Citrus Research and Development Foundation, Inc.

CRDF-Funded Projects Summary Progress Report FY 2023-2024

Period Ending June 30, 2024 (more recent reports also included)

RESEARCH TOPICS COVERED IN THIS REPORT

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CLAS PATHOGEN INTERVENTION

a. Bactericides/antimicrobials

K. Pelz-Stelinski 21-021 "CLas Inhibition with Antisense Oligonucleotides for Management of Citrus Greening Disease" – 100% Completion of Objectives (May 2024 report)

Huanglongbing (HLB) is a systemic disease of citrus caused by the bacterial pathogen Candidatus Liberibacter asiaticus (CLas) that limits citrus production worldwide. CLas is an obligate bacterial pathogen that multiplies in citrus trees and in the insect vector, the Asian citrus psyllid (ACP), Diaphorina citri Kuwayama. There is no cure for HLB currently and broad-spectrum antibiotics represent one possible therapeutic against disease symptoms. Single-stranded nucleic acid analogs, 2'-deoxy-2'-Fluoro-β-D-arabinonucleic antisense oligonucleotides (FANA ASOs), can modulate gene expression by enzymatic degradation or steric blocking of an RNA target. FANA ASOs recognize and bind to specific RNA forms, including mRNA, miRNA, and long noncoding RNA, through complementary base pairing.

Injection of oxytetracycline (OTC) into mature citrus trees with HLB ameliorated symptoms of disease, increased fruit yield, and quality of juice as compared with that produced by non-injected controls. Injection of trees with FANA ASOs also reduced CLas infection but did not improve fruit yield and quality above control levels at the injection dosage tested. Reduced pathogen titers following OTC or FANA ASO injection were coincident with lower CLas acquisition and inoculation by laboratory deployed and wild-type D. citri collected from the field, respectively.

Trunk injection of OTC in rotation with antimicrobial technologies like FANA ASOs may be useful in management of HLB by reducing CLas infection in trees and disrupting transmission. Future investigations should prioritize optimizing FANA ASO dosage in trees and exploring the potential of multiplex FANA ASOs that simultaneously target multiple mRNAs to enhance efficacy against CLas infection.

U. Albrecht 23-005 "OTC Directed Research Solicitation: Use of CRDF Rootstock Trial Locations for Testing Bactericides Inserted into Trees Through Systemic Delivery Devices" – 35% completion of objectives (June 2024 progress report)

The objective of this project is to determine the large-scale efficacy of trunk injection of OTC on citrus tree health, fruit quality, and yield in existing rootstock trials in three different commercial growing environments.

From the stated overall objective, two sub-objectives are being address within the project and are: a) test the efficacy of OTC injection in the rootstock trunk compared to injection into the scion, and b) determine whether OTC injection increases/restores root densities and if there are interactions with the rootstock.

Harvests and fruit quality analyses were completed for the trial in SW Florida. Tree care was not as desired in this location as the collaborator (Duda) has ceased citrus production in this area. Overall, trees were still relatively productive in this trial with an average of 102 boxes per acre. The best performing rootstock in this location in terms of production was UFR-2 (144 boxes/acre), and the worst was Swingle (46 boxes/acre).

Across all rootstocks OTC injection increased yield by 21% but differences were not statistically significant. The yield for trees on US-812, UFR-2, and Swingle improved the most (28-29%) while there was little to moderate improvement for trees on UFR-4 (5%) and US-942 (14%).

OTC injection increased juice quality significantly across all rootstocks. There was no significant difference between rootstock and scion injection for yield and juice quality.

Wound closure was the same on scion and rootstock. However, bark cracks were longer after scion injection than after rootstock injection. There were significant differences among rootstocks for both wound closure and bark crack length.

Year-2 injections have been completed.

This year a significant difference in the uptake rate between rootstock and scion injection was measured: trees injected into the scion took OTC up significantly faster than trees injected in the rootstock.

Similar work at the other two trial locations (Babson Park and Venus) was reported in the quarterly report ending in March 2024.

Anticipated work during the next quarter will include monitoring the trees and continue to collect leaf samples for CLas detection. Fibrous root density collections will be performed.

Spending on the budget is mostly as expected.

J. Curtis 23-010 "3. Impact of Bactericides inserted through systemic delivery on improving tree health and root density over time" -

This trial was on track and data collection on fruit drop was well under way. The crop consultant was not notified of the harvesting date and consequently was unable to obtain yield, fruit quality and fruit drop data.

H. Yonce 23-013 "8. Yield Comparison Between Bactericide and Non-treated Control Blocks on Yield and Tree Health" –

A report of the results for year 1 can be found at:
https://citrusrdf.org/wp-content/uploads/2024/07/Trials-Testing-Efficacy-of-OTC-Injection-Treatments-on-Mature-Trees-July-2024.pdf

O. Batuman 23-014 "Determining the effect of oxytetracycline when rotated with additional crop antimicrobials on citrus phytotoxicity and CLas reduction" – 60% completion of objectives (July 2024 quarterly report)

This project is working to determine the effect of oxytetracycline (OTC) when rotated with additional crop antimicrobials on citrus phytotoxicity and CLas reduction.

The project has three objectives and are as follows: Objectives of this project are to determine the phytotoxicity of OTC and other potential partner antibiotics: Objective 1) by a citrus leaf assay in the lab (completed); objective 2) by a greenhouse assay in seedling trees (completed); and objective 3) to compare OTC with other antibiotics' efficacy against CLas on mature trees in the grove (in progress).

During this quarter (Q3), all injections were completed on mature trees in the field. All phenotypic tree measurements were taken, such as trunk diameter, canopy area, and height of each tree. Data was recorded as to the visual parameters of citrus diseases that were present, which included HLB ratings, canker ratings, canopy color, and canopy density. Flower and new shoot formation percentages per tree were tracked to determine later if the treatment was potentially influencing/altering these parameters. All the points of injections were noted (injection hole), one by one, and found no phytotoxicity (in terms of oozing or cracking) as of now. In general, no phytotoxicity was found on the last visit to the trial (dated 8.13.2024). Leaf samples were taken (one month after injection) and will be processed for CLas titers to compare with time zero. We will continue to sample other time points.

Greenhouse phytotoxicity data (objective 2), collected during Q1 and Q2, were presented at the American Phytopathological Society's annual Plant Health conference in Memphis, Tennessee, in July 2024.

Per CRDF's advisory request, additional treatments were added in our ongoing field trial. On August 12, treatments were injected consisting of kasugamycin + OTC (1/2 dose each; 5500 ppm) mixed together in the same FlexInjec injector (2 FlexInject per tree; 50 ml per FlexInject), as well as kasugamycin alone (1/2 dose; 5500 ppm), and OTC alone (1/2 dose; 5500 ppm) into mature Hamlin trees at the SWFREC trial site.

Work anticipated during the next quarter will include the continuation of sampling leaves for CLas titers and process them to determine if a particular treatment significantly reduces CLas (using qPCR). Phytotoxicity will continue to be tracked every week. Visual and disease ratings, as well as canker incidence, will be tracked on a monthly basis. Flowers and shoots will also be counted together, along with the visual ratings.

Spending on the budget status is underspent as of now, but we anticipate being back on track soon. The reason that we are currently under budget is that a few of the most expensive antibiotics involved in the trial did not need to be purchased initially, as we had them in our hands to start. In the next couple of months, we will buy more of these antibiotics and use these funds.

K. Mandadi 23-018 "Truck injection-based evaluation of novel anti-CLas chemistries and OTC combinations for Florida citrus and HLB disease management" – 24% Completion of Objectives (July 2024)

This is a first year, third quarter report. The goal is to evaluate new anti-CLas compounds in Florida singly and in combination with OTC to determine their effects on HLB tree health, yield, and injury.

Objective 1: Pre-screening and quality control (purity, solubility, and bioactivity) of selected anti-CLas candidates. (Initiated).

Objective 2: Multi-year field evaluation of new chemicals, OTC, and combinations by trunk injections. (Initiated).

Based on CLas-citrus hairy root efficacy assays (Irigoyen et al., 2020), we shortlisted five new active ingredients for which synthesis and sourcing in sufficient amounts needed for the field trials were completed. All quality control assays (solubility, bioactivity, dose determination) were completed. We aimed to dissolve all compounds in water to avoid using any other solvents on the trees. Three out of five compounds were fully soluble in water. Two were partially soluble. All the molecules were shipped to Dr. Albrecht's lab. Trunk injections were conducted on ~7-year-old HLB-affected sweet orange (Valencia/US-897) trees at the UF/SWFREC, as per the experimental design.

In the next quarter, trees will be monitored for potential unintended injury or toxicity issues. General grove care and management activities will continue. Any changes in horticultural traits will be also monitored.

The budget is on track with spending.

Potential commercialization products resulting from this research, and the status of each: By the end of the study, we anticipate identifying one or more new active ingredients that improve the HLB-affected tree health and fruit yield. The new chemicals could be developed as commercial products singly or in combination with OTC for HLB management.

Y. Wang 23-027 "A High-Throughput Screen for Natural Antibacterial Agents Against CLas" –

10% Completion (May, 2024 quarterly report)

The primary goal for this phase was to optimize our screening methods while identifying promising compounds from our in-house library and published literature. 1. Screening of Natural Antibacterial Agents: We started with an extensive review of available antibacterial compounds from our internal database, as well as running a comprehensive literature search. This was supplemented with the use of a structure-activity relationship (SAR) model to predict which compounds might have a strong inhibitory effect on CLas. Based on our team's expertise and past research, we shortlisted compounds that are likely to show promising activity against CLas. The chosen compounds were prioritized for testing, as they showed potential based on structural characteristics and previously reported antibacterial activities. This systematic approach aimed to reduce the time and effort required to identify effective candidates. 2. Development of High-Throughput Screening Method: To maximize efficiency and minimize labor costs, our original plan was to adopt a high-throughput screening method using 96-well plates. This setup would allow us to test multiple compounds simultaneously while maintaining standardized conditions. Unfortunately, after 5 days, we encountered significant evaporation in the wells, even after attempting to seal the plates with different types of sealing films. In some cases, bacterial growth was also inhibited, which could have been due to insufficient oxygen levels or other factors. Despite multiple troubleshooting attempts, the evaporation and growth inhibition issues persisted, leading us to reconsider this method. 3. Transition to Centrifuge Tube-Based Assay: After abandoning the 96-well plate format, we switched to a more stable method using centrifuge tubes. The setup involved inoculating BT-1 cells in centrifuge tubes under the same incubation conditions. Over the 5-day period, the bacteria grew well, and no significant evaporation was observed, making this method much more reliable for our purposes. Given the success of this alternative approach, we have adopted it for the remainder of the project. 4. Stabilizing the Bioassay System: Parallel to the screening efforts, another critical focus was the authentication and stabilization of our BT-1 culture. Team members dedicated this time to ensuring that the culture was authentic and free from contamination. They also optimized conditions to ensure consistent growth of BT-1 in the BM7 broth.

