CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16
Quarter Ending March 31, 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: 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.
This quarter we began working with a new biopharmaceutical company developing small-molecule therapeutics for agriculture. These novel materials are considered by CRDF to be next-generation solutions because they are still in early testing stages and will require a significant period of time to test, manufacture and register. No barriers to EPA registration are expected as toxicology studies have shown these materials to have little impact environmentally.

No new materials have been identified through suggestions submitted to the "solutions inbox" (solutions@citrusrdf.org).

CRDF-funded research projects by university researchers on bactericides are all on track. The remaining three assay RSA projects are on track.

A white paper was made available in February 2016 describing bactericide use in the past and present with an emphasis on materials used traditionally for citrus greening and huanglongbing. This document addresses questions raised by growers and the public resulting from the recent compiling of a document requesting an emergency exemption from FIFRA for use of oxytetracycline and streptomycin on citrus for HLB and will be available on the CRDF website and as a supplement in the Florida Citrus Mutual Triangle newsletter.

Obj. 2- Move bactericide candidates through assay pipeline to identify promising materials for field trials.
Twenty-two materials were tested *in vitro* this quarter from a biopharmaceutical company. More than two hundred and fifty materials have been tested this fiscal year. The majority of the materials tested are microbial supernatants that may be developed into biopesticide products.

A greenhouse assay is used for testing potential bactericides. Small trees are inoculated with "hot"

psyllids until systemically infected. Bactericides are tested by one of three methods: root infusion, soil drench or foliar spray. Preliminary tests began last quarter to validate the positive controls for each application method, results can be seen below for the foliar (Fig. 1), root -drench (Fig. 2) and root infusion (Fig. 3). Controls for all application methods have now been validated. While several materials have been tested in this assay, results have not yet been available in this quarter. We are working to improve the throughput of this assay to increase the number of materials that can be tested in a shorter period of time.

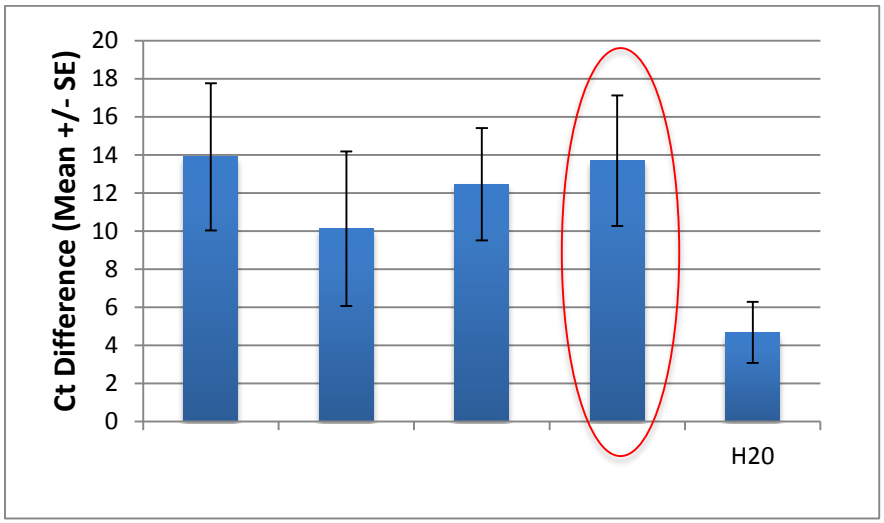

Fig 1. Foliar application. Positive Ct values reflect a decrease in plant CLas titer.

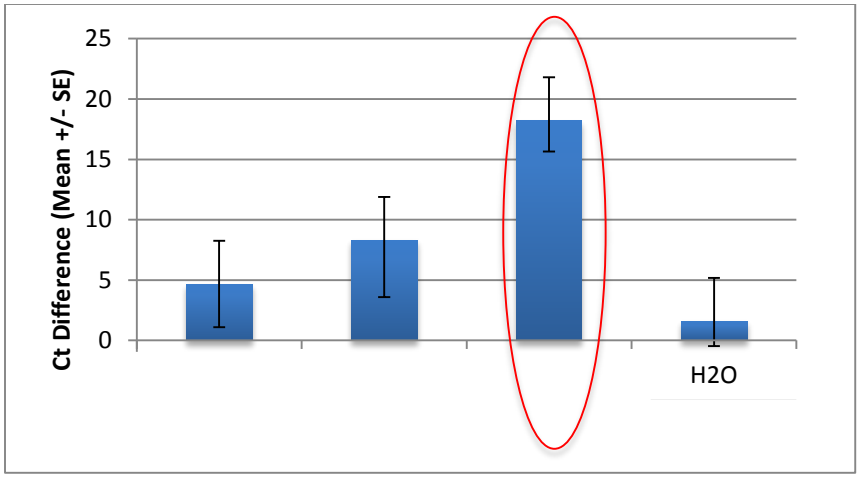

Fig. 2. Soil drench application. Positive Ct values reflect a decrease in plant CLas titer.

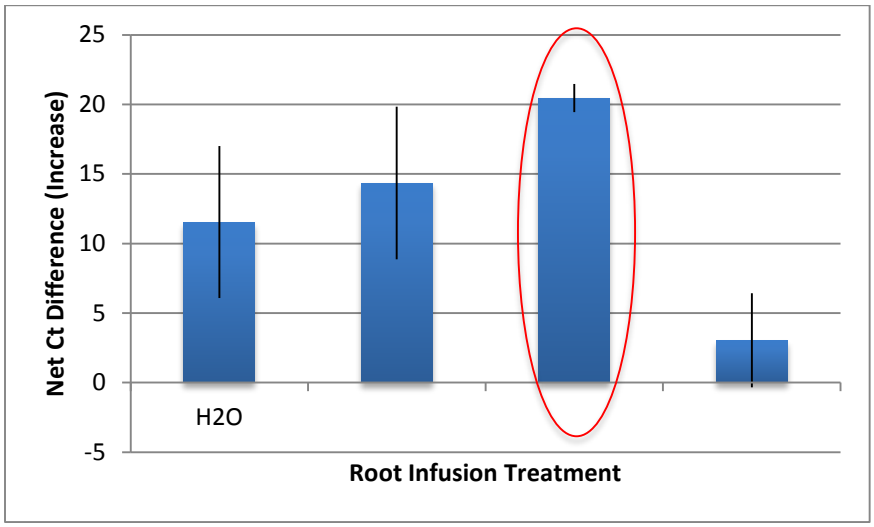

Fig 3. Root infusion application. Values are means =/- SEM. Positive Ct values reflect a decrease in plant CLas titer, negative value represent an increase.

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1.Candidatus Liberibacter asiaticus PATHOGEN INTERVENTION

Project title: 1a. Bactericide Strategies

Subproject Title: 1b. 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.
Adjuvants continue to be evaluated for use in assays. Several proprietary adjuvants are being used in the biopesticide/"minimum-risk" field trial that should increase the uptake of these types of materials.

CRDA new company has come forward with potential new adjuvants. CRDF project managers will work with this company to determine if these materials increase the absorption through the citrus leaf cuticles. It these materials are effective they will need to go through the EPA registration process and would not be immediately available for use, but this company has the resources to complete this process in the shortest time-frame possible.

Obj. 2- Track RMC and CPDC research projects relevant to the formulation and delivery of bactericides against HLB; integrate findings into project planning.
Towards the goal of developing novel delivery methods, the CPDC project (15-031C), examining the ability of a laser treatment to facilitate bactericide movement into the citrus phloem was initiated in July 2015. This one-year project has been progressing on schedule. In the next quarter project managers will evaluate the data and determine what next steps should be taken.

Project 15-048C evaluating efficacy of trunk injection as a treatment method was approved in October 2015 and was initiated this quarter. The field site of young (4-5 yo) Hamlins was treated in early March with both a trunk injection and foliar application. The first treatment applications were timed with the root and vegetative flushing period. This timing is based on new data on root flush timing of HLB infected trees as well as the timing of full bloom. A second foliar application will take place during the summer flush period.

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1. *Candidatus Liberibacter asiaticus* PATHOGEN INTERVENTION

Project title: 1a. Bactericide Strategies

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.

The first application of materials was applied in the minimum-risk/biopesticide field trial on February 29. Application frequency is every sixty days. Several

The three AgroSource projects are on track, a quarterly report was received and the quarterly meeting was held. Year two of the projects runs through June 2016.

The Nufarm residue study is making progress; a report was submitted for CRDF project manager review.

Both of these company's products are part of a Section 18 (FIFRA) exemption application that was submitted to EPA in early December. This petition is for the use of oxytetracycline hydrochloride, oxytetracycline calcium and streptomycin sulfate on citrus in Florida. EPA is in the process of reviewing the application. On March 4th, 2016 Commissioner Putnam issued a crisis declaration for use of the three bactericides. This allowed immediate use of the bactericides in Florida. Because of the lack of guidance for the use of these materials CRDF project managers developed a document outlining field trial methods for growers. This document can be found at http://bit.ly/1PQEPGT. Project managers and the CRDF field staff are also in the process of setting up small-scale trials in grower's block testing the grower's chosen application program. These trials will be set-up around the state through the next quarter and will be evaluated for as long as possible to collect data on bactericide efficacy. This data may be used to support a renewal of the Section 18 and help develop grower recommendations.

Data from the Company C field trial continues to be collected. This trial was initiated in August 2014. Treatment one and two cause severe phytotoxicity resulting in bleaching and defoliation. This resulted in significantly reduced growth as shown in the trunk cross-sectional area and canopy volume. Treatment four was not phytotoxic and was significantly improved in health compared with the control (Fig. 1 & 2). The trees used in this experiment were uninfected at the onset of this study; all of the evaluation trees were infected at a similar rate when sampled in January 2016 (Fig. 3). The materials in this study do not appear to prevent infection with the HLB bacterium, but material four does sustain or improve the health of trees with HLB.

This study will continue until August 2016 in order to collect two full years of data, at this time a full analysis of the data will be presented.

