g/m^2)

DMRT	LDW/A	N	Mulch
A	223	24	yes
A	217	24	no

East coast, Indian River site:

Valencia/Swingle trees are 6 years old. From the Jun 2016 report, the Bioflourish and Ecofriendly treated trees had a lower DI 10, so looked better) than Untreated Control trees (DI=15). There were no treatment effects however, on CV, CT or fruit yield. All trees were HLB positive having a CT less than 31 (24-26).

Leaves were sampled for nutrition analyses on 8-8-16, brought to the lab, washed, oven dried and sent to the IFAS commercial lab for analyses. Leaf samples from the 6 measurement trees within each of the 4 replicate blocks per treatment were pooled (n=4 for each treatment) plus a separate sample from the mulched trees in each block for a total of N = 8 for each treatment. In general, all leaf nutrients were near or within the optimum range (on a % dr wt basis). Leaves from the Aliette treated trees had the highest leaf N and the other treatments did not differ. Leaf P was not affected by treatment but leaves from the Bioflourish and Aliette treatments had higher K than the other treatments. On a leaf dry wt basis, no other leaf nutrients were affected by treatments. The Quantum and Ecofriendly treated leaves had greater leaf dry weight per area (LDW/A) than the other treatments such that Leaf N expressed on a leaf area basis (g/m^2) was greater in Quantum and Aliette than in the untreated control (UTC) leaves. In addition, leaf K on an area basis was lower in the UTC and Serenade treated leaves than the other treatments. LDW/A was not affected by the mulch treatment. Although well within the optimum range, mulch significantly increased leaf P by 0.01 %.

% N (dw) (Opt. = 2.5-2.8)

DMRT		% N	N	Treatment
	A	2.6	8	Aliette
B	A	2.5	8	Quantum
B	A	2.5	8	Serenade
B	A	2.4	8	EcoFrien
B		2.4	8	BioFlour
B		2.4	8	UTC

% P (dw) (Opt. = .12-.17)

DMRT	% P	N	Treatment
A	0.16	8	Aliette
A	0.16	8	BioFlour
A	0.16	8	EcoFrien
A	0.16	8	Quantum
A	0.15	8	UTC
A	0.15	8	Serenade

% K (dw) (Opt. = 1.2-1.7)

DMRT		% K	N	Treatment
	A	1.6	8	BioFlour
	A	1.6	8	Aliette
B	A	1.5	8	Quantum
B		1.4	8	EcoFrien
B		1.4	8	UTC
B		1.4	8	Serenade

LDW/A (g/m^2)				
DMRT		LDW/A	N	Treatment
	A	240	8	Quantum
	A	236	8	EcoFrien
B	A	233	8	BioFluor
B	A	228	8	Serenade
B	A	228	8	Aliette
B		224	8	UTC

LDW/A (g/m2)			
DMRT	LDW/A	N	Mulch
A	233	24	no
A	230	24	yes

DMRT	% P	N	Mulch
A	0.16	24	yes
B	0.15	24	no

Leaf N (g/m^2 leaf area)				
DMRT		N (g/m2)	N	Treatment
	A	608	8	Quantum
	A	593	8	Aliette
B	A	578	8	EcoFrien
B	A	564	8	BioFlour
B	A	561	8	Serenade
B		534	8	UTC

Leaf K (g/m^2 leaf area)				
DMRT		K (g/m2)	N	Treatment
	A	371	8	BioFlour
	A	364	8	Quantum
	A	360	8	Aliette
B	A	331	8	EcoFrien
B		315	8	UTC
B		309	8	Serenade

When nutrients were expressed on a leaf area basis (g/m^2), there were no treatment effects on P, Ca, Fe, Mg, B, Zn or Mn (data not shown). Although all values were within satisfactory ranges, after 3 years of the mulch treatment, leaf P, K, Mg, B and Zn were increased by mulch while leaf Ca was decreased (data not shown). Leaf Mn, Cu and B were not affected by the mulch treatment (data not shown). A grower field day is planned for Dec 6, 2016 to highlight treatment effects at the East coast (Indian river) site.

