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

Quarter Ending June 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: 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.

In this quarter no new companies have been identified with new candidate bactericides. This includes suggestions submitted to the "solutions inbox" (solutions@citrusrdf.org).

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

The forty-eight samples were tested *in vitro* this quarter and more than three hundred samples have been tested this fiscal year. One of the companies CRDF is working with is a company developing products to target plant pathogenic bacteria. Improved materials are being tested to identify the most active materials to be tested in the greenhouse assay and to estimate a starting concentration for treatment.

Other materials being tested are microbial supernatants that may be developed into biopesticide products. Six materials from two companies have been identified to move forward for testing in the greenhouse assay in FY 2016/17.

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

Quarter Ending June 30, 2016

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.

A new company has come forward with potential new adjuvants. CRDF project managers will work with this company in the next quarter 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. In this quarter the NDA between the company and the University of Florida and a researcher has been identified with the expertise to evaluate these materials.

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. The final report for this one-year project has been submitted to CRDF. Project managers will meet with the company developing this technology to discuss the next steps to determining if this is a method that will improve grower bactericide applications and can be made available to growers in the short term.

Project 15-048C evaluating efficacy of trunk injection as a treatment method was approved in October 2015 and treatments occurred in March 2016. The field site of young (4-5 yo) Hamlins was treated with bactericides by 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 took place in June during the summer flush period. In this quarter, a budget for a third foliar application was added to this project. This application will take place in September 2016 at the time of the second trunk injection applications.

A new project was approved this quarter to evaluate detection techniques for evaluating bactericides within the plant. This project will begin next quarter.

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

Quarter Ending June 30, 2016

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 with reapplication every 60 days. An analysis of the data collected from the first six months of this field trial will be available in the report next quarter.

The three AgroSource projects have been completed and final reports submitted to CRDF.

A draft of the Nufarm residue study has been submitted to CRDF, the completed report will be submitted in the next quarter.

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 have 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. At this time, more than 40 trials have been set-up by the CRDF field staff and more than 30 others are being monitored. In the next quarter project managers will identify new solid-set planted blocks. These blocks will be monitored for HLB infection and movement of the disease through the block.

Data from the Company C field trial continues to be collected. This trial was initiated in August 2014 and will be completed in August 2016. Data from this trial was included in the previous quarter report; an analysis of the completed trial data will be included in next quarters report.

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, which has not yet been approved, 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.

The bactericide project manager presented information on the CRDF bactericide projects at the Florida Citrus Mutual Annual Conference educational session at Bonita Springs in June.

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

Quarter Ending _ June 30, 2016 ___

1. Candidatus Liberibacter asiaticus PATHOGEN INTERVENTION

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

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

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.
3. Evaluate HLB infected citrus trees before and after thermal therapy treatments to encourage scale-up of individual tree, over-the row and root supplemental heat and evaluation of their performance in reducing disease and improving health of treated trees.
4. Continue outreach efforts to inform growers of optimized thermal treatments including CRDF sponsored field days to include thermal therapy researchers and active steaming commercial companies.

Narrative of Progress by Project Goals:

<u>2. Determine impact of thermal treatment on CLas acquisition by ACP</u>. 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.

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 imidicalopprid 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 imidcalopprid 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. Refine requirements and environmental conditions for most effective thermal treatment</u>.
The USDA, APHIS MAC group was charged to manage the federal funding to put HLB solutions in the hands of growers. 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 publicized on the CRDF web page so growers can do some self-assessments of their own thermal therapy trials and been implemented on a small scale with grower and research trials. The protocol 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>3. Encourage scale-up of individual tree, over-the row and root supplemental heat and evaluation of their performance in reducing disease and improving health of treated trees</u>.
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 18 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 15 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.

Conserve Trial 1

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, Canopy Volumes, tree height, trunk cross sectional area, Disease index, % leaf bleaching, % leaf drop and fruit drop were evaluated. Data await analyses.