In the next quarter, we will continue screening the prioritized compounds from our library using the newly developed centrifuge tube method. We will also begin exploring potential synergistic effects of natural antibacterial agents in combination with OTC as we move toward addressing Objective 2. We remain committed to optimizing the screening process and identifying effective natural antibacterial agents against CLas. Through method refinement and persistent troubleshooting, we are confident that our work will contribute valuable insights to this project.

E. Triplett 23-032 "Proof of concept for phage therapy in the reduction CLas titer and HLB symptoms in citrus" – 25% Completion of Objectives (April 2024 quarterly report)

This investigation aims to identify a bacteriophage that infects and lyses Liberibacter crescens, a close relative of the unculturable Liberibacter asiaticus. For this project, L. crescens was cultured in liquid and on solid BM7 media, with optimal growth observed at pH 6.5, incubated at 28°C, and rotated 250 rpm for liquid cultures. Given the success of growth in these conditions, BM7 will be used as the media throughout the experiment.

After establishing L. crescens growth in both solid and liquid media, preliminary phage hunt experiments began to test various methods. Soil samples were collected, suspended in media, agitated, centrifuged, and filtered using 0.2 µm filters, aiming to remove bacteria and preserve bacteriophage. Water samples were also collected and filtered at 0.2 µm. The supernatant was concentrated using centrifugal filtration and mixed with L. crescens in liquid culture and soft agar for plaque assays. Plates were incubated at 28°C for 14 days, but no plaques were observed.

Next, L. asiaticus-infected Asian Citrus Psyllids (ACP) were collected and stored at -20°C. Psyllids were processed using two digestion methods: manual crushing using a tissue homogenizer and chitinase treatment at two different volumes of chitinase (1 and 1.5 mg per 25 psyllids, as suggested by manufacturer). After digestion, samples were centrifuged and filtered with 0.45 µm filters. The larger filter was used to allow a greater variety of phages than the preliminary experiments, and will be used for the remainder of the project. The same phage plating process was used, and growth will be monitored for 10 days.

Future work depends on the outcome of these experiments. If bacteriophages are successfully isolated from the infected ACP, phages will be processed on these plates and used to reinfect L. crescens in various dilutions, then sequenced for identification. If no plaques form, we will modify our approach, possibly using different chitinase concentrations to improve digestion of the insect exoskeleton or isolating ACP abdominal contents. Additionally, we may test non-infected ACP to assess any potential differences between infected and non-infected insects. Lastly, we propose that we can process phloem and soil from infected trees as a bacteriophage source.

We are behind in spending the money having spent just 25% of the budget so far. However, our methods are now in hand and thing will progress faster which will allow a faster spend rate.

We have no products yet, but once we find phage of interest, a product is possible.

H. Yonce 23-035 "RFP 1: CRDF Study on the injection of Labeled Plant Protection Materials: Phos acid and Copper"

This project is under way with a single trial location in DeSoto County. The injections have been made. The PIs, grower representative and Brandon Page toured the trial this summer. There is phytotoxicity showing in all of the experimental treatments. Yield, fruit quality and fruit drop data will be collected in the spring of 2025.

U. Albrecht 23-036 "Testing the injection of oxytetracycline (OTC) in a pH neutral solution (RFP2)" – 20% Completion of Objectives (July 2024 quarterly)

Objective: To test OTC alone and in combination with Streptomycin and Zn at low and neutral pH (using a stabilizing adjuvant) in a commercial citrus grove.

The trial was initiated at a commercial location in SW Florida. Trees are Valencia /X-639 planted in 2017. Baseline data were collected (tree size, canopy volume, canopy health). Leaves were collected for CLas analysis. Trees were trunk-injected in June. There are 10 treatments:

1. Low pH control (acidified water)
2. Neutral pH control (adjuvant)
3. Neutral pH+Zn
4. Low pH OTC
5. Low pH STM (streptomycin)

6. Low pH OTC+ STM
7. Neutral pH OTC
8. Neutral pH OTC+Zn
9. Neutral pH STM
10. Neutral pH OTC+STM

Leaves were collected at 3 days and 1 month after injection. Trees experienced severe phytotoxicity with the neutral pH adjuvant alone (and in combination with OTC and STM). Low pH OTC injections also resulted in phytotoxicity, but less severe than the neutral pH treatments. Addition of Zn to the neutral pH adjuvant reduced phytotoxicity to the level of low pH OTC. No phytotoxicity was observed for low pH STM and the low pH control. Phytotoxicity ratings were conducted for all trees to quantify effects.

A small-tree experiment was started at the SWFREC farm, injecting low pH water and neutral pH adjuvant with and without OTC for destructive sampling after harvest to study the internal injury. The same phytotoxicity was observed after injection (more for the neutral pH treatments and less for the low pH treatments).

Therefore, additional research is being done reformulating the neutral pH adjuvant for scalability and to minimize plant toxicity. For this, several industry-grade ingredients (chloride-free) have been tested for compatibility and performance of dissolving OTC at near-neutral pH.

During the next quarter trunk wound sizes will be measured. Leaves will be collected for CLas and OTC detection. New adjuvant optimizations will be tested for toxicity on young citrus trees using the chemjet injection system. Formulation uptake, visual toxicity, and SPAD measurements will be used to determine the best candidate.

Budget status: The subcontract was finally set up allowing us to start spending funds.

b. Diagnostics

M. Irey 21-002C "Continued Support for the Southern Gardens Diagnostic Laboratory" – 100% Completion of Objectives, based on volume of testing – (June 2024)

This report is for year 3 (July 1, 2023 - May 30, 2024). This project provides HLB testing for researchers, growers, and homeowners. A total of 11,030 samples were run during the year. All samples were plant samples, mostly from research trials. To date, a total of 70,600 samples have been run for the 3 years that have been funded to date. The number of samples for year 3 represents a substantial reduction from the number of samples received in the past (averaging about 25,000/yr). This could be due to the state of the industry or to the perception that PCR analysis does not provide the resolution necessary to discriminate between treatments. As an attempt to provide more applicable data to determine if HLB mitigation treatments were working, SGDL is in the process of adapting and validating an assay developed by Dr. Ping Duan (USDA) to indirectly measure activity of the CLas bacterium. This assay, if validated, will be offered in the future as a means of evaluating treatments (e.g., OTC injection) to see if CLas metabolism and growth is being affected.

During the next quarter SGDL will continue the testing and validation of the assay developed by Dr. Duan to determine it's potential for use as a method to evaluate the effectiveness of HLB mitigation strategies. If it proves to be an effective method, this assay as well as the conventional qPCR and qPCR with copy number determination will be offered to growers and researchers going forward.

Budget – due to the reduction in the number of samples and co-funding by one of the private companies that is utilizing the laboratory, the spending did not exceed the cap that CRDF placed on spending. Although the cost per sample went up (due to the fixed cost of the technicians being spread out over a smaller number of samples), the amount funded by CRDF was approximately 62% of the not-to-exceed cap.

ASIAN CITRUS PSYLLID VECTOR INTERVENTION

a. Asian Citrus Psyllid management

K. Pelz-Stelinski 23-001 "Effects of trunk-injected oxytetracycline on tree infection and health, psyllid pathogenicity, and vector population" – 60% Completion of Objectives (May 2024 quarterly report)

Overall goal and specific objectives. The overall goal in this proposal is to evaluate the utility of trunk-injected OTC for reducing CLas infection and transmission in non-bearing and bearing citrus. In addition, we have begun also investigating the effect of streptomycin.

Objective 1) Quantify CLas reduction and tree health in response to OTC treatment.

Experiment 1.1. Mature Trees. Field trials were established with grower collaborators in groves at Wauchula and Frostproof, Florida.

Treatments were applied to 10-year-old, CLas-infected 'Valencia' trees in Wauchula on March 15, 2024. This is the second consecutive year of treatment; trees were also injected with OTC in March of 2023. Four treatments under evaluation are 1) insecticide control of ACP on productive trees (producing 80-100 fruit / box), 2) insecticides plus addition of trunk injection of OTC on productive trees, 3) insecticides control of ACP on non-productive trees (producing < 80 fruit / box), and 4) insecticides plus addition of trunk injection of OTC on non-productive trees. The insecticide treatment consists of a program that is intended to represent what certain growers are doing currently—4-6 annual sprays of formulations labeled for ACP that are rotated between the following active ingredients: (thiamethoxam, imidacloprid, spinosyn, fenpropathrin, cyantraniliprole, methoxyfenozide, clothianidin, and diflubenzuron). Rectify was injected into mature trees at 50-100mL/tree (8250 ppm solution). Each treatment was applied to four replicate groups of 0.022 ha plots that consisted of four rows, with five trees/row comprising 20 trees per plot.

Treatments at the Frostproof site were applied to 10-year-old, CLas-infected 'Valencia' or Hamlin trees on April 16, 2024. This is the second consecutive year of treatment; trees were also injected with OTC in March of 2023. Four treatments are being evaluated: 1) insecticide control of ACP on Valencia trees, 2) insecticides + addition of trunk injection of OTC (Rectify) on Valencia trees, 3) insecticide control of ACP Hamlin trees, and 4) insecticides + addition of trunk injection of OTC (ReMedium) on Hamlin trees. The insecticide treatment consisted of a program that is intended to represent what many growers are doing currently—4-6 annual sprays of formulations labeled for ACP and were rotated between the following active ingredients: (thiamethoxam, imidacloprid, spinosyn, fenpropathrin, cyantraniliprole, methoxyfenozide, clothianidin, and diflubenzuron). Rectify was injected into mature trees at 50-100 mL/tree (8250 ppm solution), according to the product label. Each treatment was applied to four replicate groups of 0.022 ha plots consisting of four rows, with five trees/row comprising 20 trees per plot. Oxytetracycline (ReMedium) was injected at 50-100 mL/tree (5,500 ppm solution) into mature trees with injectors according to the product label. Each treatment was applied to four replicate groups of 0.022 ha plots that consisted of four rows, with five trees/row comprising 20 trees per plot.

***Tree Infection Sampling*.** Monthly leaf samples are harvested from all treatment plots in both experiments. Four mature leaves are randomly selected from five trees in the second row of each replicate plot at each sample date to assess CLas infection. Collected leaves were placed into plastic bags in the field and transported to the laboratory in coolers with ice, where they were stored at −20ºC for subsequent CLas detection, as described below.

Detection of CLas* in *plants and psyllids. Dual-labeled probes were used to detect CLas in ACP and citrus plants using an ABI 7500 qPCR system (Applied Biosystems, Foster City, CA) in a multiplex TaqMan qPCR assay described in (Li et al. 2006). DNA from insect and plant samples was isolated using the DNeasy blood and tissue or DNeasy plant kits (Qiagen Inc, Valencia, CA), respectively. Las-specific 16S rDNA from psyllid and plant extracts were amplified using probe-primer sets targeting internal control sequences specific to ACP [insect wingless] or plant [cytochrome oxidase] gene regions (Li et al. 2006). DNA amplifications were conducted in 96-well MicroAmp reaction plates (Applied Biosystems). Quantitative PCR reactions consisted of an initial denaturation step of 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60 °C for 60s. Each 96-well plate containing ACP samples included a no template control, a positive control (Las DNA in DNA extractions from ACP), and a negative control (no Las DNA in DNA extractions from ACP).