<u>SW FL Duda site</u>:

Valencia/Swingle trees are 11 years old. From the Jun 2016 report, there were a few differences in DI among treatments such that none of the average DI differed significantly from that of the untreated control. Canopy volumes of the Bioflourish, Aliette, Quantum and Ecofriendly treatments were all greater than Untreated Water Control and the Serenade treatment. None of the CT values from the treatments differed from the Water control but only the Serenade treated trees would be considered HLB + as having a CT lower than 31. Fruit yield corresponded to canopy size as the smaller Water control and Serenade treated trees had lower yield than the larger trees from the other 4 treatments. There were no treatment effects on total brix in the juice or on lb solids per box.

Leaves were sampled for nutrition analyses on 8-31-16, brought to the lab, washed, oven dried and sent to the IFAS commercial lab for analyses. Leaf samples from the 6 measurement trees within each of the 4 replicate blocks per treatment were pooled (n=4 for each treatment) plus a separate sample from the mulched trees in each block for a total of N = 8 for each treatment. Results from the nutrient testing lab have not arrived yet.

Obj. 3 - Provide communication on project goals, progress and results to CPDC, CRDF and growers.

Results of these field sites are being regularly communicated to the Florida citrus industry by CRDF through written reports, a quarterly progress report to the Committees and Board of CRDF which is posted to the CRDF website, and through presentation at grower meeting as indicated below. Completion of the fruit harvest data collection and analysis in June 2016, allowed a comprehensive view of the value of these treatments over the first two years of the trial.

Significant Meetings or Conferences:

A grower field day is planned for Dec 6, 2016 to highlight treatment effects at the East coast (Indian River) site.

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3. CITRUS HOST INTERVENTION

Project Title: 3b. 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.

Tracking of research projects evaluating scion and rootstock genotypes for tolerance or resistance to HLB focused on research programs in Florida. CRDF staff met with individual researchers and with teams in the IFAS and USDA-ARS units to gather information regarding their ongoing research towards HLB resistant/tolerant genotypes. Projects were divided into two categories based on general approaches to generation of potentially HLB resistant/tolerant genotypes:

1. Conventional Breeding projects:

Biological challenges of conventional breeding in citrus have been expressed by all researchers involved in plant improvement. However, philosophical differences in conventional breeding further complicate understanding of project outcomes. During this quarter the following were identified:

a. There are different philosophies towards breeding of citrus
b. Multiple programs have overlapping goals yet work independently
c. Definitions and evaluations of important traits differ by researcher and use different protocols
d. HLB tolerance/resistance is not defined which complicates determination of success

2. Biotechnology projects:

Biotechnological approaches in citrus are relatively new areas of research as compared to other crops, and that presents unique challenges. Regulatory agencies in government provide guidance in the management of materials which can further add to the complexity working with these materials. Researchers need support to navigate the Biotechnology Regulatory Service, FDA, EPA and others to stay in compliance.

Project proposals in CRDF's portfolio reveal some overlap of project goals which has led to multiple researchers working on the same genes of interest. At this stage, CRDF staff are working to understand the status of the projects and what progress has been made towards HLB solutions. CRDF staff will continue to work with researchers to provide information regarding regulatory issues and encourage collaboration to establish common molecular and phenotypic characterization protocols.

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

Discussions with researchers regarding evaluation and planning in individual programs and collaborative efforts are ongoing.

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

Pipelines to create a common platform to evaluate, identify and advance the best performing HLB tolerant/resistant scion and rootstock candidates are being developed.

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

CRDF staff meetings with researchers in Florida to gain an understanding of current and planned field trials for evaluation of HLB tolerant/resistant rootstocks are ongoing. Thus far, we have learned there are many field trials with mixed goals and in varying stages of data collection. There are differing views among researchers about the extent of data collection, p and when/if data should be collected and protocols used to evaluate traits. Planning and resources for data collection in planned and newly planted large-scale field trials are lacking. CRDF staff will continue discussions with researchers to define work plans for data collection and evaluation of ongoing field trials for experimental materials in current and future field trials.

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:

Planting of CRDF rootstock trials at the three sites: Southwest Florida (Duda, LaBelle) site, Ridge site 1. (Peace River, Babson Park), and at the 2nd ridge site (Ben Hill Griffin, Venus) was completed in the Spring of 2016.