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, Canopy Volumes, tree height, trunk cross sectional area, Disease index, % leaf bleaching, % leaf drop and fruit drop were evaluated. Data await analyses.

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, Canopy Volumes, tree height, trunk cross sectional area, Disease index, % leaf bleaching, % leaf drop and fruit drop were evaluated. Data await analyses.

Cutrale Trial 1

There were 10 sets of 3 uniform trees of 8 year-old Hamlin on Swingle rootstock were selected for evaluation. Ten trees were non-steamed control trees, 10 trees were steamed at 127 F for 90 sec., and 10 steamed at 127 F for 90 sec followed by a bactericide spray. On 6-20-16, baseline Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. On 6-23-16, % leaf bleaching and % leaf drop were evaluated. Data await analyses.

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 Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. On 6-23-16, % leaf bleaching and % leaf drop were evaluated. Data await analyses

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 Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. On 6-23-16, % leaf bleaching and % leaf drop were evaluated. Data await analyses

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 Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. Data await analyses.

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 Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. Data await analyses

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 Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated and leaves were sampled for PCR. Data await analyses

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, 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. Data await analyses.

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, 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. Data await analyses.

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 fruit drop were evaluated. Data await analyses.

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. On 5-31-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index were evaluated. Data await analyses. Leaves will be sampled in July 2016 for nutritional analyses and leaf dry wt per leaf area ratios to be used to express leaf nutrient values on a leaf area basis.

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. On 6-17-16, Canopy Volumes, tree height, trunk cross sectional area and Disease index and fruit drop were evaluated. Data await analyses

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 6-16-16, Canopy Volumes, tree height, trunk cross sectional area, Disease index, & leaf bleaching, % leaf drop and fruit drop were evaluated. Data await analyses.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16
Quarter Ending June 30, 2016

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, a team led by UF mathematics professor Dr. Jed Keesling (Project 932.1C) continued efforts to evaluate he 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 ended 5/31/2016. Going forward, this research is being funded by a USDA Specialty Crop Grant, which will continue to run simulations comparing the new models with previously developed models.

Over the past year of the project, modelling refinements focused on two areas:

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

While progress has been made, much remains to be learned about how the presence of inoculum results in symptoms. As a result, two conjectural models for how this happens have been developed, and simulations have been run to assess the effects of RNAi treatments assuming each models holds. Work continued during the quarter in running simulations under a variety of scenarios and refining the models based on new information.

The first model, an accumulation model, assumes that the presence of CLas has a degenerative effect on the tree that is proportional to the amount of inoculum present, and that inoculum accumulates proportionally to the number of infected nymphs present. When the magnitude of this degenerative effect reaches a certain threshold, the tree is considered to be declining. The threshold was chosen by comparing model output to date from Southern Gardens.

The second model, a root model, builds on research that documents how HLB damages the root system before symptoms appear in the canopy. In the model, the inoculum enters the tree by means of the Asian Citrus Psyllid, then travels through the phloem towards the roots when the flushing period is over. Some of the bacteria make it to the roots causing damage to the root system. It assumes that the tree begins to decline only when living CLas reaches the roots of a healthy tree. As in the first model, inoculum accumulates proportionally to the number of infected nymphs present and decays at a small rate. At the end of a flushing period, when the direction of the phloem is more toward the roots, the inoculum has a chance of also being carried to the roots. If it arrives at the roots, the tree is said to be in decline. The probability that inoculum is carried to the roots also was chosen by comparing model output to data from Southern Gardens.

Simulations for both models have been run over a variety of scenarios for parameters, e.g. psyllid pressure, loss rate of RNAi within the trees, and rate of decay of inoculum over time. Simulations suggest that, under both models, there will be a significant reduction in the percentage of declining trees over a five-year period when compared with a control group.

These models will continue to be refined in the future using data from Southern Gardens and through discussions with plant pathologists to understand the phloem system and its function in symptom development. For example, recent research suggests that the current models may not adequately capture how CLas distributes in a tree over time. The understandings of bacterial migration to the roots and back into the canopy following the flow of the phloem has now been called into question. Answering this and other questions are important in determining the movement of Clas in the tree as well as the progress of the disease and the decline of production.