Fig. 1

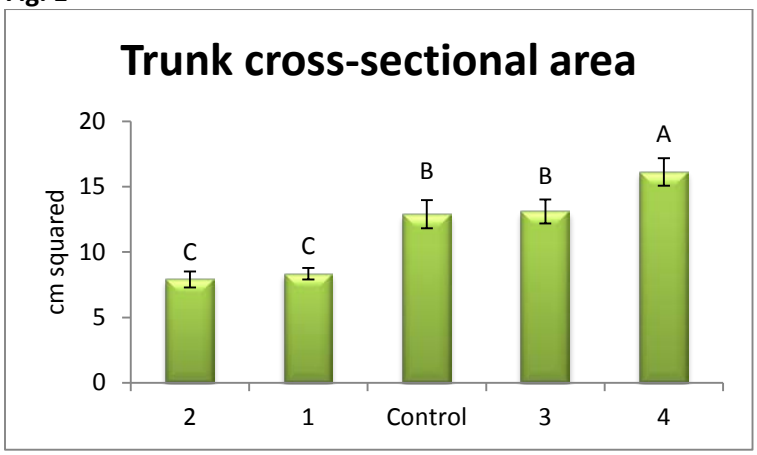


Fig. 2

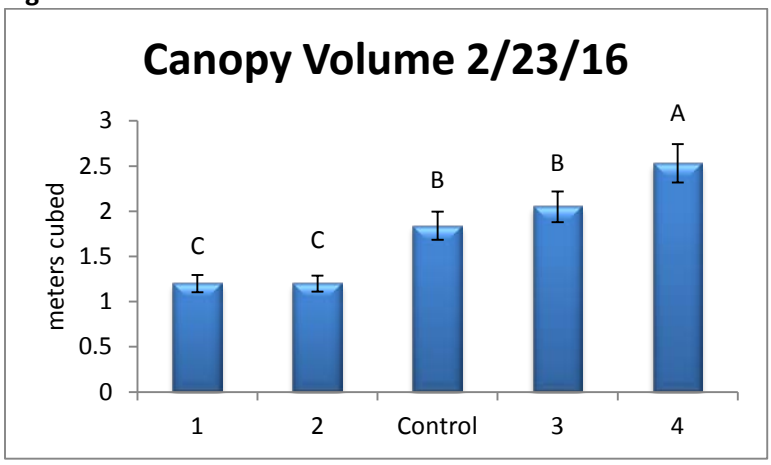
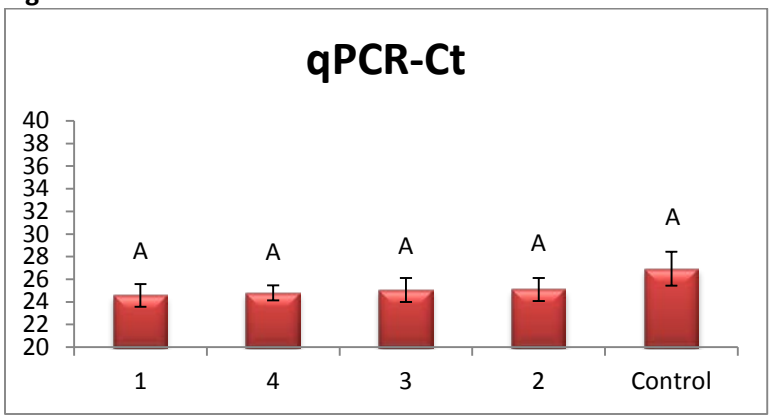


Fig. 2



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

New field trials will be developed as new bactericides are identified. EPA may require studies upon approval of the Section 18, these will be developed as necessary.

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

This report describes the progress towards the goals laid out in the bactericide project work plans. No other document has been provided this quarter, describing progress towards project goals.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16
Quarter Ending Mar 31, 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:

1. Track ongoing research on thermal therapy and its role in HLB and tree health

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 component 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.

In May 2015, bioassays were completed to quantify the rate of *Candidatus Liberibacter asiaticus (Las)* acquisition from infected citrus trees by Asian psyllids (ACP). These acquisition rates will serve 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 June because equipment was under repair, and therefore unavailable. Treatment was postponed an additional week, because trees in the experimental plot were mistakenly treated with imidacloprid. Trees were immediately irrigated for a 24 h period to reduce uptake of imidacloprid. It is critical to this study that imidacloprid is not present in trees one month (4 weeks) after thermal treatments because this is the initial time point for determining the effect of thermal therapy on Las acquisition by ACP. One week after the imidacloprid treatment, data from tap sampling indicated that the ACP population in the experimental plot was not reduced. This suggests that irrigation prevented the uptake of imidacloprid into trees. It also indicates that there will not be a negative impact of imidacloprid on ACP survival

during acquisition assays one month after thermal treatments are applied.

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 four weeks, the rate of Las acquisition by immature and adult ACP will be 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 *CLas* 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. Once qPCR assays are complete, data analysis will be conducted to determine changes in plant *CLas* titer pre- and post-treatment, and to evaluate acquisition efficiency following steam applications. Analysis should be completed in November. The next acquisition assay is also scheduled for November.

As previously reported, trees were steam-treated as described in our research proposal during July 2015. Adults and nymphs were enclosed in mesh sleeves on trees for acquisition feeding approximately 5 weeks following 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 *Candidatus Liberibacter asiaticus (CLas)* 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 *CLas* 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 *CLas* titers. *CLas* titers were significantly higher in steam-treated trees than untreated trees on day 0 as compared to day 45. *CLas* acquisition by adult psyllids enclosed on trees receiving thermal treatments did not differ significantly from acquisition by adult psyllids on untreated trees. Samples from *CLas* acquisition feeding assays with psyllid nymphs are still being processed.

Based on these results, which indicated the thermal treatments applied during July 2015 did not reduce plant *CLas* titer or psyllid acquisition, a second thermal treatment was applied during late November 2015. In early January 2016, adults and nymphs were enclosed in mesh sleeves on trees for acquisition feeding approximately 5 weeks following treatments. Insect and leaf samples were collected after 10d of acquisition feeding or upon adult emergence to assess adult and nymph acquisition, respectively. In addition, we have initiated a complementary laboratory study to evaluate the effect of thermal therapy on acquisition of *CLas* under controlled conditions. Two year old Valencia trees were inoculated with *CLas* by enclosing plants with *CLas*-infected psyllids for two weeks. Currently, plants are being held in a secure, insect-free greenhouse until they are determined to be positive for *CLas*. At that time, a controlled environmental chamber will be used to apply heat treatments to trees for use in subsequent acquisition experiments.

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

<u>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</u>

reducing disease and improving health of treated trees.
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. One grove in Lake county, however, is under organic production practices and there is one grove using conventional production practices but is under managed for comparison of TT results.

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, 11 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 will be 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 11 locations under evaluation is ahead of the plans, and we anticipate being able to conduct many additional evaluations beyond those estimated in 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 year one 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 11 sites reported on in this period are all ongoing thermotherapy projects where tree responses to different thermotherapy conditions are being monitored. 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.

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. There were no treatment

(Trtment) effects on Canopy volume (CV, m3) or Disease Index (DI) by 2/4/16. Likewise, there were no treatment differences in Cycle threshold (CT) and Copy number per DNA (CN/DNA).

Canopy volume (CV, m^3) 11_16_15

DMRT	CV	N	Trtment
A	11.2	12	130F30s
A	11.1	15	Untreated
A	10.2	8	130F15s

CV (m^3) 2_4_16

DMRT	CV	N	Trtment
A	11.6	12	130F30s
A	10.7	8	130F15s
A	10.0	15	Untreated

Disease Index (DI) 2_4_16

DMRT	DI	N	Trtment
A	26.3	15	untreated
A	25.5	8	130F15s
A	24.9	12	130F30s

Cycle threshold (CT) 2_4_16

DMRT	CT	N	Trtment
A	26.3	12	130F30s
A	25.1	15	Untreated
A	24.1	8	130F15s

Copy Number / DNA 2_4_16

DMRT	CN/DNA	N	Trtment
A	24950	8	130F15s
A	19461	15	Untreated
A	16606	11	130F30s

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. There were no treatment (Trtment) effects on Canopy volume (CV, m3) or Tree Height (TH, cm) by 2/18/16. Disease Index (DI) was significantly higher, however, in control trees (control trees looked worse, average DI = 23.8) than treated trees and the 132F for 1 sec treated trees looked the best (DI = 21.3)

Canopy volume (CV, m3)_2_15_16

DMRT	CV	N	Trtment
A	20.5	12	Untreated
A	19.8	12	128F_20s
A	18.7	12	132F_10s
A	17.9	12	132F_1s

Tree Height (TH, cm)_2_18_16

DMRT	TH	N	Trtment
A	262.2	12	Untreated
A	256.8	12	128F_20s
A	253.0	12	132F_1s
A	245.5	12	132F_10s

Disease index (DI)_2_18_16

DMRT		DI	N	Trtment
	A	23.8	12	Untreated
B	A	22.5	12	128F_20s
B	A	22.2	12	132F_10s
B		21.3	12	132F_1s

The Cycle threshold (CT) of trees treated with 132 F for 10 sec was highest (least HLB; 28.7) and the CT of the 132F for 1 sec treated was lowest (most HLB; 25.2). Although there were no significant treatment differences on Copy number per DNA (CN/DNA), the 132 F_10 sec trees had numerically the lowest values.

Cycle threshold (CT)_2_4_16						Copy No./ DNA (CN/DNA)_2_4_16			
DMRT		**CT**	**N**	**Trtment**		**DMRT**	**CN/DNA**	**N**	**Trtment**
	A	28.7	12	132F_10s		A	11997	12	132F_1s
B	A	27.5	12	128F_20s		A	11139	12	Untreated
B	A	25.9	12	Untreated		A	11107	11	128F_20s
B		25.2	12	132F_1s		A	8272	12	132F_10s

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.

Dunson

Hamlin/Swingle trees, 5 years old. Leaves were sampled on 30 trees for PCR analysis on 6/8/2015. 15 trees were steam treated 125 F for 30 seconds on 8/4/2015 and 15 remained as untreated controls. By 3/30/16, Canopy volume (CV, m^3), tree height (TH) and Disease Index (DI) were not affected by the 128 F for 30 sec treatment. On 6/18/15, the Cycle threshold (CT) of the treated trees was higher (less HLB) but by 1/25/16, this difference had disappeared as CT of treated trees declined. Copy number per DNA (CN/DNA) was not affected by treatment.