Tree health. Tree growth was assessed at the beginning of experiments to determine the effect of OTC injection over time at both locations. Tree size measurements (height, canopy, width, and trunk diameter at tree base) are taken from each tree in each treatment replicate. The measurements have been made at the initiation and mid-way through the experiment and will also be made at the end of the study. New leaf growth (flush) is assessed monthly during the growing season by quantifying the number of flushes in an open 0.3m3 cube placed into three random positions per five trees of each replicate.

Monthly collections of leaf samples were successfully made at both locations from January through May 2024 and are currently being processed for CLas infection. Additionally, flush numbers were successfully collected. Tree health measurements were done at time zero and in the middle of the experiment at both locations. This report presents results regarding the effect of OTC on CLas infections, and flush abundance obtained from January to February 2024 in both locations. Analysis of fruit and juice data from the 2023 season was completed during the last quarter.

Fruit Sampling: Fruit sampling at each location occurred November 2023-January2024. The results from injections performed in 2023 indicate that OTC treatments improved fruit and juice yield parameters but had no comparable effect on juice quality.

Wauchula site. At this field site, we are quantifying the effect of Rectify application on tree health and improving tree productivity in both productive and severely declining trees. In January 2024, no differences in CLas abundance were observed among treatments. However, in February 2024, productive and unproductive trees treated with OTC had lower CLas abundance compared to their respective controls with statistically significant reductions in bacterial titer observed at 60-90, 180-210, and 240 days after injection.

Wauchula Tree growth. Monthly flushing counts from January to May 2024 were successfully collected and are currently being processed and analyzed.

Frostproof site. We compared the efficacy of two OTC formulations in reducing CLas infection. Both Rectify and Remedium similarly reduced CLas abundance in trees as compared with their respective controls during the January and February data collections. In both cases, CLas titer was statistically reduced as compared with the controls.

Frostproof Tree growth. Monthly flushing counts from January to May 2024 were successfully collected and are currently being processed and analyzed.

Objective 2) Determine the effect of OTC injection on psyllid population abundance.

Psyllid Population Sampling. Asian citrus psyllid (ACP) adults were sampled in all plots at each location and sampling date using a 22 × 28 cm white plastic sheet placed horizontally and 30 cm underneath a randomly chosen branch. Each branch was struck three times with a 40 cm length of PVC pipe. Adult ACPs falling onto the sheet were quickly counted. All trees per replicate plot were sampled in this manner monthly.

ACP adult populations have been monitored monthly from May 2023 to May 2024 at both locations. Data correspond to 2024 was successfully collected and is currently being processed.

Objective 3) Determine the effect of OTC injection on CLas transmission by ACP.

Field trials were established at a collaborator grove at Babson Park, Florida. In this grove, treatments were applied to 10-year-old, CLas-infected 'Valencia' trees. Treatments were applied on April 16, 2024. This is the second consecutive year of treatment; trees were also injected with OTC in March of 2023. There were four treatments evaluated: 1) Rectify (8,220 ppm), 2) Remedium (5,500 ppm), 3) Fireline (70,000 ppm) (positive control), and 4) Insecticides only (negative control). For all antibiotics, trunk injections were performed using Chemjets. In this location, CLas infection in trees was evaluated by randomly collecting four leaves at time zero, 2, 7, 30, 45, 60, 90, 120, and 150 days after injection. Additionally, 40 leaves per tree (20 top and 20 bottom) were collected from 2 trees in each replicate plot to measure the translocation of treatments at the times mentioned above.

<u>Experiment 3.1. Acquisition assays.</u> Psyllid nymphs, which develop on immature leaf tissue, acquire CLas more efficiently than adults; therefore, acquisition of CLas from OTC-treated infected citrus trees was compared with acquisition from untreated infected trees, using the abovementioned treatments. ACP adults (five female and five male) from uninfected laboratory cultures were on young leaf growth (flush) of treated or control infected trees for oviposition. Each treatment was replicated three times on individual trees. Following oviposition, adults were collected and preserved for CLas detection. Egg clutches were left on trees enclosed in mesh sleeves. After nymphs reached adulthood, psyllids and leaves from test plants were collected. Transmission assays were repeated on the same trees every four months following treatments to determine the influence of treatments on pathogen acquisition over time. The effect of OTC injection on the acquisition of CLas was assessed by comparing the CLas titer in ACP caged on citrus trees before and after treatments and across time.

<u>Experiment 3.2. Inoculation assays.</u> A subsample of 10 ACP per treatment collected from the above trees was transferred to uninfected citrus seedlings in an insect-proof greenhouse. ACP was enclosed on plants for inoculation feeding for 7d. After that, ACP adults were collected for CLas detection using qPCR. Furthermore, leaves will be collected every 30, 45, 60, 90, 120, 150, and 180 days for CLas detection.

Babson Park field site. Leaves were collected to measure CLas infection and OTC translocation from day zero to 180 days after the injections of OTC. This report presents results regarding the effects of OTC treatment on CLas infection 180 and 210 days after injections were performed.

The first replication of sampling for experiments 3.1 and 3.2 was completed in June 2023. A second replication was performed in August 2023 for all experiments, and a third replication was performed in

June 2024. The upcoming report will include results regarding the effectiveness of OTC formulations on CLas transmission (CLas acquisition and inoculation).

CLas infection in trees. At 180 days, trees treated with OTC showed the lowest CLas abundance compared to non-injected trees. Trees injected with Rectify and Remedium exhibited the lowest CLas abundance, followed by Fireline, our standard positive control for OTC trunk injections. A similar effect was observed 210 days after injections were performed, although Rectify treated trees showed the lowest CLas abundance compared to the rest of the treatments. However, regardless of the formulation, OTC-treated trees exhibited statistically lower CLas abundance than control trees over 210 days.

OTC translocation in leaves. Results from our OTC translocation samples showed that OTC was abundant in leaves after 30 days of injection compared to non-injected trees. Then, CLas abundance declined at 60 days and remained low at 90 days. From 90 to 180 days, OTC abundance in leaves was similar to that of non-injected trees.

Objective 4. Compare the effects of OTC versus Streptomycin on CLas infection and tree health.

Field trials were conducted on farm in fruit-bearing, mature citrus plantings. Treatments were applied to 7-10-year-old, CLas-infected 'Valencia' trees on April 16, 2024. The treatments evaluated were: 1) non-injected trees, 2) trunk injection of OTC (Rectify), 3) trunk injection of streptomycin (FireWall) + OTC (FireLine), 4) trunk injection of streptomycin (FireWall), and 5) trunk injection of OTC (FireLine). Each treatment also receives insecticides consisting of a program that is intended to represent what growers are doing currently—4-6 annual sprays of formulations labeled for ACP, and we will rotate between the following possible active ingredients: (thiamethoxam, imidacloprid, spinosyn, fenpropathrin, cyantraniliprole, methoxyfenozide, clothianidin, and diflubenzuron). Oxytetracycline (Rectify) was injected into mature trees according to the product label using ChemJet tree injectors. FireLine and FireWall were injected at 70,000 ppm, as per Roldan et al. (2024, in press). Each treatment were applied to four replicate groups of 0.022 ha plots that consist of 3 rows, with 10 trees/row comprising 30 trees per plot. All sampling (leaves, ACP, fruit, juice), transmission assays, and CLas detection will be conducted according to the methods outlined in the full proposal for objectives 1-3.

Mature Trees. In this report, results regarding tree infection at the onset of the experiment are presented. The first replication of acquisition assays was performed at 30 days after injections were performed, as is the time when OTC is highly available on the leaves. Additionally, ACP populations and flush patterns were collected from Abril to May 2024 and are currently being processed and analyzed.

Tree health. Tree measurements such as circumference at the graft union, treewidth, and height were taken in April 2024 before the injection of antibiotics and are currently being processed and analyzed.

CLas abundance. At time zero, all trees in treatments were CLas positive and showed similar CLas abundance. Data regarding 30 days after injection were collected in May 2024 and are currently being processed and analyzed.

budget spending is constinuing as anticipated, however we are nearing overspend because we hare awaiting funding for year 2 of the project.

There are no potential commercialization products resulting from this research, as this project is evaluating registered and available products.

T. Minter 23-025 "Evaluation of PT 150, PT 159, and TPR 1 for ACP and HLB control in Florida Citrus"

A grove of two-year-old Hamlin was selected as site for the trial. The trial was sampled for PCR on October 5, 2023. These samples were to determine which trees were positive for HLB to be included in the trial. Trees that tested positive were treated with either 25 ml of 100 ppm solution of TPR 1, 25 ml of 5500 ppm solution of OTC, and an untreated control. These treatments were injected on December 21, 2023. The treated trees were covered with IPCs after injection. There were 10 trees in each treatment. Some of the trees covered with IPC's had ACP nymphs later in the trial which would indicate that adult ACP were present in the canopy at time of applying the IPCs.

Samples for PCR analysis were taken on January 16, 2024, February 14, 2024, March 19, 2024, and April 18, 2024. Samples were delivered to US Sugar laboratory for PCR analysis for HLB.

The PCR results for October 5, 2023, January 16, 2024, and February 14, 2024, were essentially the same. The PCR results from March 14, 2024, samples resulted in TPR 1 treatment with two trees negative for HLB and two trees questionable for HLB and most remaining trees in TPR 1 treatment with higher PCR values than the February samples. The March samples for OTC treatment resulted in eight trees being negative for HLB and one tree questionable for HLB. The March samples for the untreated control had ten trees positive for HLB. PCR results from the April 18, 2024, samples resulted in one negative tree and one questionable tree for the TPR 1 treatment. Eight trees were positive for HLB in the TPR 1 treatment. The OTC treatment resulted in three negative trees for HLB and seven positive trees for HLB. All ten trees for the untreated control were positive for HLB. The effect of OTC and TPR 1 on HLB was less on April 18 than March 19, 2024.

DI ratings were recorded at the beginning of the trial on January 4, 2024, and on April 18, 2024(see attached table). Due to the small size of the trees, one rating (0-5) was recorded for each side(east and west)of trees and added together for a DI rating. The average DI ratings on January 4, 2024, were 5.85 for untreated control, 6.25 for TPR 1, and 3.15 for OTC treated trees. On April 18, 2024, the average DI ratings were 6.15 for the untreated control, 4.01 for TPR 1, and 3.15 for the OTC treated trees. The DI ratings for the untreated control had increased, and the DI ratings for the TPR 1 and OTC treatments had decreased during the trial period. The reduction in DI ratings for the TPR 1 and the OTC treatments indicated a reduction in HLB during the trial period.