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 Citrus Trestiza Viral Vector (CTVvv). 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 CTVvv 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

Throughout the year, 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 simulations using the revised model, the team tentatively concludes that up to eight constructs can be simultaneously tested in a 10 acre block, while still differentiating among constructs. As further refinements are made to the model for symptom appearance, Dr. Keesling plans to run more simulations to address this issue.

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 CTVvv 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, and in May 2016 a proposal developed by Southern Gardens was approved by both CPDC and CRDF Board. (See Obj. 6)

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

To date, all of the CTVvv + RNAi work has been done under growth room conditions, using small caged plants, for a relatively short period of time. Based on the initially favorable small-scale trials, several RNAi sequences appear to be promising enough to move to a field trial situation to see if the growth room results can be replicated. CTV is the most appropriate delivery "niche" for CRDF to pursue in terms of near term 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 prepared a full proposal and budget for the Phase I trial, which was presented to CPDC in May 2016 and, with CPDC recommendation to proceed to Board consideration, the proposal was approved by CRDF Board at its May meeting. This will be a three-year trial with a targeted start in the Spring of 2017. 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 purpose 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.

CRDF is funding 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 of the indirect costs of the trial.

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. 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.

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2. Citrus Host Intervention

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

Project goal(s)

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

Narrative of Progress by Project Goals:

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 are sampled annually to determine root density. This report focuses on DI, Canopy volume, CT values from PCR, Fruit yield, and aspects of juice quality (brix and pound solids per box) through March/April 2016. Root density data are summarized below for all 3 sites across Dec 2014 and Dec 2015 for comparison.

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 Biofourish treatment resulted in larger canopy volumes than the Quantum and untreated control treatments.

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

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.

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

Fruit Yield (boxes)_4_27_16

DMRT	Boxes	N	Treatment
A	1.2	28	Quantum
A	1.1	28	BioFlour
A	1.1	28	Serenade
A	1.0	28	UT Control
A	1.0	28	EcoFriend
A	1.0	28	Aliette

Fruit samples were pooled for each of 4 mulched plus 4 no mulch within each treatment for n = 8. There were no treatment effects on brix content in juice nor on lb solids per box.

Total Brix

DMRT	Brix	N	Treatment
A	9.9	8	Serenade
A	9.7	8	BioFlour
A	9.6	8	Aliette
A	9.5	7	EcoFriend
A	9.5	8	UT Control
A	9.5	8	Quantum

Pounds of solids per box

DMRT	Lb sol/box	N	Treatment
A	5.7	8	Aliette
A	5.5	8	Serenade
A	5.3	8	UT Control
A	5.3	8	Quantum
A	5.2	8	BioFlour
A	5.2	7	EcoFriend

Root sample data from all 3 sites from both Dec 2014 and 2015 are summarized below.

East coast, Indian River site: Valencia/Swingle trees are 6 years old. The Bioflourish and Ecofriendly treated trees had a higher lower DI (looked better) than Untreated Control trees.

Tukey LS-means with the same letter are not significantly different.				
Time 9	**Treatment**	**DI**		
16-Mar-16	Untreated	14.7		A
	Serenade	11.7	B	A
	Aliette	11.3	B	A
	Quantum	10.9	B	A
	BioFluor	10.4	B	
	EcoFriend	10.4	B	

	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

There were no treatment effects on CV, CT or fruit yield. All trees were HLB positive having a CT less than 31.

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

Total brix in the juice from the Serenade treated trees lower than the Bioflourish and Untreated control trees. This resulted in lower lb solids per box in the Serenade treated trees than in the Untreated control, Ecofriendly and Aliette treated trees.

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

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

Root sample data from all 3 sites from both Dec 2014 and 2015 are summarized below.