Treatment	N	CV_3_30_16	TH_3_30_16	DI_3_30_16	CT_6_18_15	CT_1_25_16	CN/DNA_1_25_16
Control	15	5.4	206.8	20.9	23.6	23.8	5477.9
128F_30sec	15	5.4	202.7	20.9	34.2	25.0	3928.8
P of t-test		NS	NS	NS	0.0001	NS	NS

The probability (P) of significant differences as tested by t-tests.

Gapway 9

Treatment	N	CV_3_24_16	TH_3_24_16	DI_3_24_16	CT_1_25_16	CN/DNA_1_25_16
Control	6	8.8	241.2	23.2	27.0	4521.2
140F 20sec	6	6.7	219.2	23.3	27.0	2085.7
P of t-test		NS	NS	NS	NS	NS

Raley

Valencia / Carrizo, 5 years old. Pretreatment leaves sampled from 40 trees for PCR on 5/22/15. 30 trees were treated on 5/28/15 at 120 F for 20 sec. while 10 trees were untreated as controls. By 3/18/16, average canopy volume (CV, m^3) tree heights (TH) were smaller in the 120F_30 sec treated trees than in the control trees but and disease index (DI) did not differ. Cycle threshold (CT) was lower in 122F_20sec treatment trees (more HLB) on 5/22/15 but by 1/25/16, CT did not differ. Copy number per DNA (DNA) was also not affected by treatment on 1/25/16 even though treated trees had numerically larger values.

Treatment	N	CV_3_18_16	TH_3_18_16	DI_3_18_16	CT_5_22_15	CT_1_25_16	DNA_1_25_16
Control	10	15.1	291.8	23.5	32.8	28.2	1706.6
120F_20sec	30	10.7	250.4	24.1	25.1	26.6	3381.8
P of t-test		0.001	0.001	NS	0.001	NS	NS

Shinn

Valencia /Swingle, 3 years old, double set. Pretreatment leaves sampled for PCR 8/7/2015 18 trees treated 8/7/2015 at 122-127 (avg 125) for 30 sec. and 18 trees were untreated as controls. By 3/8/16, there were no treatment effects on average Canopy volume (CV, m^3), tree height (TH) or Disease Index (DI). Cycle threshold (CT) was lower in 125F_30sec treatment trees (more HLB) on 8/7/2015 and 1/25/16 but Copy number per DNA (DNA) of 125F_30sec treatment trees was lower than in control trees on 1/25/16.

Treatment	N	CV_3_8_16	TH_3_8_16	DI_3_8_16	CT_8_7_15	CT_1_25_16	DNA_1_25_16
Control	18	2.5	147.8	20.3	31.2	26.2	3725.6
125F30sec	18	2.3	145.5	21.2	24.6	25.0	3136.5
P of t-test		NS	NS	NS	0.001	0.003	0.010

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 initial CT from PCR on 10/5/15. By 3/26/16, average canopy volume (CV, m^3) was smaller in the 131F_30 sec treated trees than in the control trees but tree heights (TH) and disease index (DI) did not differ. Trees in this trial uniformly had HLB disease but the treatment did not affect Cycle threshold (CT) on 10/5/15 or on 1/26/16. Copy number per DNA (DNA) was not affected by treatment on 10/5/15 but by 1/26/16, DNA was significantly higher in treated trees.

Treatment	N	CV_3_28_16	TH_3_28_16	DI_3_28_16	CT_10_5_15	DNA_10_5_15	CT_1_26_16	DNA_1_26_16
Control	24	10.8	224.3	16.5	24.8	54979.7	25.1	1610.0
131_30sec	24	10.0	222.8	16.7	24.1	72327.6	24.5	3662.4
P of t-test		0.036	NS	NS	NS	NS	NS	0.025

Wheeler

Valencia/Swingle, 4 years old. Wheeler's Lake Wales block was the site of the December 3, 2015 field day for growers. Leaves were collected for pretreatment PCR on 6/9/2015. 15 trees were steam-treated on 6/12/15 at 122-128 F (avg 125) for 20 seconds, with 15 trees serving as untreated as controls. By 3/14/16, average canopy volume (CV, m^3) and tree heights (TH) were still smaller in the 125F_20sec treated trees than in the control trees but disease index (DI) did not differ. Cycle threshold (CT) on 6/9/15 lower (more HLB) in 125F_20sec treated trees but CT and Copy number per DNA (DNA) did not differ by 1/26/16.

Treatment	N	CV_3_14_16	TH_3_14_16	DI_3_14_16	CT_6_9_15	CT_1_26_16	DNA_1_26_16
Control	15	6.8	201.3	17.7	26.3	25.3	2778.2
125F_20sec	15	5.2	181.1	18.5	20.3	24.5	6734.2
P of t-test		0.008	0.009	NS	0.009	NS	NS

BHG

Murcott /Cleo trees, planted 2007-2008, 7 to 8 years old. The 25 trees to be steam treated were hedged and topped to a height of 6 ft. (182 cm) prior to treatment, so they would fit inside the tent, while 5 control trees were left at heights of about 10-12 ft (305-365 cm). Canopy volumes (CV) and tree heights (TH) of treated trees are still much smaller than control trees. Disease index (DI) on 4/1/16 were not different. The 25 treated trees were treated at 126 F (52 C) for 30 seconds (sec). The 5 untreated control trees were not steamed. Leaves for post-treatment PCR were sampled from all 30 trees on 3/13/15 and 1/25/16. The average cycle threshold (CT) values from PCR were not different on 3/13/15 but by 1/25/16, CT values of treated had decreased to levels below the control trees as HLB infection progressed. Copy number per DNA (CN/DNA) was higher in previously treated trees than in control trees.

Treatment	N	CV_4_1_16 *	TH_4_1_16*	DI_4_1_16	CT_3_13_15	CT 1/25/16	CN/DNA1_25_16
Control	5	25.1	317.6	24.4	36.1	30.0	1050.5
126F30s	25	9.6	246.5	26.2	30.2	23.6	7238.0
P of t-test		0.002	0.00001	NS	NS	0.060	0.001
		*Treated trees were hedged and topped					

LeeJones

These tree were selected for uniformity and CV, TH, DI and HLB status will be followed through time.

Treatment	N	CV_3_9_16	TH_3_9_16	DI_3_9_16	CT_1_5_16
Control	22	4.5	230.5	21.9	31.5
128F_30s	22	4.2	233.3	21.5	31.6
P of t-test		NS	NS	NS	NS

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.

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2. Asian Citrus Psyllid VECTOR INTERVENTION

Project Title: 2b. RNAi Molecules/Psyllid Shield

Narrative of Progress against Goals:

Obj. 1- Continue to refine the mathematical model with vector entomologists and epidemiologists.
During the quarter, modelling refinements focused on two areas:

a. Understanding the relationship between transmission of CLas and eventual development of HLB symptoms.

A team led by UF mathematics professor Dr. Jed Keesling (Project 932.1C) continues efforts to evaluate the Psyllid Shield concept by accurately modeling its performance over different spatial dimensions, neighboring psyllid and disease pressure, and RNAi performance. This model is being refined to bring the necessary precision to a field trial design. This two-year project ends 5/31/2016.

Previously the model for the appearance of symptoms was based on the assumption that inoculum accumulates at a rate proportional to the number of infected of infected nymphs present in the citrus trees. Comparisons of these simulations with data from Southern Gardens showed that this model was not accurate. The team has now implemented a model where the rate at which symptoms develop is proportional the amount of inoculum in the tree, and that inoculum decays over time at some rate.

During the quarter, efforts continued to refine this revised model for the appearance of symptoms to further understand the carrying capacity of the inoculum. This includes investigating the impact of a local carrying capacity at the flush level as well as the whole tree. This model will continue to be refined using data from Southern Gardens and through discussions with plant pathologists to understand the phloem system and its function in symptom development.

b. Refining the psyllid movement and migration portion of the model

Dr. Keesling continued to work with scientists at the USDA in Gainesville to understand how psyllid population density and citrus tree flush quality impact psyllid dispersal. These results continue to be incorporated into the model as refinements.

Obj. 2- Continue to experimentally evaluate candidate protective effects of selected RNAi in CTV inoculated plants.

Dr. Dawson's CRDF-funded project (618C), that ran from 4/2013 to 9/2015, tested 14 selected dsRNAs separately for activity against psyllids when expressed in plants using the CTV vector. The evaluation was conducted against adults and nymphs feeding on citrus in caged greenhouse experiments. The study found dsRNAs of specific psyllid genes that reduce the survival of these psyllids. In an effort to find an effective and economical method to deploy this strategy, these sequences were cloned into the Citrus

tristeza virus (CTV) vector, to test whether production of RNAi molecules in citrus will prevent the survival and reproduction of psyllids. This has led to identification of sequences of certain psyllid genes when expressed using the CTV vector, greatly reduce the production of psyllid progeny in citrus seedlings.

Going forward, this research is being funded by a USDA Specialty Crop Grant, which will continue to identify the most effective target sequences, test multiple sequences, and optimize delivery methods.

Obj. 3- Continue to evaluate new RNAi for improved activity with CTV vectors

During the quarter, the search for new RNAi candidates continued through nuPsyllid and related research. The challenge is to advance what might be "good enough" into the regulatory pathway while continuing to understand what might also be worthwhile further improvements. It is therefore a priority to advance the most promising current candidates into field trials.

Obj. 4- Continue to model performance of best RNAi for field trials and complete scale-up feasibility analysis.

During the quarter, Dr. Keesling continued discussions with statisticians on the design of field trials to answer questions regarding how many constructs can be used in the field trial while still differentiating between constructs with high confidence. Based on initial simulations from the previous model, it appears that at least four constructs can be used while still differentiating among constructs. As changes are made to the model for symptom appearance, Dr. Keesling will need to run more simulations to determine how many constructs can be used.

Obj. 5- Pursue a corporate partnership to carry this project forward toward field trials and commercialization

The Psyllid Shield idea is a spin-off of nuPsyllid and RNAi research in combination with CTV vector development, and therefore cuts across several areas of Intellectual Property (IP). This is both a challenge and opportunity because it involves multiple stakeholders, IP owners and licensees.

Prior discussions in 2015 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.

Southern Gardens (SGC) has agreed to partner with CRDF to develop plans for and implement a Phase 1 field trial, subject to CPDC and CRDF Board approval. (See Obj. 6)

Obj. 6- Make key decisions regarding initiation of field trials and regulatory approval process.