Results from the PCR analysis and the DI ratings infers that TPR 1 has an positive effect in reducing HLB in citrus trees from an injection application. Higher rates should be evaluated for the control and/or suppression of HLB in citrus from an injection application; the PIs were not successful with their request for a second year of funding.

b. New technologies, e.g., RNAi, attract and kill traps, reflective mulch

S. Santra 22-002 "Management of tree health and huanglongbing disease pressure using advanced Zn formulations" – 90% Completion of Objectives (May 2024 quarterly)

Project objectives this quarter: Valencia fruit was harvested by the end of April in trial site 1. Similar to the last year, 400 ppm of both Fertizink and Nuzink (treatments applied in October 2023, January 2024 and February 2024) increased yield as compared to control (180 Kg of fruit per 3 trees replicate as compared to 120 kg per three-tree replicate, 33% increase) with no significant differences between both compounds and by 17% as compared to trees treated on the same dates with Zn sulfate as the grower standard. Internal quality was not significantly affected this year. Additionally, the fruit juice and rinds have been digested for Zn content (analysis pending). Analysis of auxin content in fruit flavedo continued. After three applications of both compounds, NuZn and FertiZn, auxin increased as in last year by similar levels. Noticeably, trees this year are in worst shape, with very thin canopies, which may explain the lower yields, even though physiologically trees responded to the treatments by increasing endogenous auxin content. Next year's study will be very important as trees with better health after receiving 2 years of OTC injections could serve as a bridge for the gap year.

The greening severity was evaluated in trial site 2 (ridge) for both Valencia and Hamlins (400 ppm and 800 ppm Zn). In this site, we observed a dose dependent effect on the greening severity for both Hamlin and Valencia. Over 80% of trees treated with 800 ppm of NuZn and FertiZn are rated as healthy compared to those treated with 400 ppm, the later rate displayed some symptoms of greening. Nevertheless, NuZn and FertiZn at these rates significantly increased the percentage of healthy rated trees compared to Zn sulfate. The greening evaluation correlates with the yield results. Valencia plot treated with NuZn and FertiZn (800 ppm), demonstrated a yield increase of 25% and 45%, respectively, compared to the untreated control and an increase of 8% and 25% compared to Zn sulfate.

Fruit and juice quality was assessed for Hamlin and Valencia (trial site 2) harvested in Fall 2023. FertiZn at 800ppm performed the best in terms of fruit size and weight in comparison to all other treatments including controls at both rates. None of the treatments have any significant impact on juice quality.

The rain fastness, Zn absorption and in planta mobility were evaluated using Swingle liners. On average NuZn and FertiZn demonstrated a 20% and 130% increase of bound Zn, respectively, over Zn sulfate. These findings correlate with the percentage of ionic to insoluble Zn found in the formulations. A week after treatment, the Zn content in the plants was analyzed. It was found that FertiZn increased the Zn content in roots by 160% compared to Zn sulfate. This might explain the reduction in greening severity and the impact on overall physiological response.

In the next quarter, we will quantify the Zinc content in juice of fruit harvested trial site 1. Additionally, fruit shelf-life data will be analyzed to determine if the treatments possess other benefits. In year 3 trial Zn leaf residue, will be analyzed for field samples. Research findings will be reported through peer-reviewed publications. Budget status: Spending is on track

R. Turgeon 22-020 "Protecting citrus trees from citrus greening with anchored, single-chain antibodies" – 100% completion of objectives (March 2024 quarterly progress report)

1. The goal of this project is to protect citrus from CLas by producing in the phloem anchored, single-chain antibodies that will bind and immobilize the bacteria, allowing the plant to destroy them by natural defense mechanisms.

2. Transgenic Carrizo plants expressing these anchored antibodies were produced in Cornell and send to the Levy lab at Lake Alfred where they are being prepared for HLB assays. The Turgeon has improved their transformation approach by using a stronger promoter and using antibodies attaching to two bacterial sites instead of just one. These constructs will be transformed into grapefruit.

3. In the next quarter, the Levy lab will test whether the transgenic Carrizo prevent CLas movement within the phloem and the Turgeon will transform grapefruit and Valencias with the new agro constructs. Funding for the second year of the project was approved.

CITRUS HOST INTERVENTION

a. Deployment of disease resistant or tolerant citrus rootstocks and scions

U. Albrecht 21-005 "Comparison of field performance of citrus trees on rootstocks propagated by seed, cuttings, and tissue culture" – 100% Completion of Objectives (June 2024 final report)

This project was a continuation of a previous CRDF project (18-028C) in which we collected growth data from field trials in different citrus production areas and assessed root architectural differences during the first three years after planting. Continuation allowed us to gather additional information on the potential influences of the rootstock propagation method on tree performance during the early production years under HLB-endemic growing conditions. The specific objectives were as follows: Objective 1: Assess rootstock propagation method and rootstock cultivar effects on growth, health, and productivity of grafted Valencia trees during the early production years (years 4-6 after planting) in two commercial citrus production environments. Objective 2: Assess rootstock propagation method effect on tree performance, root architectures and uprooting resistance of Valencia trees after 5 years of growth. The project involved two commercial trials, one in Hendry County near Felda (managed by Duda & Sons), and one in Polk County near Fort Meade (managed by Peace River Packing). A third trial was conducted at the SWFREC research farm. The Hendry County trial included four rootstocks (US-802, US-812, US-897, US-942) and the Polk County trial includes three rootstocks (US-802, US-812, US-942) propagated by seed, by cuttings, and by tissue culture. Both trials included additional rootstock cultivars that were propagated from seed, intended to compare the commercial performance of eight rootstocks (Cleopatra, Swingle, X-639, US-1516, US-802, US-812, US-897, US-942) in the two different production environments. Unfortunately, business decisions forced Duda & Sons to reduce their efforts in managing the trial near Felda, resulting in tree decline and poor fruit production. Peace River continued managing the trees according to their regular standards. This included injecting all trees with OTC in year 3 (2023). The third trial at SWFREC, designed to assess root architectures after 5 years of field growth by whole-root system excavation was terminated two years after planting due to heavy HLB pressure from inadequate vector management at that location and resulting in tree decline. However, we conducted a detailed root analysis from two other trials which had been started under the previous project. The results from all these studies showed that trees grafted on cuttings and tissue-culture propagated rootstocks behave similar in terms of tree growth, health and productivity. Although different methods of propagation create different types of root systems (i.e., a tap root system with seed propagation and an adventitious root system with cuttings and tissue culture propagation), these differences disappear soon after field establishment under the current production conditions. Our detailed uprooting studies and root analyses confirm that the rootstock has a larger influence on the root system architecture than the propagation method. Accordingly, in all our studies, in contrast to the propagation method, considerable differences were always found among rootstocks. This reaffirms that the rootstock cultivar has more influence on the performance of a grafted citrus tree than the propagation method. It must also be noted that nursery production methods are variable, especially for tissue culture propagation, as new technologies are available, and protocols are being fine-tuned. Hence, any potential differences among trees may not be due to the propagation method but due to varying production practices. For example, we have seen root deformations in field-ready nursery trees because the young rootstock liners (produced by tissue culture) were kept in the paper pots when they were transplanted to the larger nursery containers. The restrictions of the paper pots lead to J-rooting and circling of the roots in some of those trees, but not the tissue culture production method per se. Poor transplanting practices can also affect root structures and growth considerably. These include leaving trees out in the sun for extended periods before transplanting, adding granular fertilizer in the planting hole, injuring roots when removing the nursery container, and stamping on the roots when filling the hole and compacting the soil. In conclusion, based on all our studies thus far, there does not seem to be any major disadvantage when propagating rootstocks by cuttings or tissue culture as opposed to by seed, provided the young trees are handled properly and received good tree before, during, and after transplant to the field. Our detailed uprooting studies and root analyses conducted in two field trials after two years of field growth confirm that the rootstock has a larger influence on the root system architecture than the propagation method (https://citrusindustry.net/2024/06/05/standing-up-against-hurricanes/).

The reduced tree care of one of the grower collaborators and the inadequate tree care at our center resulted in tree decline in two of our trials. However, we still collected a wealth of data from these trials and the previous trials. It would be desirable to have multiple field trials in place at different, well-managed production sites to assess long-term production trends of trees with differently propagated rootstocks.

K. Bowman 21-008 "Development of Next-Generation SuperSour rootstocks with tolerance to HLB" – 67% Completion of Objectives (April 2024 quarterly)

This three-year project is completing the end of the second year and has been working on two major objectives and are as follows:
Objective 1. Collect field performance data from replicated rootstock field trials and release new rootstock cultivars as justified by superior performance in multiyear field trials.
Collect field performance data. Thirty USDA replicated rootstock trials are active. For February-April 2024, tree survival, cropping and juice data was collected from eight Valencia rootstock trials. Juice analysis of frozen samples was conducted for early trials from the 2023-24 season.
During this quarter, planting of stored seed began on candidate SuperSour rootstocks (SSR) that will be used for morphology and assessment of trueness-to-type from seed for selections tentatively considered for release over the next three years. Additional work on trueness-to-type assessment and salinity tolerance of new rootstocks was also continued. Cooperative work was continued to assess US rootstocks for resistance/tolerance to citrus nematode, sting nematode, and other traits.
Release of new rootstocks. New rootstocks will be officially released by USDA for commercial use when justified by superior performance in multi-year replicated field trials. The most promising USDA rootstock selections are provided to the FDACS-DPI program for shoot tip grafting (STG) and disease testing in advance of potential release. The two newest USDA rootstocks, US SuperSour 4 and US SuperSour 5 released in September 2023, were propagated for planting in upcoming demonstration plantings and field trials. Plans are also being made to include these newest rootstocks in the next phase of CRAFT. Eight other promising new USDA rootstocks are planned to be provided to DPI for STG and testing in 2024, in preparation for possible release in future years.

Objective 2. Create hybrid rootstocks that combine germplasm from parental material with good rootstock traits and HLB tolerance, propagate the most promising of these hybrids, and establish replicated field trials with commercial scions.
Create hybrids. About 230 hybrid seedlings from 2023 high priority crosses are being grown in the greenhouse this quarter for preliminary evaluation and further testing.
Propagate hybrids. About 1,500 trees for two new stage 2 rootstock trials with 35 advanced selection SuperSour rootstocks and Valencia scion continue growing in the nursery, with a plan for field planting in June 2024.
Establish replicated field trials. No new trials were field planted this quarter, but two are planned for planting in the next quarter.
Tree care in trials. Periodic care was applied in the 30 field trials to maintain tree health and productivity and manage weeds. Two young rootstock trials are being grown with IPCs. One rootstock trial is being used for a comparison of tree performance of different rootstocks with and without OTC injection.

For the coming quarter, work will focus on processing brix, acid, and color for about 5,000 juice samples from rootstock trials in the 2023-24 season, continuing propagation of trees for future trials, collection of data on tree size and health in trials, and care of existing trees in trials. Two new Valencia rootstock trials with the advanced SuperSour selections are planned for field planting in the next quarter.
Data analysis of tree performance in the 30 trials is underway, to evaluate SuperSour candidates for official release in 2025-26, and to compare performance among commercially available rootstocks.
Cooperative work continues to evaluate USDA rootstocks for tolerance/resistance to sting nematode, citrus nematode, salinity, cold hardiness, nursery performance, and other traits. Work continues to coordinate with other laboratories in mapping of important rootstock traits that will facilitate more effective citrus breeding in the future.
The budget is on schedule and spent out all second-year funds at the end of the second year of the project. Work is progressing as planned.