SW FL Duda site: Valencia/Swingle trees are 11 years old. Although there were a few differences in Di among treatments, 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.

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

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.

	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

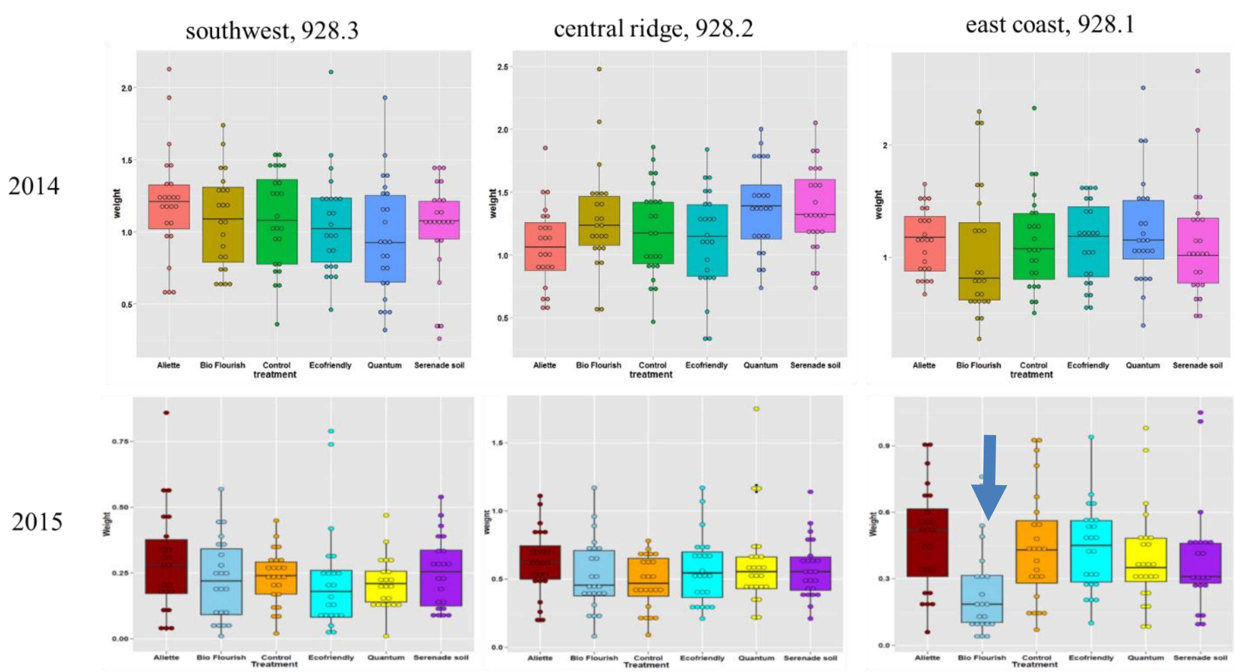
Fruit Yield (boxes) 3_15_16			
+ & - mulch combined			
DMRT	**Boxes**	**N**	**Trtment**
A	3.5	28	Biofluor
A	3.5	28	Aliette
A	3.2	28	Quantum
A	3.2	28	Ecofrien
B	2.7	28	WatCont
B	2.7	28	Serenade

There were no treatment effects on total brix in the juice or on lb solids per box.

Total Brix			
DMRT	**Brix**	**N**	**Treatment**
A	11.7	8	Aliette
A	11.7	8	Biofluor
A	11.7	8	Wat Cont
A	11.6	8	Serenade
A	11.6	8	Ecofriend
A	11.5	8	Quantum

Lb Solids per Box			
DMRT	**Lb sol/box**	**N**	**Treatment**
A	6.4	8	Biofluor
A	6.2	8	Ecofriend
A	6.2	8	Serenade
A	6.1	8	Aliette
A	6.1	8	Quantum
A	6.1	8	Wat Cont

Root comparisons. Data are from 8 soil cores (1 x 6") per tree separated with a 2 mm sieve. There were no mulch effects on root mass per tree so ± mulch data were combined for 7 trees x 4 reps so n=28 trees per treatment. There were no treatment effects on root mass per tree at any of the 3 sites in Dec 2014. The Bioflourish treated trees at the east coast site in Dec 2015 (arrow) had significantly less roots per tree than the other treatments. This was the site with the least HLB and these data imply that the fewer roots produced by the Bioflourish may have been more efficient than the other treatments in that they supported larger trees than the other treatments.