The science and modeling efforts have progressed to the point where it is feasible to proceed with field trials on RNAi for psyllid control in 2016. CTV is the most appropriate delivery "niche" for CRDF to pursue in terms of 2016 field trials. Five RNAi constructs were identified in Dawson, et.al research as particularly effective in killing psyllids in greenhouse trials, and are therefore recommended for further evaluation in

the field trials.

At the December 2015 CPDC meeting, a proposal was presented by CPDC staff recommending a two phased approach to field trials. Phase 1 would be a small-scale trial, followed by a Phase 2 area-wide trial that captures the Phase 1 learning and experience from both a scientific and regulatory point of view and establishes relationships with regulatory agencies. CPDC authorized SGC and CPDC staff to proceed to the next step, a formal proposal with detailed plan and budget, as well as a recommendation for a proposed CRDF-SGC relationship to implement the project.

At the February 2016 CPDC Meeting, there was continued discussion about the scope and nature of the trial, and the roles and responsibilities of CRDF and Southern Gardens in this effort.

Based on that discussion, Southern Gardens is putting together a full proposal and budget for the Phase I trial, which will be presented at an upcoming CPDC meeting.

Significant Meetings or Conferences:

None.

Obstacles Encountered and 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. Based on the results of the February CPDC review, appropriate decisions will be made as to the next steps to advance the Psyllid Shield concept to commercial use.

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3. Citrus Host Intervention

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

Narrative of Progress by Project Goals:

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.
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**. The project's 2nd year anniversary will come up 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 are sampled annually to determine root density.

Site Results to Date:

Ridge Site: Balm, FL. Valencia/Swingle trees are 17 years old. There were 4 replicates (blocks) of 20 trees in each of the 6 treatments. There were 6 measurement (sentinel) trees with no mulch in each of the 4 blocks in each treatment (n=24). There was 1 measurement sentinel tree, of three mulched trees with supplemental mulch, in each of the 4 blocks in each treatment (n=4). In 2016, mulch (n=24) vs no mulch (n=144) comparisons revealed that overall, there were no mulch effects on Disease index (DI)

rating (14.5-15.8) or on Canopy volume (34-35 m^3). Treatment comparisons can, therefore, pool results from mulch + no mulch (n=28).

Disease index (DI) 2-22-16

DMRT	DI	N	Mulch
A	15.8	24	yes
A	14.5	144	no

Canopy volume CV (m^3) 2-16-16

DMRT	CV (m^3)	N	Mulch
A	35.7	24	yes
A	34.1	144	no

DMRT (Duncan's multiple range test) is a statistical test to determine if treatment means are different. Average values adjacent to the same letters from a DMRT are not statistically different; values adjacent to different letters are statistically different. There were no significant treatment effects on average DI (13.4-15.6) but the Untreated control trees (UTControl) trees had numerically the lowest DI (had fewer visible symptoms). The Biofluorish trees had the largest average canopy volume (41.4 m^3) and the UTControl trees had the smallest canopies (29.8 m^3), Biofluorish and Serenade treated trees were larger than the UTControl trees.

DMRT for Disease index (DI)

DMRT	DI	N	Treatment
A	15.6	28	Serenade
A	15.5	28	Quantum
A	15.4	28	EcoFriend
A	14	28	BioFlourish
A	14	28	Aliette
A	13.4	28	UTControl

DMRT for Canopy volume CV (m^3)

DMRT		CV (m^3)	N	Treatment
	A	41.4	28	BioFlourish
B	A	38.3	28	Serenade
B	C	32.9	28	Aliette
B	C	32.6	28	EcoFrnd
	C	30.7	28	Quantum
	C	29.8	28	UTControl

Leaves were sampled for PCR January 2016. There were no mulch effects on CT values (25-26) so data from all trees were pooled to test for treatment effects. Regardless of treatment, CT values were similar as all trees were infected at about the same level.

Cal_CT + vs. – Mulch 11-13-15 NS

DMRT	**CT**	**N**	**Mulch**
A	26.4	24	yes
A	25.2	144	no

Cal_CT + and – Mulch. No treatment effects.

DMRT	**CT**	**N**	**Treatment**
A	26.8	28	Aliette
A	26	28	Ecofriend
A	25.9	28	Serenade
A	25.4	28	Quantum
A	24.2	28	Biofluorish
A	24	28	UT Control

Trees have not yet been harvested (as of 4/22/16) so there are no yield or juice quality data.

East Coast, Indian River, Premier: Valencia/Swingle trees are 6 years old.
There were 4 replicates (blocks) of 20 trees in each of the 6 treatments. There were 6 measurement (sentinel) trees with no mulch in each of the 4 blocks in each treatment (n=24). There was 1 measurement sentinel tree, of three mulched trees with supplemental mulch, in each of the 4 blocks in each treatment (n=4). In 2016, mulch (n=24) vs no mulch (n=144) comparisons revealed that overall, there were no mulch effects on Disease index (DI) rating (11.5-11.7), Canopy volume (11 m^3), CT from PCR (25) or fruit yield (1.2-1.3 boxes). Treatment comparisons can, therefore, pool results from mulch + no mulch (n=28).

Disease Index (DI) 3_16_16			
DMRT	**DI**	**N**	**Mulch**
A	11.7	24	Yes
A	11.5	144	No

Canopy Volume (m^3) 3_16_16			
DMRT	**CV**	**N**	**Mulch**
A	11.2	24	Yes
A	11.0	144	No

Cycle threshold (CT) 1_7_16			
DMRT	**CT**	**N**	**Mulch**
A	25.5	24	Yes
A	25.1	144	No

Fruit Yield (boxes) 3_9_16			
DMRT	**Yield**	**N**	**Mulch**
A	1.31	144	No
A	1.26	24	Yes

DMRT (Duncan's multiple range test) is a statistical test to determine if treatment means are different. Average values adjacent to the same letters from a DMRT are not statistically different; values adjacent to different letters are statistically different. There were no significant treatment effects on average DI (8.6 -10.5) on 12-11-15 but by 3-16-16 all DIs had increased and Serenade treated trees looked the worst (DI= 13.5) and Ecofriendly and Aliette looked the best (DI= 10.1). There was no effect of treatment on average canopy volume (10-11 m^3) and the UTControl trees were intermediate in size.

Disease Index (DI) 12_11_15 NS			
DMRT	**DI**	**N**	**Trtment**
A	10.5	28	Serenade
A	10.3	28	UTCont
A	10.3	28	Quantum
A	9.9	28	EcoFrien
A	8.8	28	BioFluor
A	8.6	28	Aliette

Disease Index (DI) 3_16_16				
DMRT		**DI**	**N**	**Trtment**
	A	13.5	28	Serenade
B	A	11.8	28	BioFluor
B	A	11.8	28	UTCont
B	A	11.7	28	Quantum
B		10.1	28	EcoFrien
B		10.1	28	Aliette

Canopy Volume (m^3) 3_16_16			
DMRT	**C V**	**N**	**Trtment**
A	11.7	28	EcoFrien
A	11.5	28	Serenade
A	11.2	28	BioFluor
A	11.0	28	UTCont
A	10.6	28	Aliette
A	10.3	28	Quantum

Leaves were sampled for PCR on 1-7-16. Regardless of treatment, CT values were similar as all trees were infected with HLB at about the same level (24-26). Fruit yield also was not affected by treatments as yields averaged 1.2-1.4 boxes per tree.

Cycle Threshold (CT) 1_7_16 NS					Fruit Yield (boxes) 3_9_16 NS			
DMRT	**CT**	**N**	**Trtment**		**DMRT**	**Yield**	**N**	**Trtment**
A	26.3	28	BioFluor		A	1.4	28	BioFluor
A	25.5	28	EcoFrien		A	1.4	28	Quantum
A	25.4	28	Aliette		A	1.3	28	Serenade
A	24.9	28	Serenade		A	1.3	28	UTCont
A	24.5	28	UTCont		A	1.2	28	Aliette
A	24.1	28	Quantum		A	1.2	28	EcoFrien

Indian River Juice Quality

There were 4 replicates (blocks) of 20 trees in each of the 6 treatments. There were 6 measurement (sentinel) trees with no mulch and 1 measurement tree, of three mulched trees with supplemental mulch. Fruit samples were pooled from each of the 6 measurement trees and tested separately from the single mulched tree in each of the 4 blocks in each treatment. Thus, there were 2 fruit samples from 4 blocks = 8 from each treatment. In 2016, there were no mulch vs no mulch effects on any juice quality parameters so ± mulch were pooled (n=8).

Serenade had the largest average fruit size (.51 lbs) and Aliette had the smallest fruit (.45 lbs). Fruit size from the Untreated Control (UTC) trees was intermediate. Percent juice in the fruit from the UTC, Aliette and Ecofriendly treatments (.63-.64 lbs) was higher than % juice in the Quantum, BioFluorish and Serenade Treatments (.6-.61 lbs).

East Coast, Ft Pierce. Average Fruit Weight					Percentage juice in Fruit			
DMRT		**Avg Fr Wt**	**N**	**Treatment**	**DMRT**	**% juice**	**N**	**Treatment**
	A	0.51	8	Serenade	A	0.64	8	UTControl
B	A	0.49	8	BioFluorish	A	0.63	8	Aliette
B	A	0.47	8	Quantum	A	0.63	8	EcoFriend
B	A	0.46	8	UT Control	B	0.61	8	Quantum
B	A	0.46	8	EcoFriend	B	0.6	8	BioFluorish
B		0.45	8	Aliette	B	0.6	8	Serenade

The same trend occurred for Lbs juice per box where fruit from the UTC, Aliette and Ecofriendly treatments had the highest average Lbs of Juice per Box (56.8-57.3 lbs) while the Quantum, BioFluorish and Serenade treatments had lower Lbs of Juice per Box (54.1-54.9 lbs).

There were no treatment effects on % Acid (.6-.66) or juice color (38.2 -38.; data not shown). Total brix was higher in fruit from the BioFluorish and UTControl treatments (9.4) and lowest in the Serenade treatment (8.9).