Standardized CRDF protocols for data collection on horticultural traits have been developed and are in use for horticultural evaluation for HLB disease index (DI), PCR for *Candidatus* Liberibacter asiaticus bacterial titer, tree height, trunk cross-sectional area, canopy volume as well as leaf sampling, dry weight, and leaf area for nutritional analyses.

<u>Data collection and analyses:</u>

During this quarter horticultural trait data were collected and analyzed statistically within each site. Preliminary statistical analyses of horticultural data reveal some differences in rootstock performance within sites, the significance of which may become more apparent as the trials mature. Comparison of rootstock performance among sites at this stage has confounding effects due to different completion dates of planting of some rootstock entries at the two ridge sites (Peace River and Ben Hill Griffin). Horticultural performance data including HLB disease incidence and severity will be collected in each quarter as the trees mature. This data may further the understanding of the

impact of HLB infection on new plantings as well as the degree of tolerance or resistance to HLB of these selected rootstocks. Leaf nutrition data is in the process of being analyzed and will be reported in the next report.

Obstacles Encountered and Breakthroughs:

Determining the current status of plant improvement efforts by many researchers with different approaches and research philosophies is challenging. This challenge is further is underscored by reluctance in some to provide information which would further our understanding of progress and challenges encountered.

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3. CITRUS HOST INTERVENTION

Project Title: 3c. Genetic Technology (MCTF): Deploying Canker-Resistant Genes

Narrative of Progress Against Goals:

Obj. 1a - Continue Agrobacterium and biolistic transformation with genes to confer disease tolerance to HLB and canker as a service

During the quarter, the facility continued to provide transgenics to Drs. Dutt, Grosser, McNellis, Mou and Wang. An additional 12 independent, transgenic mature Valencia and Hamlin scions (some events were duplicated to equal 15) were shipped to Dr. McNellis at Penn State University during the quarter. Because *in vitro* plants are easier and cheaper to ship than large citrus trees, the plants were small and had not undergone the secondary graft. Ten transgenic Hamlin and Valencia scions (with duplicates and triplicates) were produced for Dr. Mou. These also had not undergone secondary grafting. Secondary grafts were performed on all plants for Drs. Grosser, Dutt and Wang, to enhance the growth of the transgenics. Six additional transgenics were transferred to Dr. Wang. Approximately 21 transgenics were produced for Drs. Dutt and Grosser and were transferred during the quarter, of which 16 had rooted.

Obj. 1b - Biolistics: progress will be made in optimizations for mature citrus scion

During the quarter, efforts continued to optimize the amount of DNA per shot, time of bombardment and helium pressure (psi) to coincide with organogenesis. Optimizations are being made using green fluorescent protein reporter and PMI selectable marker. The goal is to complete optimization and minimal cassettes transfer by June 2018.

Obj. 1c - Determine which of the micro-grafting steps can be bypassed altogether by growing explants in bioreactors for elongation of shoots and secondary grafting

During the quarter, efforts continued to shorten the time involved in transgenic plant production. Transgenic explants and shoots with GUS reporter were sub-cultured through the mature citrus tissue culture process, then placed in liquid medium with kanamycin for 2 weeks to elongate shoots for secondary grafting and eliminate escapes. Rootstock shoots have been shown to elongate easily for secondary grafting, but it is unknown whether scion shoots will elongate for grafting. The goal is to achieve this objective by June 2018.

Obj. 1d - Compare genes thought to enhance shoot production/transformation efficiencies and apply pre-treatments to increase organogenesis in mature rootstock

During the quarter, work continued to sub-clone candidates into the same plasmid backbone with a GPF reporter and use in transformation for comparisons. Different genes are being evaluated for different purposes, e.g. use of Poncirus FLT cDNA to both enhance flowering and shoot production; Arabidopsis ELP 3 and 4 genes to increase plant immunity and also as well as shoot production and transgenesis.

Obj. 1e - Determine efficiencies of PMI selection in biolistics and Agrobacterium-mediated transformation compared to nptII.

Phosphomannose isomerase (PMI) selection has been shown to work well after biolistics in immature citrus, increasing the number of transgenics while significantly decreasing the number of non-transformed escapes when compared with nptII selection. During the quarter, MCTF continued its investigation of whether PMI will be useful for mature citrus transformations. Initial observations indicate that mannose is toxic to mature shoot development, but tests continue to determine the effect of mannose after the shoots have formed on sucrose medium.