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

Significant Meetings or Conferences:

Obstacles Encountered and Breakthroughs:

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 was from February to April 2016. A field day will be planned for final quarter 2016 to highlight treatment.

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3. HOST PLANT INTERVENTION

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

Project goal(s) for this project:

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 early release rootstocks and scions to growers 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 5. Implement and evaluate Phase I and II grower field trials of most promising candidate HLB tolerant rootstocks using standard varieties as scions. 6. Communicate progress and results of evaluation of rootstocks to industry

Narrative of Progress for Project Goals:

2. Encourage early release of new commercial rootstocks and other strategies to make these rootstocks available to growers

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.

Tissue culture. Tissue culture plants started on 3-09-2016 were subjected to the same 4 durations of relative humidity (RH; 2,3,4 and 5 days) as previously. Plants were harvested on 28 April for root and shoot growth. Citrus cultivars consisted of C-35, C-54 and sour orange. Dry mass measurements are continuing. The last set of tissue culture plants for hardening were started on 25 May 2016. These will be harvested on 13 July. 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. Cuttings of Kurharske, C-54, C-35, US 812 and sour orange were treated with 4000 ppm Dip&Gro on 8 April, 28 April, 20 May and 10 June. This completed 1 year of initiating the rooting of cutting every 3 weeks. Concurrently stem cutting stuck previously were harvested for set #13 on April 6, with the remaining harvested on May 3, May 20, June 10 and the last replication harvested on June 30. With the harvest of the April 8 cuttings, root development and growth was similar to that early summer 2015, and was continued until the end of the experimental year. All plants were dried at 70C. Measurement of root growth and dry mass is continuing.

On 25 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 the 4000 ppm auxin and stuck in seedling trays. On 25 April cuttings were selectively harvested from MREC block and stuck. Total HLB resistant cuttings stuck this spring were UFR7 – 216, UFR9 – 473, UFR10 – 153, UFR11 – 374 and UFR12 – 143. Additional cuttings will be propagated as required by Dr. Grosser.

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

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 2 or 3 from the USDA breeding program (US-812, US-942 & at one site US-897), along with 3 standard rootstocks (Swingle, Carrizo, Sour orange,) for comparison at individual sites for comparison.

5. Evaluate ongoing grower plantings of candidate rootstocks at 3 different sites: 2 on Central Ridge and one in Southwest Florida. All rootstocks were budded with '1-14-19 Valencia' for scion uniformity and all have now 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 the 2nd ridge site (BHG, Venus) in June 2015. Trees on UFR16, were not large to be planted in the 2 ridge plantings in Spr/Sum 2015 so gaps were left to be planted in 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 and will be sampled again in August 2016. Leaves for qPCR have been sampled and analyzed for treatment (rootstock) effects on HLB status and will be resampled this winter (Dec 2016).

CRDF DUDA Rootstock Trial. SW FL (Flatwoods) Valencia. All trees on all 7 rootstocks were planted March 18,19, 2015 at 10' x 21.4 ft. The rootstocks were US-812, US-942, UFR-2, UFR-3, UFR-4, UFR-16 and Swingle (as a standard) 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.

Measured canopy volumes (CV in m^3) on 4/21/16 ranked rootstocks similarly as tree cross sectional area (TCSA, in cm^2) with larger US rootstocks, smaller UFR rootstocks and trees on Swingle intermediate in CV and TCSA.