Lbs of juice per box

DMRT	LbJuice Box	N	Treatment
A	57.3	8	UT Control
A	56.8	8	Aliette
A	56.8	8	EcoFriend
B	54.9	8	Quantum
B	54.2	8	BioFluorish
B	54.1	8	Serenade

Total brix

DMRT		Brix	N	Treatment
	A	9.4	8	BioFluorish
	A	9.4	8	UT Control
B	A	9.2	8	EcoFriend
B	A	9.2	8	Quantum
B	A	9	8	Aliette
B		8.9	8	Serenade

Thus, there were no treatment effects on the Brix/acid ratio (13.9-15) and the ratio of the UTC fruit were in the middle (14. 8). The UTControl, EcoFriendly, Aliette, BioFluorish and Quantum all had higher Lb Solids per Box (5.1-5.4) than Serenade (4.8).

Brix / acid ratio

DMRT	Ratio	N	Treatment
A	15	8	BioFluor
A	14.9	8	Serenade
A	14.8	8	UT Control
A	14.2	8	Quantum
A	14	8	EcoFrien
A	13.9	8	Aliette

Lbs solids per box

DMRT		LbSolid/ Box	N	Treatment
	A	5.4	8	UT Control
	A	5.2	8	EcoFriend
	A	5.1	8	Aliette
B	A	5.1	8	BioFluorish
B	A	5.1	8	Quantum
B		4.8	8	Serenade

SW FL site: Duda, Valencia/Swingle trees are 11 years old.

There were 4 replicates (blocks) of 20 trees in each of the 6 treatments. There were 6 measurement (sentinel) trees with no mulch in each of the 4 blocks in each treatment (n=24). There was 1 measurement sentinel tree, of three mulched trees with supplemental mulch, in each of the 4 blocks in each treatment (n=4). Canopy volume and DI have not been evaluated yet in 2016. On 3-15-16, mulch (n=24) vs no mulch (n=144) comparisons revealed that fruit yield was increased by the mulch treatment (3.5 boxes) compared to the no mulch treatment (3.1 boxes). There were no mulch effects CT from PCR (33-35) sampled on 1-12-15 or on CT about 12 months later on 12-2-15. Likewise, there were no mulch effects on Disease Index (DI) or Canopy volume (CV)measured on 3-30-16. Thus, treatment effects on CT, DI and CV can be pooled across $\pm$ mulch treatments (n = 28 trees).

+ Mulch vs. No mulch

Frt Yield (boxes) 3-15-16

DMRT	Boxes	N	Mulch
A	3.5	24	Yes
B	3.1	144	No

Cycle theshold (CT) 1_21_15

DMRT	CT	N	Mulch
A	35.4	24	Yes
A	33.1	144	No

Cycle theshold (CT) 12_2_15

DMRT	CT	N	Mulch
A	35.9	24	Yes
A	33.2	144	No

Disease index (DI)_3_30_16

DMRT	DI	N	Mulch
A	16.2	144	No
A	14.8	24	Yes

Canopy volume (CV, m^3) _3_30_16

DMRT	CV	N	Mulch
A	32.8	24	Yes
A	29.8	144	No

Within the no mulch treatment (n=24 trees per treatment), the Biofluorish and Aliette treatments had the greatest average fruit yield (3.4 boxes per tree) while the Water control and the Serenade treated trees yielded the least (2.7 boxes per tree). Within the mulch treatment (n=4 trees), the Serenade and the Water control treated trees had lower yields (2.7-2.8 boxes per tree) than the other treatments (3.7-4.1 boxes) that did not differ from each other. Combining the yields from the ± mulch treatments (n=28), the Water control and the Serenade treated trees yielded less (2.7 boxes per tree) the BioFluorish, Aliette, Quantum and EcoFriendly treatments which did not differ from each other and averaged 3.2-3.5 boxes per tree.

Fruit Yield (boxes) 3_15_16 — No mulch

DMRT		Boxes	N	Trtment
	A	3.4	24	Biofluor
	A	3.4	24	Aliette
B	A	3.2	24	Quantum
B	A	3.0	24	Ecofrien
B		2.7	24	WatCont
B		2.7	24	Serenade

Fruit Yield (boxes) 3_15_16 — + mulch

DMRT	Boxes	N	Trtment
A	4.1	4	Ecofrien
A	4.0	4	Aliette
A	3.9	4	Biofluor
A	3.7	4	Quantum
B	2.8	4	Serenade
B	2.7	4	WatCont

Fruit Yield (boxes) 3_15_16 — + & - mulch combined

DMRT	Boxes	N	Trtment
A	3.5	28	Biofluor
A	3.5	28	Aliette
A	3.2	28	Quantum
A	3.2	28	Ecofrien
B	2.7	28	WatCont
B	2.7	28	Serenade

When + and – mulch treatments were combined (n=28), leaves sampled for PCR on 1-21-15 had no effect of treatment (CT = 31-34) but the average CT of Serenade treated trees would be considered HLB positive. By 12-2-15, Alliette treated trees had the highest average CT values (35.5, lowest HLB) while Serenade treated trees had the lowest CT (30.4) and would be considered HLB positive. None of the other average treatment CTs differed significantly from the water control treatment.

Cycle theshold (CT) 1_21_15			
+ & - mulch combined			
DMRT	**CT**	**N**	**Trtment**
A	34.2	28	Aliette
A	34.1	28	WatCont
A	33.6	28	Biofluor
A	33.5	28	Quantum
A	33.4	28	Ecofrien
A	31.7	28	Serenade

Cycle theshold (CT) 12_2_15				
+ & - mulch combined				
DMRT		**CT**	**N**	**Trtment**
	A	35.5	28	Aliette
B	A	34.3	28	Quantum
B	A	34.2	28	Biofluor
B	A	33.5	28	Ecofrien
B	A	33.5	28	WatCont
B		30.4	28	Serenade

By 3-30-16, only the Quantum treatment had a lower DI (looked better) than the water control but several treatments had developed larger canopy volumes than the water control. The Biofluorish and Aliette treated trees were larger than the Quantum and Ecofriendly treated trees while the water control and Serenade trees were the smallest.

Diseasae Index (DI)_3_30_16				
DMRT		**DI**	**N**	**Trtment**
	A	17.0	28	Serenade
	A	16.8	28	Ecofriend
	A	16.5	28	Biofluor
B	A	15.7	28	WatCont
B	A	15.3	28	Aliette
B		14.4	28	Quantum

Canopy volume (CV, m^3)_3_30_16			
DMRT	**CV**	**N**	**Trtment**
A	37.6	28	Biofluor
A	36.4	28	Aliette
B	31.7	28	Quantum
B	28.5	28	Ecofriend
C	23.6	28	WatCont
C	23.5	28	Serenade

Duda juice quality.

There were 4 replicates (blocks) of 20 trees in each of the 6 treatments. There were 6 measurement (sentinel) trees with no mulch and 1 measurement tree, of three mulched trees with supplemental mulch. Fruit samples were pooled from each of the 6 measurement trees and tested separately from the single mulched tree in each of the 4 blocks in each treatment. Thus, there were 2 fruit samples from 4 blocks so n = 8 from each treatment.

In 2016, there were no mulch vs. no mulch effects on any of the measured juice quality parameters. Only the non-significant plus and minus (+) mulch treatments for average fruit weight (.54-.55 lbs) and Lb solids per box (6.17-6.23) are shown.

Avg Fruit Weight, + vs. - mulch. NS.

DMRT	Mean	N	Mulch
A	0.55	24	Yes
A	0.54	24	No

Lb Solids per Box, + vs. - mulch. NS.

DMRT	Mean	N	Mulch
A	6.23	24	Yes
A	6.17	24	No

Juice quality responses for ± mulch were pooled (n=8 for each treatment) for analyses. Fruit from the Untreated Control (UTC) trees had the largest average fruit size (.58 lbs) and those from the Quantum treatment were the smallest fruit (.52 lbs). There were no treatment effects on Percent juice (.58-.61), Lbs juice per box (52.5-54.8), total brix (11.5-11.7), Lb Solids per Box (6.1-6.4) or juice color (37.6 -37.7 nm) so these data are not shown. Fruit from the Aliette treatment had the highest percent acid (.72) whereas the Water Control and Serenade treatments had the lowest % acid (.65-.66). Since there were no significant differences in total brix (11.5-11.7), differences in % acid resulted in differences in the Brix/acid ratio where Serenade had the highest ratio (18.1) and Aliette the lowest ratio (16.3).

Average Fruit Weight

DMRT	Avg Fruit Wt		N	Treatment
large	A	0.58	8	Wat Cont
B	A	0.55	8	Serenade
B	A	0.55	8	Ecofriend
B	A	0.53	8	Biofluor
B	A	0.53	8	Aliette
small		0.52	8	Quantum

Percentage acid in juice

DMRT			% Acid	N	Treatment
high	A		0.72	8	Aliette
B	A		0.7	8	Biofluor
B	A	C	0.68	8	Ecofriend
B		C	0.67	8	Quantum
low		C	0.66	8	Wat Cont
low		C	0.65	8	Serenade