R. Shatters 21-025 "Transgenic capable field site to assess HLB-resistant and other improved citrus" – 42% Completion of Objectives (June 2024 quarterly)

The Picos farm test site received routine horticultural care, and trees were exposed to HLB and psyllid vectors to assess their resistance in a real-world environment. All BRS permits were upheld, and the site underwent inspections to ensure compliance with regulatory requirements. In addition, Dr. Zhonglin Mou planted a total of 23 transgenic rootstock lines in the farm on July 24, 2024. These lines include 14 NPR3 RNAi rootstock lines, three NPR3 RNAi rootstock lines top-grafted with Hamlin, and six NPR1 rootstock lines. These transgenic lines were generated through mature tissue transformation. They were planted in the field for fruit/seed production. Dr. Mou's existing transgenics were also assessed. The NPR1 scion lines: 69 trees were planted in May 2019, 98 in May 2021, and 27 in October 2021. Among those planted in 2019, 15 were produced through mature tissue transformation, and 11 survived. These trees have flowered and are producing fruits. Two lines were chosen for propagation. Budsticks from these two lines have been submitted to DPI on July 15, 2024. Leaf and fruit samples were taken from the mature trees on July 26, 2024, for CLas titer assays. One tree from juvenile transformation has flowered this spring and currently has three fruits on the tree. Other juvenile trees have not flowered.
2) Work planned for next quarter:
Next quarter, the team will analyze the data collected from the current field trials to evaluate the efficacy of different HLB-resistant candidates. Researchers will continue to have access to the test site for data collection and analysis purposes. Discussions with Soilcea are continuing as the USDA continues to review contract documents for the planting of CRSPR trees for trial at this site in a true Florida citrus growing region. Upcoming meetings with Dr. Manjul Dut will be held at the end of August to discuss additional plantings at the site and plans for evaluating existing plantings.
3) Budget status: Spending is on track.
4) Please show all potential commercialization products resulting from this research, and the status of each:
The transgenic test site is consistently monitored and researchers are contacted when their population(s) of trees show promising results for commercialization. This research has the potential to yield HLB-resistant transgenic and non-transgenic citrus cultivars suitable for commercial production. Some materials may be immediately suitable for commercial citrus production. The University of Florida has shown increased interest in the commercialization of some transgenic scions planted in the field that show promising single tree data. The data gathered from the field trials will guide recommendations for advancing materials to larger replicated trials, ensuring the development of commercially viable HLB-resistant citrus varieties.

J. Chater 22-011 "Using high-throughput phenotyping to screen germplasm and ongoing field trials for promising citrus accessions in HLB-endemic Florida" – 94% Completion, (May, 2024 report)

The purpose of this directed research project was to 1) to determine if there are selections in current evaluation trials that stand out as selections that could be sufficiently HLB tolerant or resistant to allow the citrus industry to achieve profitability, much as is hoped with the 'Donaldson' selection from the USDA; 2) to identify gaps and redundancies in breeding selections and populations to assist plant breeders in deciding future crosses; and 3) to create data that can be used by researchers to help determine future crosses of breeding parents. The original aim of this project was to provide growers and other stakeholders with **inventories** of accessions and rootstock-scion combinations in trials and other plantings and **lists of top performers.** The previous final report includes these inventories and top performers as requested by stakeholders. This report includes 2023 and 2024 top performers, 23-027 and oxytetracycline findings. This project was extended to reevaluate a limited number of sites flown previously and to further identify candidates for conservation, plant transformation, and breeding. One of the primary reasons for the second year of flights was the potential effects of Hurricane Ian on the data acquired from year 1 flights.

This final report represents a second snapshot of ongoing trials and plantings. A significant number of accessions are only identifiable by a code or number, many are not identifiable by map. This final report was created to provide stakeholders with the requested information as soon as possible. Most of these second drone flights occurred in December 2023. The data sets presented in this interim report were gathered by drone and the metrics reported include two phenotypic traits: 1) **tree size**; and 2) normalized difference vegetation index (**NDVI**). **Tree size** is represented in area in square feet. **NDVI** is multispectral imagery data represented by a unitless value used to quantify vegetation greenness and characterize a plant's biomass. It is reported to be useful in understanding vegetation density and determining status and/or changes in plant health. Hyperspectral and multispectral imaging captures data from across the electromagnetic spectrum instead of just red, green, and blue (RGB), and utilizes a wider range of wavelengths (400-1100 nm, often in steps of 1 nm). These traits were recommended by the drone company for this project.

Tree size often determines age and bearing capacity of citrus, so a cutoff was set at 50 square feet in area with the intent to analyze trees that are of bearing age. Several stakeholders indicated that making decisions on young trees with no fruit would be a mistake and that is why this cutoff was set and recommended by the drone company. The drone company decided that NDVI, which they call 'plant vigor' was a better metric for this task than normalized difference red edge index (NDRE) which is dubbed 'plant health' by the drone company and represents a plant's ability to photosynthesize (and build sugars for pounds-solids in citrus). Outcomes differ greatly between these two different drone metrics determined by multispectral imagery. Commercial groves in a country that does not have the HLB pressure exhibited in Florida were used as comparators to set a cutoff for multispectral imagery. It was determined that a plant vigor (NDVI) value of 0.85 and higher would be the cutoff based on these functional, commercial groves.

For this report, drones were flown over 94,697 citrus trees, representing hundreds of acres of grove material. These flights covered approximately 62 sites comprised of scion and rootstock trials, plantings, commercial groves, breeding populations and germplasm repositories. There were plantings in proximity to each other and many that were in different counties, all in the state of Florida. Genetically modified organisms (GMOs), also known as transgenics, were included in this project. This report represents an effort to present as much information as possible in a relatively short timeframe. There are still grower sites that require ground truthing due to lack of grower availability and maps that do not align with drone images. Please consider that these results are based primarily on drone metrics from a bird's eye view. Just because a drone indicates a selection is healthy does not mean that it is healthy nor will be healthy at all sites and environments under different cultural care. This report does not include fruit quality and yield data, so it is recommended for growers to **NOT** make selections for grove plantings based solely on this document.

Year 2 Site Reports

Each site contains a "site report" that provides top performers that meet the cutoffs described previously (> 50 sq ft and > 0.85 NDVI*). The reports also include comparisons in tree size and tree vigor between the two years. For some IFAS/UF sites, maps are not available to the Plant Improvement Team because the site belongs to another faculty member, but in most cases, the trees did not meet cutoffs for further investigation.

Part 2: Oxytetracycline

Two sites had trees that were injected with oxytetracycline. One site was a sweet orange site (Site A) and one site was focused on fresh market selections (Site B). At the sweet orange site, OLL selections had injected and control trees within the same 10-tree set. At Site B, there were varying numbers of trees selected, often seemingly at random, by the injection crew. For Site A, statistics were straightforward, at Site B, the design was unbalanced, so only selections with enough biological replicates were included in statistical analysis. All data for all selections is included for transparency. Vigor and canopy area were followed from year to year.

Conclusions: Oxytetracycline appears to have an effect in some cases and no effect in others. Seeing that there was evidence for no effect, negative effects, and positive effects of injection, it is good to encourage growers to be careful with injections and consider rootstock and scion interactions and effects. Not injecting all trees multiple years may be a precautionary method to reduce risk. There are many instances where the injected trees appear to be in better health or are growing off better, but these data are only for one year and not too much can be learned from these preliminary results. When taken together, there seems to be an effect on the whole on the fresh market fruit and sweet orange site (Site B), but not for the sweet orange site (Site A), which adds to the confusion. There has been so much variation in what is being reported in commercial groves and there are differing results at these two sites. Site B was rather unbalanced in nature, but there were some selections where there was enough biological replication to run statistics. The most important factors to growers, pounds-solids per box and boxes per acre, are beyond the scope of this report, but there are data on juice quality (and some data on yield) and related findings. That information will be disseminated at a later date in a separate report.

This report represents an attempt to deliver as much trial plant health and tree size data to the stakeholders as possible in the shortest duration of time. Below is an appended list of all top performing selections from the '23/'24 flights as measured by drone using traits recommended by the drone company to evaluate trees of bearing age. Additionally, germplasm block maps are complicated as plantings have occurred continuously for decades. These germplasm blocks will be characterized, and candidate trees will be tagged, documented, propagated, and conserved for industry.

M. Dutt 22-016 "Preliminary Field Trial to Evaluate the Ability of HLB Tolerant Interstocks to Protect Commercial Scions Against HLB" – 95% Completion of Objectives (March 2024 progress report)

Interstock grafted trees are being maintained in the greenhouse and the plants are increasing in size for the next round of grafting, in the early summer.

All rootstock liners have been stick-grafted with the HLB tolerant interstocks and budding with sweet orange and grapefruit scion will commence in the next quarter.

We are on track with the budget spending as funds have been committed for plant propagation.

M. Dutt 22-019 "Understanding the HLB tolerance and reduced fruit drop in Parson Brown and evaluation of other early season sweet oranges" – 50% Completion of Objectives (March 2024 progress report)

`Hamlin', `Roble' and `Parson Brown' sweet orange trees were exposed to psyllid infected with Candidatus Liberibacter asiaticus for over 3 months to established infection then plants were cleaned from the insects. Seven months after exposure, PCR analysis showed infection with a Ct of 25. Roble and Hamlin showed slight symptoms, but Parson Brown showed no symptoms. Currently, biochemical and metabolomic analysis are being carried to investigate specific responses of Parson Brown to the infection in comparison with Hamlin and Roble. This elucidation will be accomplished by mid-June. Biochemical and physical data collected from samples of the abscission zone and peel are being analyzed. Based on the data collected, sampling methods and plans for the upcoming crop are being formulated.
Fruit was collected from a Roble sweet orange grove in Zephyrhills and from several survivor trees from a grove in Lake County have been collected. HLB status of these trees and the biochemical profile of the leaf samples are being analyzed. Preliminary data shows Roble has higher brix compared to Hamlin.
Leaf samples will be collected from the Hamlin survivor trees for detailed biochemical analysis. Also, root samples will be collected for HLB evaluation. data and sample collection on this season's crop starting in May 2024 will be initiated. Spending is on track with the budget.

M. Ritenour 23-003 "Evaluation of Potential HLB Tolerant Grapefruit Rootstock/Scion Combinations in Florida" – 75% completion of Objectives (April 2024 progress report)

The overall goal is to discover which of the seven (7) grapefruit scion and six (6) rootstock combinations planted in 41 blocks perform well under the endemic HLB disease pressure in Florida. The objectives are to 1) measure tree growth (using drone technology) and ratings of canopy thickness, yellowing, and HLB disease symptom severity, and 2) estimate fruit drop and tree yields as they come into production through preharvest fruit counts and measurement of fruit weight, size distribution and fruit quality from representative fruit samples.