Canopy Volume (CV, m^3)_4_21_16

DMRT		CV	N	Rootstock
	A	1.5	40	US_942
	B	1.3	40	US_812
	B	1.3	40	UFR_4
	C	1.1	40	SWINGLE
D	C	1.1	40	UFR_2
D		1.0	40	UFR_16
	E	0.7	40	UFR_3

Trunk X Sect. Area (TCSA, cm^2)_4_21_16

DMRT		TCSA	N	Rootstock
	A	11.7	40	US_942
	B	10.1	40	US_812
	C	9.0	40	UFR_4
	C	8.9	40	SWINGLE
D	C	8.4	40	UFR_2
D		7.6	40	UFR_16
	E	5.4	40	UFR_3

Trees were still too small to rate Disease index (DI) on a 40 point (score 1-5 in 8 canopy section) scale so trees were rated overall for visible symptoms on an overall 5 point scale. Trees on UFR-3 had the highest DI (the most visible symptoms) as none of the trees on the other rootstocks differed. Average tree heights (in cm) measured on 4/21/16 revealed that trees on US-942, US-812, UFR-4 and SWINGLE rootstocks were the tallest and those on UFR-3 were the shortest. Thus, rootstock tree height ranked identically to CV and TCSA.

Disease Index (DI)_4_21_16

DMRT		DI	N	Rootstock
	A	0.4	40	UFR_3
B	A	0.3	40	SWINGLE
B	A	0.2	40	UFR_16
B		0.2	40	UFR_2
B		0.1	40	US_812
B		0.1	40	US_942
B		0.1	40	UFR_4

Tree Height (TH, cm)_4_21_16

DMRT	TH	N	Rootstock
A	139.3	40	US_942
A	135.8	40	US_812
A	135.0	40	UFR_4
A	134.6	40	SWINGLE
B	128.4	40	UFR_2
B	124.4	40	UFR_16
C	112.0	40	UFR_3

Peace River CRDF Rootstock Trial, Babson Park, FL (Ridge). Valencia trees on 7 of 8 rootstocks (US-897, US-942, US-812, UFR-2, UFR-4, UFR-3 (short half of the trees), & Carrizo (as a standard) were planted on Apr 27, 2015. All trees on UFR-3 trees have now been planted and trees on UFR_16 were big enough to be planted 4/8/16. There were 8 sentinel trees in each of 5 replicate blocks (n=40) of each of the 7 rootstocks (RtSt).

Canopy Volume (CV) 4_19_16			
DMRT	CV (m^3)	N	Rootstock
A	0.23	40	US-897
A	0.23	40	US-942
A	0.20	40	US-812
B	0.15	40	Carrizo
B	0.15	40	UFR-4
B	0.12	40	UFR-2
B	0.11	40	UFR-3
C	0.05	40	UFR-16

Trunk Cross sectional Area (TCSA)				
DMRT		TCSA (cm^2)	N	Rootstock
	A	58.3	40	US-942
	B	44.4	40	US-812
C	B	41.5	40	US-897
C		39.3	40	UFR-4
C		37.8	40	Carrizo
	D	32.8	40	UFR-2
	E	18.3	40	UFR-3
	F	3.9	40	UFR-16

Tree Height (TH) _4_19_16				
DMRT		TH (cm)	N	Rootstock
	A	115.7	40	US-897
	A	115.5	40	US-942
	A	112.1	40	US-812
	B	100.9	40	Carrizo
	B	100.5	40	UFR-4
C	B	95.8	40	UFR-2
C		91.2	40	UFR-3
	D	69.2	40	UFR-16

Average canopy volumes (CV, in m^3) measured on 4/19/16 revealed trees on the US rootstocks were larger than the standard Carrizo or UFR-4 and UFR-2 trees that planted at the same time. The smaller trees on UFR-3 and UFR-16 were planted later complicating valid comparisons. Trunk Cross Sectional Area (TCSA in cm^2) of rootstocks followed a similar ranking order as CV with larger US rootstocks than UFR rootstocks. Likewise, average tree height (TH in cm) of trees on US-897, US-942 and US-812 were the tallest (112-115 cm) while the more recently planted UFR-3 and UFR-16 were still the shortest. Trees were very small to evaluate Disease index (DI) as there were no differences among rootstocks (data not shown).