Brix / acid ratio

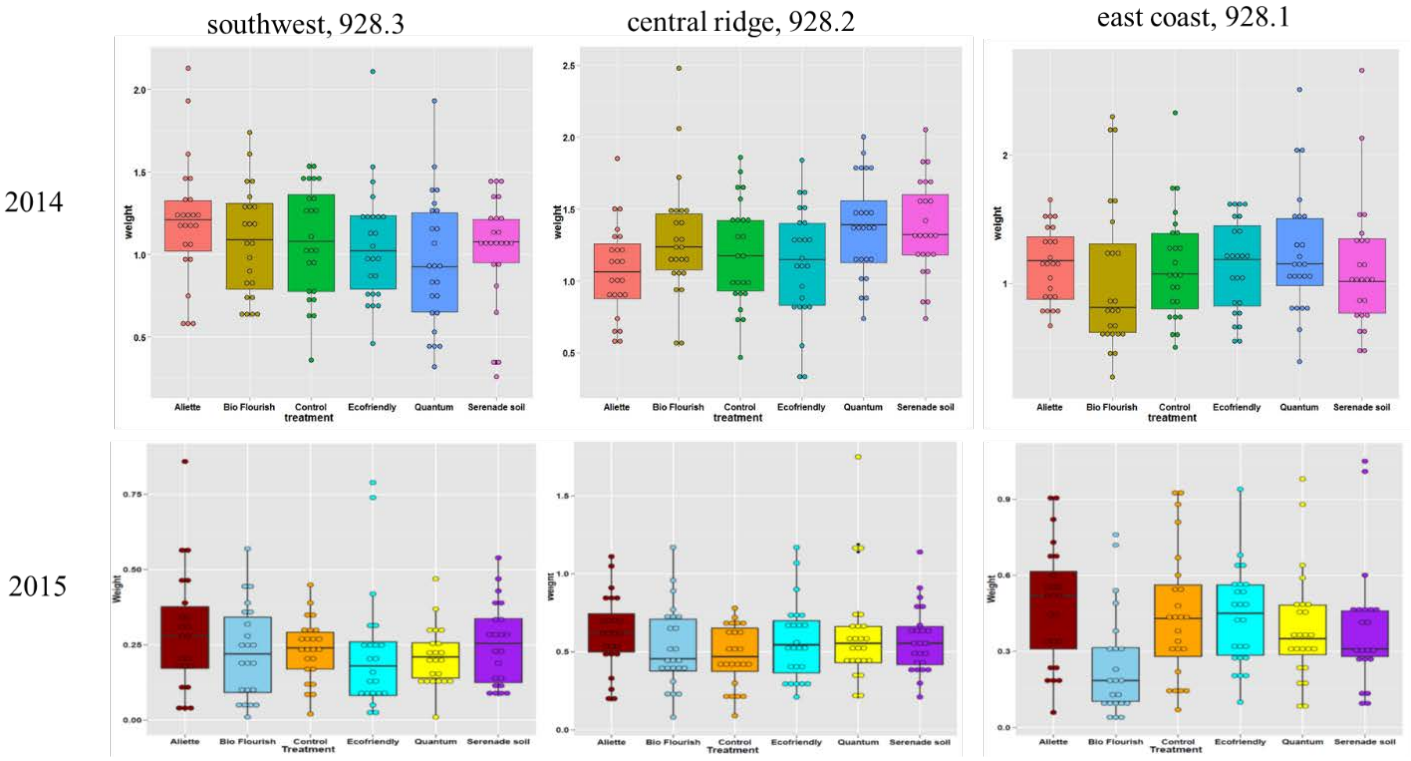
DMRT			Ratio	N	Treatment
	A		18.06	8	Serenade
B	A		17.76	8	Wat Cont
B	A	C	17.15	8	Quantum
B	A	C	17.03	8	Ecofriend
B		C	16.67	8	Biofluorish
		C	16.33	8	Aliette

Root density. All 3 sites.

The root density was determined in December 2014 and December 2015. Roots were extracted from 500 cc aliquant of each soil sample over a 2 mm mesh sieve by hand. The dry weight of roots was measured. There were totally 504 samples (3 sites*6 treatments*4replicates*7 trees).

Based on the data from two years, the root mass did not show any significant difference between most of the treatments (ANOVA, *P*>0.05) (Fig. 1). However, the root mass of Bio flourish treatment in East coast site

928.1 was numerically lower than other treatments in 2014, but it was significantly lower than other treatments when measured in Dec 2015 (LSD, P < 0.05). This trend was only observed in site 928.1 but not in other two sites (Fig. 1).



Al BioF Contl Eco Qua Ser

Fig. 1. Root mass of different treatments from the 3 sites for 2014 & 2015. Each box plot [interquartile range (IQR)] represents the 25–75% range, with the thick line placed at the sample median. Lines below and above the box show the range of quartile 1 − 1.5 × IQR and quartile 3 + 1.5 × IQR. The dots represent outlying data points.

Mulch treatments added: When the mulch treatments were taken into account, the results suggested mulch treated trees tended to have higher root masses, but there was no significant effect (ANOVA, *P*>0.05) (Fig. 2).

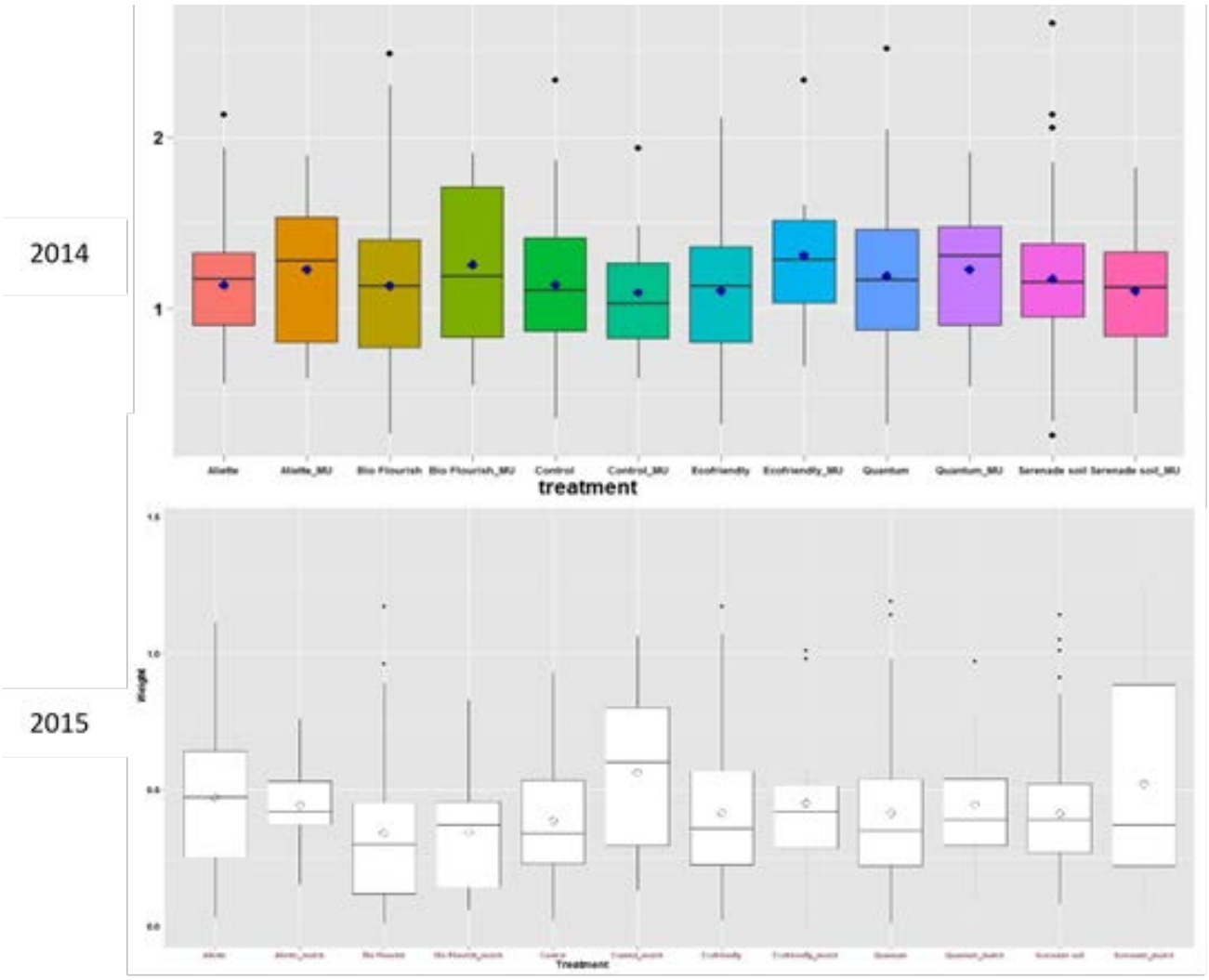


Fig. 2. Pooled Root mass samples from the different treatments from the three sites. Each box plot [interquartile range (IQR)] represents the 25–75% range, with the thick line placed at the sample median and square placed at the sample mean. Lines below and above the box show the range of quartile 1 − 1.5 × IQR and quartile 3 + 1.5 × IQR. The black dots represent outliers of this range. '_MU' or '_mulch' means treated with mulch. There were 72 trees for treatments without mulch while 12 trees for treatments with mulch.

The bacterial survival and effects of treatments on the soil bacterial community will be determined soon.

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 will allow a comprehensive view of the value

of these treatments over the first two years of the trial. Valencia crop harvest will be from February to April 2016. A field day will be planned for 1st half 2016 to highlight treatment effects as the second year of this trial comes to a close.

Significant Meetings or Conferences:

Obstacles Encountered and Breakthroughs:

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16
Quarter Ending March 31, 2016

3. HOST PLANT INTERVENTION

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

Narrative of Progress for Project Goals:

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

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.

Progress in development of techniques for nursery management of new citrus rootstocks emerging from UF and USDA breeding programs is reported here from the project being conducted by Dr. Richard Beeson of the UF, IFAS MFREC, Apopka. CRDF approved funding to Dr. Beeson to investigate barriers to propagation of new rootstocks and supported the construction of an addition to existing facilities at MFREC.

Tissue cultivation

Tissue culture plants started on 12-14-2015 were subjected to the same 4 durations of relative humidity (RH; 2,3,4,5) as previously. This group also included 3 reps of each cultivar hardened-off on heated benches (80 F). On 1 Feb. these plants were harvested for root and shoot growth. Citrus cultivars consisted of C-35, C-54 and sour orange. The next set of tissue culture plants for hardening were started on 9 March 2016. These will be harvested in late April. This set also includes 3 replications on 80 F heated benches. This set includes Kuharske, Swingle, C-35, sour orange and US 812. For the most part, survival rates have remained high on most cultivars, with perhaps more growth under the shorter hardening-off periods.

Stem cuttings

Kuharske cuttings stuck in the propagation benches under mist produced using the same protocols as in the past produced no roots or shoots when stuck on 1-4-16, nor when stuck on 1-12-16. This was independent whether there was bottom heat or not. In contrast, cutting similarly stuck on 2-5-16 once again began to produce some roots over the 6 week rooting period that ended on 3-28-16. Continuing in the sequence, cuttings of Kuharske stuck on 2-23-16 produced roots when harvested on 3-29-16. Cuttings of both X639 and C-54 were also stuck with this group of Kuharske. They also produced roots. These plants were harvested, but dry weights have not been measured. The last set of cuttings were stuck on 3-18-16. These consisted of Kuharske, X639, C-35 and US812.