In February 2024 we continued with the fruit sampling for sizing and external and internal fruit quality analysis, to calculate fruit yield, plus the estimation of fruit drop by counting fruits on the tree and on the ground. In March and April 2024, we processed and analyzed the data collected in the previous months, and completed collection of disease index ratings, and initiated documentation of tree growth. A program has been developed to extract the data from the drone spreadsheets.

Yield and disease index data were presented to Florida Citrus Show attendees on April 3, 2024.
Preliminary results were presented by Dr. Zambon at the Polytechnic University of Valencia on May 22, 2024, and to the Valencian Grower's Association on the May 23, 2024.
Drone data was collected on May 29, 2024.

Next quarter will comprise of the drone data analysis, continuation of the ground documentation of tree growth and initiating the HLB based on a quadrant-evaluation rating (disease index).
Spending was slightly delayed to allow for the second round of drone flights and maintain personnel until year two funds are available. A no-cost extension was approved. Expenses for the other half of the drone measurements will be paid upon receipt of the invoice, expected early June.

Y. Duan 23-028 “Exploring the HLB control potential of a new citrus-infecting virus, NMV-M/CFL and its expression system” – % Completion of Objectives (quarterly report not received)

J. Chater 23-029 “Consolidation of citrus breeding plant material to vacate space for Stage I and Stage II field trials and to exploit tolerant germplasm for gene editing strategies” – 10% Completion of Objectives (June 2024 quarterly)

This project has two objectives related to the consolidation and conservation of plant material located at various UF CREC locations. The objectives and progress thus far on each objective are as follows:

Objective 1: Destroy declining plant material with no direct use to the citrus industry to make space for Stage I and Stage II trials (Years 1 and 2) at CREC locations. Since project planning and the beginning of the project period, several thousand citrus trees have been pushed and destroyed at various CREC locations, a portion of which is for making space for future Stage I and Stage II trials. Of these several thousand trees, approximately a thousand are slated to be pushed and destroyed since the beginning of the last reporting period. Hundreds of trees and up to 1,000 have been identified as being suitable for conservation, movement with the tree spade (or other means) and sourced for budwood to allow for conservation of those selections for planting in future sites within CREC property. Trees were identified by both drone and by visual inspection. The nursery is aware of the need to collect and propagate uncertified budwood and they have given the go-ahead to transfer budwood immediately to begin propagation. UFarm charges have conserved and saved all materials needed for data mining for tolerant genes and other variants.

Objective 2: Conserve, move, consolidate, and propagate tolerant and resistant citrus trees to a centralized location. (Years 1 and 2). The block where consolidation will take place has been reserved for this purpose. The nursery received the first budwood on Tuesday 8.6.24. When the buds take, efforts will move forward with tree movement with tree spade and other means. Currently, the tree spade has not been delivered to the CREC for future use. However, it has been repaired and waiting for the UF/IFAS payment to be processed before bringing it to the CREC to begin practicing moving trees of the appropriate size.

During the next quarter: once the tree spade is delivered, it will be utilized to begin moving the first trees and transfer budwood to the nursery for propagation. 1,000 bud eyes from 84 accessions are planned to be transferred to the nursery. It is anticipated to remove thousands of additional citrus trees with no immediate value to the citrus industry to continue to make space for Stage I and II trials as well as breeding populations during the next quarter.
Spending on approved budget indicates the project is no longer underspent (29% time spent versus 40% funded expended) according to the UF/IFAS grants tracking system. All funding will be utilized by the end of the project period. The payment has been made to the contractor, and we are on track for spending.

M. Zhonglin 23-030 "Evaluate new transgenic rootstocks for HLB tolerance" – 5% Completion of Objectives (May, 2024 quarterly report)

Objective 1 Evaluate transgenic rootstocks over-expressing AtNPR1 – Ten cuttings of 4-5 inches with at least 2-3 nodes were collected from each of the eight selected AtNPR1 transgenic rootstock lines. All leaves were removed. The cut ends were dipped in rooting hormone powder. The cuttings were planted into potting mix in 10-inch pots and covered with plastic bags. Each pot has five cuttings. The pots were kept in the greenhouse and were sporadically checked for new shoot and root formation.

Objective 2 Characterize transgenic rootstocks expressing CsNPR3-hpRNA – Total RNA was extracted from leaf tissues collected form the CsNPR3-hpRNA transgenic rootstock lines. The RNA was reverse-transcribed into cDNA. Expression of the CsNPR3 gene was analyzed by quantitative PCR. CsGAPDH and CsActin genes were used as the internal controls. Six lines with significantly reduced CsNPR3 expression were selected. As for the AtNPR1 transgenic rootstock lines, ten cuttings with at least 2-3 nodes were collected from each selected line and treated with rooting hormone powder. The cuttings were then planted into potting mix and covered with plastic bags. They were maintained in the greenhouse for rooting.

In the next quarter, water and fertilize the cuttings to promote rooting. Hormone solutions may be used. The budget is on track.

W. Johnson 23-031 "Accelerate Establishment of Stage 2 Citrus Trials to Combat Citrus Greening Disease" – 50% Completion of Objectives, August, 2024

<u>Objectives:</u>
1. Phenotype the top 250 Sweet Orange & Reticulata hybrid accessions from USDA & University of Florida breeding programs. **Complete**
2. Identify the top 50 for a stage 2 replicated trial. **Complete**
Sweet Orange: Hamlin, Valencia, Bahia 48, Laranja Bahianinhase, 10 unreleased accessions from UF Lake Alfred.
Mandarin-Type: Sun Dragon, Sugar Belle, 14 unreleased from USDA, 11 unreleased from Gainesville, 19 unreleased from UF Lake Alfred.
3. Graft new accessions onto X-639, US 812, and Swingle. Budwood was grafted onto X-639 & US 812 in August.
In the next quarter, budwood will be grafted onto Swingle in October.

The budget is on track. Brite Leaf's final invoice will be in 2025.
Commercialization: We requested USDA release FF 1-5-213 and that University of Florida release C4-10-42 and RBA 13-18. These were the top performers for tree health, fruit quality, and juice quality from Objective 1 above. USDA & UF committees are in process of reviewing the request and DPI is working on budwood cleanup. We anticipate release sometime in 2025. Other commercialization products will be revealed during the stage 2 trial in coming years--i.e. 2028-2030.

M. Mattia 23-037 "Fruit Yield and Quality Assessment for Advanced Selections and Drone Re-flight for Hurricane-Affected Sites" – 100% Completion of Objectives (final report)

This project was initiated at the request of citrus growers to measure trees at field sites affected by Hurricane Ian and collect fruit data from the most promising selections for evaluation. The project utilized drones operated by the company Aerobotics, and data on Brix, acid, and color were collected from the most promising selections. The sites for drone re-flight included all field sites south of Interstate 4 and west of US 27. Four sites matched the criteria for evaluation and were re-flown in December 2023. Subsequently, fruit data were collected. The data from the re-flown sites were analyzed, revealing that the hurricane did not impact the top trees at these sites, nor did the ranking of the top individuals change as a result of Hurricane Ian. The top candidates from the sites were assessed and ground-truthed. The technician assigned to the project processed 331 samples for Brix and titratable acidity. The top candidates across locations were the mandarins FF-5-51-2, FF-1-24-48, and US SunDragon. US SunDragon continues to demonstrate good quality across locations and can be transported to juice facilities for use in NFC (Not From Concentrate) orange juice. Additionally, Early Season Donaldson fruit were also evaluated and had an average Brix of 10.5 and a TA (titratable acidity) of 0.6. Donaldson remains an early season orange variety of interest.

The USDA Citrus Scion Breeding Program will continue the momentum of this project as part of the Citrus Breeding Grand Plan, which will fund repeated measures on these trees. This will provide growers with added confidence in newly developed varieties under HLB (Huanglongbing) conditions.

The project was underspent due to a reduction in costs compared to project estimates.

This research has the potential to yield HLB-tolerant citrus cultivars suitable for commercial production. Materials like US SunDragon and Donaldson are already commercially available and may be immediately suitable for commercial citrus production, pending validation by commercial growers.

Z Pang, Silvec Biologics 23-044 A combined approach to reduce CLas and reverse symptoms in orchard trees" – 25% Completion of Objectives (July, 2024 quarterly report)

Objective 3.1: Evaluate the modified SoD2* for enhanced expression levels and antimicrobial activities using the CTV vector. Five CTV constructs (CTV-SoD2*, CTV-Cmpp16-SoD2* CTV-SoD2*-Cmpp16-SoD2*, CTV-p13-SoD2* and CTV-SoD2*-p13) were proposed for testing SoD2* expression levels and antimicrobial activities. This quarter, all five constructs were successfully generated, introduced into Agrobacterium, and inoculated into Nicotiana benthamiana. Systemic infection of all five constructs in N. benthamiana was confirmed via RT-PCR. Western blot (WB) analysis and enzyme-linked immunosorbent assay (ELISA) for SoD2* levels are currently in progress. To prepare for bark-flap inoculation of CTV constructs into citrus, Citrus macrophylla plants are currently being propagated in the greenhouse.

During the next quarter: We will conduct Western blot (WB) analysis and enzyme-linked immunosorbent assay (ELISA) to quantify SoD2* levels in Nicotiana benthamiana and Citrus species. We will also extract virions from CTV positive N. benthamiana plants, followed by bark-flap inoculation of CTV constructs into Citrus macrophylla. Subsequent testing will be performed to confirm CTV infection in citrus. CTV-positive citrus plants will then be propagated for in-house trials to assess their resistance to HLB challenge.

The budget is currently on track, with expenditures proceeding as planned. All necessary resources have been procured on schedule, and project milestones are being met within the allocated budget.

Commercialization status: The CTV vectors expressing modified SoD2* will be potential commercialization products. Silvec has received a permit from USDA to apply its products in all Florida counties and is expecting regulatory approval from EPA in late 2025 which would allow full commercial sales in Florida in 2026.

b. Gene technology: deploying resistance genes, antimicrobial peptides, CRISPR

D. Manker 16-026C "Establishment and application of tools to allow a systematic approach to identify and characterize hits with confirmed in planta HLB activity" – (August 2023 update).

A no-cost extension has allowed for continued work in field, greenhouse and analysis of these activities beyond the original planned end of grant period of August 2023. During this past year, the final thirty-four microbial strain extracts were run through the hairy root assay at Texas A&M by Dr. Mandadi's lab and several strains were found to be active at a concentrated level. The six active strains were fermented, concentrated and extracted to provide both organic and aqueous soluble materials for plant testing in the HLB greenhouse assay at UF.

Work continues in the greenhouse to determine the efficacy of a number of analogs of the lead synthetic H-class of Plant Defense Inducers. For these assays, citrus trees are sprayed several times with PDI candidates before repeated inoculations with HLB infested psyllids. Several candidates have been shown to delay HLB infection for up to six months. Greenhouse work on three-year-old Valencia trees was also carried out to assess control of citrus canker with these PDI compounds. Two PDI candidates were found to give significant protection against canker compared to the untreated control when applied 48 hours before inoculation with Xanthomonas and assessed ten days after inoculation. Other standards including sage oil and Actigard (drench, spray or injection) did not separate from the untreated control in this greenhouse assay.