Obj. 2 - Test a more sensitive, non-destructive screening process to increase throughput

Currently the lab uses a GUS histochemical assay that is labor intensive, tedious and destructive to tissue. It produces a visible blue stain as a marker. A new screen was developed that is more sensitive and less destructive, using MUG as an alternative substrate to GUS for fluorometric detection. During the quarter, this protocol continued to be tested in mature citrus to determine if shoots survive the MUG application and subsequent grafting steps. Also of interest is whether there will be auto-fluorescence in non-transformed shoots, i.e. false positives. Timeframe for completion of this objective is June 2017.

Obj. 3 - Test new breeder lines using standard tissue culture protocols to determine whether optimizations are necessary.

The facility continues the process of introducing new breeder lines in which to produce transgenics. MCTF recently introduced Kuharski and Glen Naval cultivars for Drs. Grosser and Dutt through shoot-tip grafting (STG). Kuharski is a rootstock similar to Carrizo, but it has some nematode tolerance, and Glen Navel sweet orange is pollen sterile, so it will provide a contained system to prevent transgene flow. Some of the budwood from FDACS in Chiefland was contaminated with the yeast endophyte, so it was essential that STGs be conducted on all introduced material prior to tissue culture. Mandarin and pummelo were also introduced for Dr. Wang.

Obj. 4 - Increase throughput of budded plants in the growth room

This remains a major team effort. Measures are being pursued such as increasing planting density using citrus pots where possible, and, after budding, leaving the bud stick attached to scion to accelerate growth.

Significant Meetings/Conferences/Publications

Dr. Hao Wu, MCTF Biologist, made a presentation at the Annual Conference of the American Society for Horticultural Science in Atlanta in August. The presentation discussed MCTF development of the first reported particle bombardment transformation system for citrus.

Obstacles Encountered

Production from the mature transformation pipeline during the last quarter was interrupted due to the move to the packinghouse while the AC in the permanent facility was being repaired. The facility lost time due to unforeseen delays in the physical move and set up in the temporary laboratory. The move back to the permanent facility is anticipated to occur the end of October 2016.

Other Information

In June 2016, CPDC and the CRDF Board approved a two-year project continuation. (Project 15-045C).

MCTF's mission is to develop protocols for mature transformation of citrus that can be used to incorporate genes of interest, when available, into Florida cultivars. Through MCTF, CREC will generate the first mature sweet orange transformants with development protocols adjusted in the lab and in the growth room for Valencia, Hamlin and other commercial cultivars.

MCTF remains an important element of the overall pipeline encompassing both conventional breeding and genetic transformation, from inception, to field testing, to scale-up and delivery to growers. MCTF's role in this overall process is tied to CRDF efforts address the overall process for HLB host resistance and tolerance, including side-by-side field testing of the most promising candidates and delivery to Florida growers.

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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

Post-Bloom Fruit Drop Survey

A CRDF Research Management committee meeting was called on June 29, 2016 to discuss post-bloom fruit drop (PFD) caused by *Colletotrichum spp.* in Florida. In this meeting a suggestion was made 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 the CRDF field crew has since surveyed twenty-one blocks. Data was collected from twenty trees per site; the trees were of similar age, variety and disease severity within the site. Canopy surface areas were measured for standardization purposes. Fruit and residual fruit calyx buttons within a 0.5 square meter frame was counted twice on each side of the tree (4x total).

Grove data is being collected to be used in the analysis is the following:

- The history of PFD in specific groves
- Production history
- Previous PFD treatment programs
- Flowering timing
- Past weather conditions recorded by grower weather stations
- Weather data from the local FAWN weather stations.

Analyzed data will be shared back to the industry by way of a summary that shows the general trends associated with weather, disease pressure and resulting fruit load. All groves have been surveyed at this time; project managers are presently waiting for grove information to be returned from growers and are determining the best method to analyze the data. Results are expected to be available early in quarter three and before spring PFD fungicides are applied.

Obj. 2 - Evaluate PFD management tactics under field conditions

Planning is underway for supplementary research objectives to complement ongoing field work during winter/spring of 2016-17.