In late March, all available cuttings of the HLB resistant trees in Citra were taken and brought to the citrus propagation bay at MREC Apopka. These were treated with an auxin blend of 4000 ppm auxin and stuck in seedling trays.

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

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

Nine candidate rootstocks were selected and propagated in large numbers: 5 experimental rootstocks: 4 from the UF breeding program (Orange 4 (UFR-2), Orange 15 (UFR-3), Orange 19 (UFR-4), 46 x 31-02-

13 (UFR-16) and 1 from the USDA breeding program (US 942), along with 3 standard rootstocks (Sour orange, Carrizo, Swingle) for comparison at individual sites for comparison.

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

All rootstocks were budded with '1-14-19 Valencia' for scion uniformity and most have been planted at the 3 sites: Southwest Florida (Duda, LaBelle) site in March 2015, ridge site 1. (Peace River, Babson Park) at the end of April, at the 2nd ridge site (BHG, Venus) in July 2015. Trees on UFR16, were not large enough to be planted in the 2 ridge plantings in Spring/Summer 2015 so gaps were left to be planted this spring 2016.

Grove site evaluations include soil type, soil and water pH, and cultural practices including irrigation scheduling, fertility programs and pest/psyllid control. Best management practices will be determined by the individual cooperator and will be uniformly applied to all trees at each site. Cultural practices include:

- Aggressive psyllid management according to current CHMA recommendations or equivalent for young trees and early mature trees. Active participation in a CHMA or cooperative treatment area is encouraged as relevant.
- Irrigation, nutrition and grove floor management consistent with best management current practices to promote root health and growth in the presence of HLB
- Freeze protection should be a component of the planting plan.

Record-keeping on the field trial plantings has begun and includes dates, materials, rates and application methods for all practices. Grower cooperators and CRDF coordinate data collection on these field trials and will share information gained from the trials. CRDF and the local SW FL Extension agent hosted the first CRDF Rootstock Field Day at the Duda SW FL site on 10 Nov 2015. This site has a full complement of the 7 rootstocks in the March 2015 planting; the field day also included 3 additional ongoing rootstock trials by Dr. Grosser at the same site.

Standardized CRDF protocols for tree evaluation: Each tree has been assigned a unique treatment and replicate number. Tree evaluations initially included tree height and trunk diameter; Digital Photographs and Disease Index which are summarized below for each of the 3 trial sites. Leaf nutrition was evaluated August 2015 and data were summarized in the Sept 2015 report. Leaves for qPCR have been sampled and analyzed for treatment (rootstock) effects on HLB status.

CRDF DUDA Rootstock Trial. SW FL (Flatwoods) Valencia planted March 18,19, 2015 at 10' x 21.4 ft. 7 rootstocks (UFR3, US942, UFR2, UFR4, UFR16, Swingle & US812) in 5 reps of 126 trees (7 x 18) in each rootstock plot = 630 trees minus buffers: 5 x 14 = 70 evaluation trees in each rootstock plot. CRDF has 8 measurement trees (sentinel trees) in each plot. The 8 sentinel trees in each of 5 replicate plots means there were N = 40 trees of each of the 7 rootstocks (RtSt).

Average tree heights (in cm) measured on 10/1/15 revealed that trees on US942 were the tallest and those on UFR3 were the shortest. All trees had grown such that by 1/4/16, tree on US942 were still the tallest but those on UFR2 were smaller followed by the smallest trees on UFR16 and UFR3. Measured canopy volumes (CV in m^3) ranked rootstock size similarly as tree heights with larger US rootstocks, smaller UFR rootstocks and trees on Swingle intermediate in canopy volume.

Tree Height (Tr.Ht., cm) 10/1/15						Tree Height (Tr.Ht., cm) 1/4/16						Canopy volume (CV, m^3) 1/4/16				
DMRT		**Tr.Ht.**	**N**	**RtSt**		**DMRT**		**Tr.Ht.**	**N**	**RtSt**		**DMRT**		**CV**	**N**	**RtSt**
	A	106.9	40	US942			A	124.7	40	US942			A	0.95	40	US942
	B	101.8	40	Swingle		B	A	120.2	40	US812		B	A	0.86	40	US812
C	B	100.9	40	UFR4		B	A	119.3	40	Swingle		B	A	0.85	40	UFR4
C	B	100.8	40	UFR16		B	A	119.1	40	UFR4		B	C	0.80	40	Swingle
C	B	100.3	40	UFR2		B		115.7	40	UFR2			C	0.71	40	UFR2
C	B	100.1	40	US812			C	109.0	40	UFR16			D	0.60	40	UFR16
C		96.8	40	UFR3			D	100.8	40	UFR3			E	0.47	40	UFR3

Trees were still too small to rate Disease index (DI) on a 40 point scale so trees were rated overall for visible symptoms on a 5 point scale. Trees on UFR3 had the highest DI (the most visible symptoms) and UFR4 looked the best but these symptoms may have been nutrient symptoms from the nursery as they disappeared by 1/4/16.

Disease Index (DI) 10/1/15						Disease Index (DI) 1/4/16			
DMRT		**DI**	**N**	**RtSt**		**DMRT**	**DI**	**N**	**RtSt**
	A	0.33	40	UFR3		A	0.05	40	UFR16
B	A	0.30	40	Swingle		A	0.05	40	UFR3
B	A	0.23	40	US812		A	0	40	Swingle
B	A	0.13	40	UFR2		A	0	40	UFR2
B	A	0.10	40	US942		A	0	40	UFR4
B	A	0.10	40	UFR16		A	0	40	US812
B		0.03	40	UFR4		A	0	40	US942

Leaves were sampled on 1/13/16 for PCR. Average CT (cycle threshold, 39-40) indicated that all trees were HLB negative (>32). Since some CTs were less than the 40 cut-off, values from 2 trees on US942 to 8 trees on Swingle for example, could be used to calculate the copy number per DNA (CN/DNA). Although the rootstocks have not yet segregated with respect to HLB status, it is interesting that PCR is apparently already detecting the bacterial DNA in a number of trees, from 2-8 of the 40 measurement trees throughout the grove. The relatively high average CN/DNA from the 5 trees on UFR16, came from a single tree that had a CT of 22 and was the only positive tree (CT less than 32) in the grove. The CN/DNA may be a more reliable number for indicating the presence of HLB than the CT as the CN/DNA is corrected by the amount of DNA extracted in the sample.

PCR Cycle threshold (CT) 1/13/16					Copy No. per DNA 1/13/16			
DMRT	**CT**	**N**	**RtSt**		**DMRT**	**CN/DNA**	**N**	**RtSt**
A	40	40	US942		A	11760	5	UFR16
A	40	40	UFR3		A	124	8	Swingle
A	40	40	UFR4		A	124	2	US942
A	40	40	US812		A	83	4	UFR3
A	39	40	UFR2		A	58	8	US812
A	39	40	UFR16		A	41	7	UFR4
A	39	40	Swingle		A	31	6	UFR2

Peace River CRDF Rootstock Trial, Babson Park, FL (Ridge): Valencia trees on 7 of 8 rootstocks (US897, US942, UFR3, UFR2, UFR4, US812 & Carrizo were planted on Apr 27, 2015. All trees on UFR3 trees have now been planted but trees on UFR_16 were not yet big enough to be planted but will be planted on 4/8/16. There were 8 sentinel trees in each of 5 replicate blocks (n=40) of each of the 7 rootstocks (RtSt). Average tree height (Tr.Ht. in cm) of trees on US897, US942 and US812 measured on 12/29/16 were the tallest (99 cm) while the more recently planted UFR3 were still the shortest (88 cm). Average canopy volumes (in m^3) revealed similar trends as trees on US942 were the largest (.47 m^3), Carrizo and UFR2 were smaller and the younger UFR3 were the smallest (.15 m^3). Trees were very small to evaluate Disease index (DI, here on a 0-10 scale) as there were no differences among rootstocks. It is interesting that the smallest trees had numerically the most visible symptoms, perhaps due to nutrient deficiency, while larger trees had the least.

Tree height (cm) 12_29_15			
DMRT	**Tr. Ht.**	**N**	**RtSt**
A	99.8	40	US897
A	99.7	40	US942
A	99.0	40	US812
B	94.2	40	UFR4
B	93.4	40	Carrizo
B	93.1	40	UFR2
C	88.0	40	UFR3

Canopy Volume (m^3) 12_29_15				
DMRT		**Can Vol**	**N**	**RtSt**
	A	0.47	40	US942
	B	0.37	40	UFR4
C	B	0.36	40	US897
C		0.32	40	US812
	D	0.27	40	Carrizo
	D	0.26	40	UFR2
	E	0.15	40	UFR3

Disease Index (DI) 12_22_15			
DMRT	**DI**	**N**	**RtSt**
A	0.45	40	UFR3
A	0.3	40	UFR2
A	0.3	40	Carrizo
A	0.2	40	US897
A	0.2	40	US812
A	0.1	40	UFR4
A	0	40	US942

Leaves were sampled on 1/12/16 for PCR. Average CT (cycle threshold, 39) indicated that all trees were HLB negative (>32). Since some CTs were less than the 40 cut-off, values from 5 trees on UFR_3 to 9 trees on US942, could be used to calculate the copy number per DNA (CN/DNA). The CN/DNA may be a more reliable number for indicating the presence of HLB than the CT as the CN/DNA is corrected by the amount of DNA extracted in the sample. Although the rootstocks have not yet segregated with respect to HLB status, it is interesting that PCR is apparently already detecting the bacterial DNA in a number of trees, from 5-9 of the 40 measurement trees throughout the grove. The relatively high CN/DNA in UFR4 and UFR3 are due to a single HLB+ tree on each rootstock with a CT = 27. There are no other trees on any rootstock that have a CT value less than 32.