<u>BHG CRDF Rootstock Trial</u>, 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, US-942, US-812 and Sour orange as a standard. Trees on UFR-3 were planted 8-18-15 and trees on UFR-16 were just planted in June 2016 complicating valid comparisons.

There were 8 sentinel trees in each of 5 replicate blocks (n=40) of each of the 7 rootstocks. On 5/9/16, the young trees were large enough to estimate canopy volumes. Trees on US_812 had the largest canopy volume and that of the more recently planted UFR_3 were the smallest. Similarly, on 5/9/16 average tree heights of trees on US_812 were tallest and UFR_3 were the shortest.

Canopy Volume (CV, m^3)_05_09_16			
DMRT	CV	N	Rootstock
A	0.33	39	US_812
B	0.29	40	US_942
C	0.22	40	SOUR
C	0.19	40	UFR_4
C	0.18	40	UFR_2
D	0.07	40	UFR_3

Tree Height (TH, cm)_05_09_16				
DMRT		TH	N	Rootstock
	A	103.1	39	US_812
B	A	99.1	40	US_942
B	C	95.0	40	UFR_4
D	C	93.0	40	UFR_2
D	C	91.8	40	SOUR
D		88.9	40	UFR_3

Trunk X Sect. Area (TCSA, cm^2)_05_(			
DMRT	TCSA	N	Rootstock
A	3.3	40	US_942
B	2.9	40	US_812
C	2.5	40	SOUR
C	2.4	40	UFR_4
D	2.1	40	UFR_2
E	1.1	40	UFR_3
6/15/2016	0.3	40	UFR_16

Trunk cross sectional area of trees on US_942 was the largest, followed by US-812 with UFR-2 and UFR-3 the smaller and the most recently planted UFR-16 the smallest. Thus, the generally larger trees on US rootstocks than on UF rootstocks was consistent across all 3 sites.

5. 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 complicate valid comparisons using the later planted UFR-3 and the most recently planted UFR-16 at two sites. The effect of planting some of the replications a year later will have implications on data collected from juvenile trees.

CRDF Commercial Product Delivery Sub-Project Progress Report FY 2015-16 Quarter Ending June 30, 2016

3. CITRUS HOST INTERVENTION

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

Project goal(s) for this project area for FY2015-2016

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.

Narrative of Progress Against Goals:

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.

Over the past quarter, the Mature Citrus Facility continued to make steady progress in producing Agrobacterium-mediated transgenics for clients as a service. Since July 1, 2015, approximately **100 transgenics** were produced with Agrobacterium, a roughly **4-fold increase** over rates in the previous funding period. The increase was due to several factors: use of superior vectors with linked reporter genes, stronger promoters driving expression of the nptII selectable marker, and an increase in micro-grafting efficiencies. Molecular analyses were conducted using qPCR and Southern gel blots of transgenic plants to confirm gene transfer.

<u>Outreach to additional research institutions and industry</u>
To increase the number of high quality genetic constructs, over the past quarter MCTF continued its outreach to additional research institutions and industry. Researchers have been encouraged to submit vectors with linked reporter genes and with all plant sequences and no pest sequences, which will lessen regulatory hurdles. Over the course of the past year, transformations were performed on constructs provided by 5 scientists: Drs. Dutt (UF); Wang (UF); McNellis (Penn State); Mou (UF); and Louzada (TAMU). At this time the goal is to produce 5 to 10 events for each genetic construct and deliver back to the scientist.

<u>Transformation efficiencies continue to improve</u>
One of the project objectives is to increase transformation efficiencies. 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, mature scion transformation efficiencies more than doubled to 7.6% over mid-2015 levels.