Results from one small scale field trial initiated in 2023 at UF have demonstrated that foliar applications of a PDI at 3-4 month intervals after OTC injection of 5 year old HLB infected Valencia trees give reduced HLB levels compared to OTC injection alone. Ct values measured at one month after PDI spray showed significantly lower HLB levels compared to OTC and at lower levels than OTC alone (not statistically significant) at 3 and 4 months after application. Trials are continuing with a second OTC injection made in 2024 and ongoing PDI sprays to allow for collecting yield and harvest data. A second trial was set up at the UF site to repeat this work at this scale (three trees and three replications for each treatment).

From the positive results in this first UF trial, two larger scale trails were arranged with Florida Agco in two grower fields with Valencia trees on US812 rootstock. For these trials (9 trees, six replicates), the trial design included comparison of a lead PDI compound at three rates, a commercial PDI (isotianil) at three rates and three integrated programs with a new insecticide (several formulations) from Bayer that will be registered for use on psyllids in citrus in 2025 (active ingredient Spidoxamat). The OTC injections for these trials were made in November 2023 with foliar PDI applications made in March and June 2024. All trees (2 to 3 years old) in the two trials were HLB positive before applications were made and leaf samples are being collected and analyzed by qPCR and metabolomics on an ongoing basis.

Status of PDI chemical class at Bayer:
The two lead compounds in the PDI class were discovered to have different human safety adverse effects in the ongoing toxicity testing for this class. Replacement of the two advanced synthetic plant defense inhibitors has been initiated and one hundred new analogs were synthesized, and multiple studies were initiated to decipher metabolization pattern and identify compounds with favorable regulatory profile. New backups are under investigation in field trials in vegetable crops against bacterial diseases to more quickly validate their performance and select a new potential development

candidate. The UF is also evaluating the performance of the new backups against HLB and citrus canker. Estimates of cost of goods for these compounds have been made showing that they could theoretically be produced for around $10/acre, depending on the final compound selected. The fate of this chemical class will depend on identifying an analog with a clean toxicity profile while still providing efficacy in the field against bacterial diseases in crops.

M. Leslie 20-015C "Vismax™: A novel peptide-based therapeutic for mitigation of citrus diseases, including HLB" – 100% Completion of Objectives (February 2024)

The objective of this project was to determine whether Vismax® treatment promotes resistance to major citrus diseases in addition to HLB, specifically Phytophthora root rot and citrus canker.

The greenhouse work is being done by Dr. Megan Dewdney (U. Florida IFAS CREC). Her team has performed Phytophthora root rot greenhouse cone-tainer and Rhizotron trials to evaluate rates of drench-applied Vismax® in combination with and comparison to Foliar-applied Vismax® for ability to promote resistance to phytophthora root rot in susceptible orange seedlings. Plant measurements and scoring of disease symptoms is complete for the Spring 2023 and Fall 2023 Phytophthora cone-tainer trials, and preliminary analysis indicates that both soil drench and foliar applications of Vismax® improve root health. Dr. Dewdney also conducted a series of citrus canker trials that were initiated to further explore the effects of Vismax® application timing, rate, and method (root drench vs. foliar) on canker disease progression in a susceptible orange variety. Data has been collected and is currently being analyzed.

Vismax® received full US EPA section 3 registration as a biochemical pesticide with a new active ingredient, Flg22-Bt Peptide. A supplemental distribution label was subsequently granted in the state of Florida to Nutrien Ag Solutions under the product name, Aura Citrus, for applications targeting citrus greening/HLB and citrus canker.

N. Wang 21-028 "Generation of non-transgenic HLB-resistant sweet orange varieties using CRISPR-Cas technology" – 83% Completion of Objectives (July 2024 quarterly)

The purpose of this project is to generate non-transgenic HLB resistant Valencia and Hamlin sweet orange plants using CRISPR-Cas technology. In total, six putative S genes were proposed to be edited and two more targets have been added. Constructs needed for CRISPR genome editing are being made. To speed up the process, they developed a selective process using the herbicide chlorsulfuron.

Among the 8 target genes, they continued protoplast editing with 7 more attempts and resulting transformants are under regeneration. They successfully generated non-transgenic lines for at least three of the S genes. In addition, they have generated Ray Ruby embryogenic protoplasts which are being tested for efficacy of transformation, editing, and regeneration.

More and more regenerated shoots have been generated. We will conduct more micrografting which will be reported in the greenhouse. We will propagate and conduct CLas inoculation after we have acquired 6 biological replicates per line for the non-transgenic genome edited plants.

Z. Deng 23-006 "Evaluating Novel Gene-edited Duncan Grapefruit Mutants for Resistance to Huanglongbing (HLB)" – 60% Completion of Objectives (July 2024)

Knocking out disease susceptibility genes (*S* genes) has resulted in broad disease resistance in multiple crops. The bottleneck to using CRISPR for engineering HLB resistance in citrus has been the lack of suitable and validated gene targets. Identifying suitable gene targets has been the most urgent task for achieving HLB resistance through gene editing. We are the first group in the world that have knocked out two S genes, DMR6 and SWEET1, in HLB-susceptible `Duncan' grapefruit and produced *dmr6* and *sweet1* mutants. The objectives of this project are to evaluate the resistance of four `Duncan' mutants to HLB after graft inoculation (Objective 1a) and exposure to infected Asian citrus psyllids (ACP) (Objective 1b,) and to assess potential side effects of these mutations on citrus plant growth and morphology (Objective 2). The overall goal of this project is to determine the effectiveness of editing DMR6 and SWEET1 for engineering HLB resistance in citrus.

Experiment 1 is being conducted to achieve Objective 1a. Four `Duncan' mutants and one control wildtype line were grafted onto HLB-free sour orange rootstock in two batches. The first batch of clonal plants was graft-inoculated with CLas-positive buds or CLas-free buds (mock inoculation) on June 17, 2023, resulting in 29 graft-inoculated plants and 10 mock-inoculated plants. These plants have been grown in a temperature-controlled greenhouse. Data on plant height, trunk diameter below and above graft union, and CLas titer were collected on June 6 (before inoculation), September 21 (3 months post inoculation), and December 21, 2023 (6 months post inoculation). The Ct value of these mutants and wildtype was approximately 40, indicating undetectable CLas in these inoculated plants. This result was unexpected, and it suggested that the graft inoculation did not perform as expected, even though the CLas inoculum source plants continued to test positive, with the Ct value of 27 to 28. Consequently, these plants were re-inoculated in December 2023; tender shoots were fed upon by 10 female and 10 male hot Asian citrus psyllids (ACP) per plant for 10 days, then the ACPs were collected and analyzed for CLas titer. On average, 63% of these ACPs tested CLas-positive. These re-inoculated plants were tested for CLas titer on March 24, 2024 (3 months post re-inoculation, and 9 months post initial inoculation), and June 24, 2024 (6 months post re-inoculation, and 12 months post initial inoculation). In March 2024, CLas was still not detected in the two dmr6 mutants, but it was detected in both sweet1 mutants and wildtype `Duncan' grapefruit plants. The Ct value in one sweet1 mutant plant was as low as 19, indicating high titers of CLas in this plant. Data on plant height, trunk diameter below and above graft union were also collected on March 20, 2024, but they did not show any specific trend of changes. In June 2024, more wildtype `Duncan' plants (5 out of 7, or 71.4%) became CLas-positive and had low Ct values (as low as 18) or high CLas titers. These wildtype plants showed severe yellowing symptoms. The dmr6 mutants shower lower CLas positivity (40.0% or 66.7%) and higher Ct values (31 to 34) or lower CLas titers. One of the dmr6 mutant did not show any significant changes in plant height and trunk diameter; the other dmr6 mutant showed slower trunk diameter increase compared to the wildtype. Two sweet1 mutants had greater trunk diameter increase. These dmr6 and sweet1 plants will be analyzed for Ct positivity in late September 2024. The second batch of clonal plants (total 34, with 4 to 8 per mutant) were propagated onto sour orange on July 24, 2023. These plants have grown to a height of 16 to 49 inches and were repotted into bigger pots on April 11, 2024. These plants were re-inoculated with CLas using hot ACPs on July 15, 2024. The re-inoculated plants are being grown in a temperature-controlled greenhouse.

Experiment 2 and Experiment 3 are being conducted for Objective 1b and 2, respectively. These experiments require 20 clonal plants of similar stem diameter for each mutant or wildtype line. To produce the required clonal plants, 50 to 75 cuttings were taken from each mutant stock plant and stuck into potting mix in early July 2023. The cuttings were rooted under an intermittent misting system in a secure greenhouse for 3 months. For each mutant, six to 37 cuttings have rooted and produced new shoots. To produce additional clonal plants for Objective 1b and 2, another batch of cuttings were taken on November 3, 2023. These cuttings were rooted in Oasis Rootcubes. Now, enough numbers of rooted cuttings have been produced for all four mutants and wildtype `Duncan'. Twelve to 15 rooted cuttings were potted up in late June 2024 and have been grown in a greenhouse at the GCREC to produce tender shoots for ACP-based inoculation. These young plants on average are 7 to 15 inches tall, with some having new lateral shoots. Ten additional rooted cuttings were potted up in late August 2024, and these plants are being grown in a greenhouse at the CREC for plant morphological comparison.

Next quarter: Objective 1a (Experiment 1) -1st batch of inoculated plants: This batch of plants was re-inoculated in December 2023 by exposing them to infected ACP. All plants have been grown in a secure air-conditioned greenhouse. Data on plant height, trunk diameter, and HLB symptoms will be collected in September 2024 (9 months post re-inoculation, 15 months post initial inoculation). Leaves from each plant will be sampled for DNA isolation and qPCR analysis to quantify CLas titer 9 months post re-inoculation or 15 months post initial graft inoculation.
Objective 1a (Experiment 1) - 2nd batch of clonal plants: This batch of plants was repotted into bigger pots on April 11, 2024 and inoculated with CLas by 10 days' feeding of hot ACP on July 15, 2024. These plants are being grown in a temperature-controlled greenhouse. Data on plant growth, trunk diameter, and CLas titer will be collected 3 months post inoculation (i.e., October 2024).

Objective 1b (Experiment 2): These plants have been pushed to grow rapidly for ACP inoculation. Our initial plan was to perform the inoculation in April 2024. However, due to unforeseen ant infestation of the ACPs, this inoculation was re-scheduled to July 2024. Due to extreme high demand for CLas-infected ACPs and shortage of technical support, our collaborating entomologist lab has not been able to provide hot ACPs. We have been actively seeking other sources of hot ACPs, but in vain so far. If reared ACPs are not available, we may be forced to collect ACPs from citrus groves and use them to inoculate the mutant plants. Regardless of the source of the ACPs, the inoculated plants will be grown in a temperature-controlled greenhouse. Data on plant growth, trunk diameter, HLB symptom, and CLas titer will be collected 3 months post inoculation.
Objective 2 (Experiment 3): Non-inoculated mutants have been in containers in a secure greenhouse at the CREC. Plant growth and leaf and shoot morphology of mutants will be monitored closely and compared with the wildtype to determine potential side effects from the edited S genes. These traits include plant height, number of shoots, shoot internode length, trunk diameter, leaf length, leaf width, leaf thickness, petiole length, wing size, midvein size, and chlorophyll content. Data will be collected at two time points, one time point being mid November 2024, and then in mid January 2025. Plants will be given enough space to grow out normally in order to collect the above morphological data.