PCR Cycle Theshold (CT) 1_12_16

DMRT	CT	N	RtSt
A	39.6	40	US812
A	39.4	40	Carrizo
A	39.4	40	US897
A	39.3	40	UFR3
A	39.3	40	UFR2
A	39.2	40	UFR4
A	39.0	40	US942

Copy no. per DNA_1_12_16

DMRT	CN/DNA	N	RtSt
A	1889	7	UFR4
A	413	5	UFR3
A	158	9	US942
A	64	8	US897
A	43	7	UFR2
A	36	6	US812
A	24	7	Carrizo

BHG CRDF Rootstock Trial, Venus, FL (Ridge): Valencia trees on 5 (of 7) rootstocks were planted July16-20, 2015, in 12 double set rows = 24 rows X 6 = 144 trees of each rootstock in each plot. 144 trees/plot X 7 rootstock X 5 reps = 5040 total trees. Only trees on 5 rootstocks were initially planted: UFR-2, UFR-4, US942, US812 and Sour orange but the missing trees on UFR3 but became available and were planted 8-18-15. Trees on UFR16 will not be ready from the nursery until spring 2016.

There were 8 sentinel trees in each of 5 replicate blocks (n=40) of each of the 6 rootstocks. On 9/28/2015, average tree heights of US_812 were tallest and that of the recently planted UFR_3 were shortest. By 11/27/15, all trees had grown and only the younger trees on UFR_3 were shorter than the others. At this time, the young trees were large enough to estimate canopy volumes. Trees on Sour and US_812 had the largest canopy volume and UFR_3 were the smallest.

BHG Tree height 9_28_15

DMRT		Tr. Ht.	N	Rootstock
	A	87.4	39	US_812
B	A	86.9	40	US_942
B	A	85.0	40	UFR_4
B	A	84.7	40	SOUR
B		84.0	40	UFR_2
	C	68.6	32	UFR_3

Tree height (cm) 11_17_15

DMRT	Tr. Ht.	N	Rootstock
A	90.1	39	US_812
A	89.6	40	US_942
A	89.1	40	SOUR
A	88.4	40	UFR_4
A	87.5	40	UFR_2
B	78.2	32	UFR_3

BHG first Canopy volume 11_17_15

DMRT		Can Vol	N	Rootstock
	A	0.10	40	SOUR
	A	0.09	39	US_812
B	A	0.08	40	US_942
B		0.07	40	UFR_2
B		0.07	40	UFR_4
	C	0.02	40	UFR_3

Leaves were sampled on 1/19/16 for PCR. Average CT (cycle threshold; 37-38) indicated that all trees were HLB negative (>32). Since some trees had CTs less than the 40 cut-off, values from 16 trees on UFR_3 to 25 trees on sour orange, could be used to calculate the copy number per DNA (CN/DNA). The CN/DNA may be a more reliable number for indicating the presence of HLB than the CT as the CN/DNA is corrected by the amount of DNA extracted in the sample. Although the rootstocks have not yet segregated with respect to HLB status, it is interesting that PCR is apparently already detecting the bacterial DNA in a number of trees, from 16 -25 of the 40 measurement trees throughout the grove. There was a single tree on UFR2 that had a CT = 31.5 and a high CN/DNA.

BHG first PCR CT_1_19_16

DMRT	CT	N	Rootstock
A	38.7	40	UFR_3
A	38.5	40	US_942
A	38.4	40	UFR_2
A	38.3	39	US_812
A	38.2	40	UFR_4
A	37.8	40	SOUR

Copy no. per DNA_1_19_16

DMRT	CN/DNA	N	Rootstock
A	81.3	19	UFR_2
A	46.2	18	US_812
A	45.6	25	SOUR
A	38.7	18	US_942
A	28.9	22	UFR_4
A	21.9	16	UFR_3

6. Communicate progress and results of evaluation of rootstocks to industry
CRDF had the first Rootstock Field Day at the Duda SW FL site on 10 Nov 2015.

Significant Meetings or Conferences:

Obstacles Encountered and Breakthroughs: Availability of sufficient numbers of contracted trees to be ready to plant continues to be a delaying factor.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16
Quarter Ending March 31, 2016

3. CITRUS HOST INTERVENTION

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

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

Narrative of Progress Against Goals:

1. Make measurable progress toward producing transgenic citrus lines from mature tissue transformation of commercially available cultivars for the Florida citrus growers. 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 follows:

Obj. 1- Continue *Agrobacterium*-mediated genetic transformation of mature citrus rootstocks and scions to confer tolerance to HLB and canker, and conduct molecular analyses to show transgene expression and copy number.

During the quarter, MCTF continued to focus on increasing the number of high quality genetic constructs used in transformations and improving the rate of successful transformations.

Outreach to additional research institutions and industry

To increase the number of high quality genetic constructs, MCTF has continued its outreach to additional research institutions and industry. They have been encouraged to submit vectors with linked reporter genes and with all plant sequences and no pest sequences, which might lessen regulatory hurdles. During the quarter, transformations were performed on constructs provided by 5 scientists: Drs. Dutt (UF), Wang (UF), McNellis (Penn State), Mou (UF), and Louzada (TAMU).

Transformation efficiencies continue to improve

During the quarter, transformation efficiencies have continued the trend of steady increase since the facility began using vectors with linked reporter genes in July 2015, with mature scion transformation efficiencies in 1Q2016 more than doubling to 7.6% over mid-2015 levels.

Made arrangements for inter-state transport of materials

To manage out-of-state transport of transgenics, USDA APHIS permits were obtained in the prior quarter by scientists shipping materials to MCTF, and during this quarter certification was obtained through UF to ship transgenic, mature citrus to Dr. McNellis at Penn State.

Continued development work on PMI selectable marker from Syngenta

During the quarter, MCTF continued efforts to optimize the PMI selectable marker for use in biolistics and agrobacterium transformation in immature and mature citrus transformation. PMI is being evaluated as an alternative to the previously used NPTII selectable marker. PMI was obtained from Syngenta in 2015. Syngenta has authorized use by MCTF for research purposes only. PMI has never before been used in mature citrus. The results so far look promising and shoot growth doesn't appear to be negatively impacted.

Continued development work on high throughput MUG assay

Development work is also continuing on a rapid, high throughput, non-destructive MUG (Fluorescent Beta-Galactosidase) Assay to screen transgenic citrus shoots for GUS expression. The potential advantage of this approach over the currently used X-Gluc substrate in the GUS reporter system, is that it is quantifiable and more sensitive. Fluorescence can be quantified on a plate reader, or visualized on a gel doc with known controls. It is anticipated that GUS expression will correlate to a copy number similar to NPTII expression. It remains to be determined whether the shoots will survive immersion in the MUG substrate and subsequent micro-grafting. Data is being collected describing the method for potential publication.

Obj. 2- Increase micro-grafting efficiencies or root mature citrus scion.

Steady improvements in efficiencies recorded

One of the reasons transformation efficiencies have been low is that ~40-60% of transformed shoots were lost due to micro-grafting failures. By adding a dedicated micro-grafting station in the growth room upon Dr. Zale's arrival and continued practice, the percentage of successful micro-grafts of scion have steadily increased, achieving 77% by end of 1Q 2016.

Transfer to scientists after grafting.

During the quarter, MCTF continued to increase production of mature citrus trees, and to transfer them to scientists directly without secondary grafting or propagation, unless otherwise directed. The transfers occur as soon as the primary or secondary grafts heal.

Reduce potential micro-grafting incompatibilities

To improve efficiency and lessen potential micro-grafting incompatibilities, sweet orange continues to be micro-grafted onto sweet orange rootstock. As previously reported, MCTF has found that micro-grafting losses in mature rootstock have significantly decreased when young shoots are micro-grafted onto rootstock grown in high sucrose solution.

Evaluate bioreactor alternative to micro-grafting

The facility is continuing to evaluate whether it can avoid the micro-grafting step altogether by growing the explants with developing shoots in bioreactors. If the shoots grow large enough, it may be possible to use the directly in secondary grafting, which is 100% effective.

Obj. 3 Continue plant propagation and budding events.

Transfer to Dawson Lab

Over the past two quarters, approximately 154 transgenics have been transformed with Dr. Mou's NPR1 sequence, propagated and transferred to Dr. Dawson's lab for additional testing. Approximately 50 more are planned to be transferred in April. This includes all of Dr. Mou's primary transgenics, and some budded or rooted vegetative progeny originally intended for field tests.

New breeder lines

The facility continues the process of introducing new breeder lines in which to produce transgenics. These sweet orange varieties and one grapefruit are being introduced via shoot-tip grafting. Once plants are established, they will be used in budding mature citrus onto rootstock to obtain budstick for transformations.

Obj. 4- Streamline operations, reduce expenses and secure additional external funding.

High operating expenses for staff, materials, equipment and facilities continue to be addressed, in part, through external funding sources. One method is to increase revenues through paid service charges from

customers. In addition, Dr. Zale is waiting for the USDA SCRI citrus call for proposals which is expected in Spring 2016 to see which areas the federal government has given priority for funding.

Obj. 5- Biolistics transformations

Biolistic transformations are being pursued as time and resources permit. During the quarter, the facility continued to successfully transform both immature and mature citrus with biolistics and demonstrated that the protocol is reproducible. As a result, the lab can now offer plant production using biolistics as a transformation alternative.

Significant Meetings/Conferences:/Publications

During the quarter, Dr. Zale, et.al. completed a manuscript that was submitted to Plant Cell Reports describing biolistic transformation of immature citrus rootstock, never previously reported in the literature. Biolistic transformation to produce transgenics will augment those produced with Agrobacterium.

Obstacles Encountered

As the facility moves forward there are a number of issues and challenges that have been identified and are being addressed with support from the MCTF Steering Committee:

- Continue to increase the number of high quality genetic constructs for evaluation by the facility.
- Leverage the knowledge and experience of Dr. Pena (IVIA Spain) to continue to increase transformation efficiencies of the facility.
- Take measures to ensure a stable supply of healthy, viable rootstocks, including a steady supply of disease free rootstock seed. During the quarter sour orange and Volkameriana seed were purchased for the growth room because seed of preferred rootstock varieties were sold out. It remains to be determined if these varieties perform well in the growth room.
- Securing external financing through grants and charges to customers remains an ongoing challenge.

Breakthroughs:

Successful biolistic transformation of immature or mature citrus has not previously been reported. This will be an important technology to transform citrus without pest sequences, which might lessen regulatory hurdles.

Other Information:

Funding for MCTF is provided in Project 15-045C, which expires 6/30/2016. 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 and Hamlin.

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 deliberations of the CRDF knowledge mapping exercise for HLB host resistance or tolerance, and associated efforts to develop side-by-side field testing of the most promising candidates and delivery to Florida growers.