Speed growth of mature scions

Over the past year, protocols have continued to evolve in an effort to speed the growth of mature scions for use in transformations. For example, after budding mature buds, rootstocks are left attached for the two flushes of stem growth. Mature buds will break and stems can be used in transformations within 6-8 weeks rather than 12 to 16 weeks specified in the earlier protocol. Other potential protocol changes are being investigated, including cold treatments and hormone applications.

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 the previously used nptII selectable marker that uses antibiotics (kanamycin) to suppress Agrobacteria.

PMI was obtained from Syngenta in 2015, which 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. Specifically, the number of non-transformed, escaped shoots appears to be significantly lower than with nptII as a selectable marker.

Testing improvements in assays to identify transgenic shoots.

Currently the lab uses histochemical colorimetric dye assay (GUS) that is labor intensive, tedious and destructive to tissue. As a potential alternative, development work is also continuing on a rapid, high throughput, non-destructive assay based on a fluorescent dye substrate (MUG) to screen transgenic citrus shoots for GUS expression.

The potential advantage of this approach over the currently 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 fluorescent 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.

<u>Steady improvements in micro-grafting efficiencies recorded</u>

One of the reasons transformation efficiencies have been low is that ~60% of transformed shoots were lost due to micro-grafting failures. Dr. Zale added a dedicated micro-grafting station in the growth room upon her arrival, and hired a dedicated skilled person with attention to detail perform the task. With continued practice, the percentage of successful micro-grafts of scion have steadily increased from ~43% three years ago to ~77%.

<u>Transfer to scientists after grafting.</u>

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.

<u>Made arrangements for inter-state transport of materials</u>

Over the past several quarters, steps were taken to manage out-of-state transport of transgenics, USDA APHIS
permits were obtained by scientists shipping materials to MCTF, and certification was obtained through UF to
ship approximately 10 transgenic, mature citrus to Dr. McNellis at Penn State. First shipments were made in
March, with more to follow once transgenes are confirmed.

<u>Reduce potential micro-grafting incompatibilities</u>

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.

<u>Evaluate bioreactor alternative to micro-grafting</u>

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 to form replicates for disease testing.

<u>Transfer to Dawson Lab</u>

From primary mature transgenics produced during an earlier funding period, vegetative progeny were produced by budding mature scion onto Carrizo, or rooting mature rootstock cuttings. Over the past two quarters, approximately 200 transgenics were transformed with Dr. Mou's NPR1 sequence, and transferred to
Dr. Dawson's lab for additional testing. This includes all of Dr. Mou's primary transgenics, as well as 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. Three sweet orange varieties and one grapefruit were introduced via shoot-tip grafting and are being budded for transformations. Protocols initially are following those used for Hamlin and Valencia, but might still have to be optimized for these new cultivars.

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 used to increase revenues is 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. During the past year, focus has been on optimizing the process, e.g. amount of DNA per shot, time of bombardment and helium pressure to coincide with organogenesis.

Significant Meetings/Conferences:/Publications

During the quarter, a manuscript prepared by Dr. Zale, et.al. was published in ***Plant Cell Reports*** describing biolistic transformation of immature citrus rootstock, never previously reported in the literature. An additional manuscript is in preparation describing the development of a quantitative in fluorescent assay for transgenic, mature shoots.

Obstacles Encountered

Some clients have asked for each transgenic event to be budded onto immature rootstock into replicates, which appears to delay flowering. An experiment is being conducted to determine how many months flowering is delayed by grafting flowering tissue onto immature immature rootstock. This result will influence recommendations to clients.

The lab will be moving to the packinghouse in July 2016 in order to fix the AC in the current lab. The move will cause disturbances to plant production, and efforts will be made to minimize disturbances to the mature citrus transformation pipeline.

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 will lessen regulatory hurdles.

Other Information:

During the past fiscal year, funding for MCTF was provided in Project 15-045C, which expired 6/30/2016. In June 2016 , CPDC and the CRDF Board approved a two-year project continuation.

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 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.