The total spendings by far add to $59,044.13, about 59.34% of the total budget. This is a little bit below the expected spending (72%) for the project. The primary reason was the difficulty we experienced in the first quarter of this project in propagating the mutants and the wildtype to produce clonal plants for Objective 1b and 2 during the hot summer and fall months and the unexpected ant infestation of the ACP colonies in the third quarter. Without enough numbers of clonal plants and without hot ACPs, the planned CLas inoculation, DNA extraction, qRT-PCR, and horticultural experiments had to be postponed.

c. Gene delivery, e.g., plant transformation technologies, CTV vector

C. El-Mohtar 21-014 "CTV-T36 vectors as a tool to induce efficient flowering in citrus seedlings" – 57% Completion of Objectives (March 2024 Quarterly)

Objective: Induce efficient flowering in citrus seedlings by overexpressing FT3 and knocking out negative regulators of flowering.

Two out of the three constructs infected into citrus where FT3 is replacing p33 were positive by ELISA. It prompted us to check the construct in which the FT3 from Hamlin was replacing the p33 gene for stability by RT-PCR and sequencing. RT-PCR as well as sequencing revealed a stable insertion of the FT3 as replacement of p33. The ELISA positive plants did not flower in the first flush but are starting to push new flush which we are monitoring for flowering.

The double gene constructs failed to infect citrus until the writing of this report.
The transgenic Carrizo lines that flowered have fruited and we plan to isolate seeds from these rooted lines to enable us to infect these lines with more and different combinations CTV-RNAi vectors in the future.
In the next quarter we plan to repeat infection of the double gene constructs into citrus.
We plan to inoculate by grafting the FT3 transgenic Carrizo lines with the different CTV-RNAi constructs targeting negative regulation of flowering.
The project is on budget with 57% completed, and 48% $ remain.

C. Messina 23-034 "Accelerate Establishment of Stage 2 Citrus Trials to Combat Citrus Greening Disease" – % Completion of Objectives (quarterly report not received)

d. Horticultural Practices

D. Kadyampakeni 20-004 "Organic acids compared to conventional acidification for improved nutrient uptake and root physiology" – 100% Completion of Objectives (June 2024 quarterly)

Recent work demonstrated that irrigation and soil acidification can improve the health and productivity of huanglongbing (HLB)-affected citrus. The purpose of this project was to understand the physiological response of HLB-affected roots to soil acidification and to determine if organic acids can effectively acidify the root zone and improve nutrient uptake and tree productivity.
Objective 1. Determine effects of lowered soil pH on Candidatus Liberibacter asiaticus (CLas) populations and root physiology including internal root apoplast and vascular tissue pH.
Objective 2. Field test multiple acidification materials including organic acids for tree response, CLas suppression, nutrient uptake, and root and vascular pH changes.
In the greenhouse study, acidic soils (pH 5.5) stimulated root growth. The field study showed a positive correlation between soil pH and soil calcium (Ca), magnesium (Mg0, zinc (Zn), manganese (Mn), and boron (B), possibly suggesting that the availability of these soil nutrients increased as pH increased. A negative correlation was observed between soil pH and soil iron (Fe), sulfur (S), potassium (K), and phosphorus (P), indicating decreased nutrients in soil solution as pH increased. There was no significant canopy difference between treatments but there were differences through time due to trees deteriorating due to the bacteria or loss caused by natural hazards, especially in hurricane-prone Florida. Results showed no significant difference in fruit yield and fruit quality parameters in all treatments. In both sites, soil pH positively correlated to soluble solids and soluble solids to acid ratio but negatively to titratable acidity.

T. Vashisth 20-011 "Right Leaf Sampling-The first and most critical step to good nutrition program" – 97% Completion of Objectives (June 2024 quarterly)

Objectives: 1. To determine how many leaf nutrient sampling per year are required to effectively capture the tree nutritional status and adjust fertilizer accordingly.
2. To establish the relationship of leaf nutrient concentration with yield, fruit drop, and canopy density
3. To determine how the leaf nutrient (all 14 nutrient) levels change in the tree throughout the year.
4. To evaluate how the leaf age affects the leaf nutrient status.

In this quarter our major focus was on analyzing the data. We have 3 years of data from 4 sites which was collected every quarter. It is an enormous undertaking. Another focus this year has been a small-scale fertilizer trial based on the leaf nutrient results of fruiting and non-fruiting branches.

The major findings so far are:
1. The results from nutrient profile comparison of fruiting and non-fruiting branch show that fruiting branch have severe deficiency of macro nutrients as the fruit matures whereas the micronutrient depletes in non fruiting branch as the season progresses. It is interesting to find that in many countries across the world, nutrient analysis is performed on fruiting branch as the growing fruit is major sink of nutrients. Thus, we believe it is important for Florida citrus industry to know if fertilizing based on nutrients in fruiting leaf can benefit fruit production on HLB-affected trees.

2. Fertilization based on spring emerged leaf nutrient analysis influences yield. We have found that the fertilization based on spring emerged yield improved the yield by more than 20% and reduced the tree canopy decline by 15%. The data also suggests that yield is directly correlated with potassium and boron content of spring emerged 6-9 month old leaves. This suggests that grower should emphasize on potassium and boron fertilization starting in July. Also, preliminary analysis suggests that age of leaf is more critical than time of year. Overall, it is quite obvious from this data that leaf nutrient analysis is a excellent tool available to growers for precision nutrient management.

In the next quarter we will continue data analysis and interpretation, and develop recommendations for nutrient analysis.

The budget is being spent as per the plan where major funds have been used for nutrient analysis.
We have also been able to hire staff for proper analysis and interpretation of data.
Changes in leaf sampling recommendation will occur as a result of this research. We anticipate to have recommendations by December 2024 including whether to sample fruiting or non-fruiting leaves.

A. Schumann 21-024 "Determine optimal timing for application of fertilizer to improve fruit quality and reduce preharvest drop" – 80% Completion of Objectives (June 2024 Quarterly)

The overall goal of the project is to develop fertilization strategies to best match nutrient supply and demand and develop recommendations for optimal nutrient application timing as compared to a simple constant supply, which will improve fruit yield, quality, and reduce fruit drop. The project has two objectives and are stated below.
Objective 1) Test if a reduced N-P-K nutrient supply in the fall is safe for sustaining HLB-affected citrus, and whether it can improve fruit quality to facilitate earlier maturity / harvesting and reduce fruit drop: In this quarter, fertilization treatments were applied (2nd and 3rd application) in the third year of the experiment. Routine leaf sampling was done in May and processed for nutrient analysis. Nutrient data from leaf sampling in March 2024 is described below, and leaf data from May 2024 samples is still pending. Fruit diameter measurements were started with fruit tagging, and dropped fruits counts began after the physiological drop for the 2024 season, and then will be continued to measure every 2 weeks until harvesting. Destructive root sampling in the Hamlin experiment was done in January and scanned using a flatbed scanner to estimate total root length and volume (presented below). Additionally, total root biomass per unit soil area was also calculated.
Fruit growth and drop: These measurements were started after the physiological drop in May. Eight fruits were tagged in each experimental unit to measure the fruit growth recording over time, measured at every 2-week interval until November. Similarly, fruit drop counting from each plot was started after removing physiological drop fruits. Fruit dropped in both Hamlin and Valencia varieties were less in June than May, and fertilizer treatments had no significant effect on fruit drops to date. Fruit drop counting data will be used to calculate the cumulative fruit drop per tree and fruit drop rate.
Leaf nutrient status in March 2024: Leaf sampling was done one month after the first 2024 season fertilization in the experiment. Most of the macronutrients and micronutrients in leaf samples were in the

optimum concentration range in the Hamlin trial except Mn and Zn, which were in the low range. The status of leaf nutrients was not significantly different between fertilizer treatments. This might be due to an almost equal proportion of fertilizer applications in February and efficient nutrient uptake by the Hamlin trees during leaf flushing and bloom growth stages. However, leaf nutrients N, K, Zn, and Fe were deficient in the Valencia trees. Contrasting results might be due to the later ripening stages of the 2023 crop for the Valencia variety, where internal physiology controls nutrient uptake and distribution.
Root dynamics: Root growth measurements for the 2023 season root samples were calculated using Rhizovision® root analysis software, and the total root biomass at two soil depth intervals (topsoil 0-20 cm and subsoil 20-40 cm) was measured. Total root length, total root volume, and root dry biomass per unit soil volume of fibrous (2 mm size) roots were calculated. Total root length, volume, and biomass were significantly higher in topsoil than in subsoil. The measured parameters of fibrous roots were significantly higher than those of coarse roots in surface topsoil except total length of fibrous roots which was higher in subsoil. In contrast, root biomass and volume of coarse roots were measured higher in subsoil (20-40 cm). There were no significant differences in root parameters in response to sigmoidal fertilizer management compared to the control linear fertilizer management treatments in 2023. However, overall root parameters were higher in treatment 3 (100% sigmoidal supply with 25% fall application in August) in subsoil.

Objective 2) Develop an optimized, practical fertilizer timing management profile to boost fruit quality and reduce fruit drop for HLB-affected citrus based in part on the sigmoidal nutrient demand curve defined by four physiological growth phases (0=bloom/fruit set; 1=cell division; 2=cell enlargement; 3=maturation): Fruit growth measurement and drop counting of year 3 were started in June and will be reported in the next quarter. Initial data on fruit drop showed no significant difference in fertilizer management treatments. Leaf nutrient status during March was also not significantly different from fertilizer management treatments; however, suboptimal, or deficient leaf nutrients N, K, Fe, and Zn were recorded in Valencia. Sigmoidal fertilizer management treatments were not significantly affecting root parameters like total root volume, root length, and root biomass compared to the linear fertilizer management (control) treatments.

Work anticipated during the next quarter will include:
Routine lysimeter sampling, leaf sampling, processing and analysis will be ongoing, as will tree size, root growth and fruit drop and growth measurement. Plan for July to September 2024
Fertilization treatment applications (4th or 5th application)
Routine leaf sampling every 2-month interval (July 30, September 30)
Root scanning via Minirhizotron
Trunk size measurement
Routine lysimeter leachate collection
Schedule Aerobatics flight for tree health, vigor, canopy volume, and area estimation

Budget spending rate is slightly below the target by about 15%. This is considered within the normal deviations of budgeting. The departure of a TEAMS employee from our lab has caused an unexpected reduction in salary expenses. We will request a 6-month no cost extension in September in order to complete the collection and analysis of the third-year field data (Hamlin harvest in December, Valencia in February / March 2025).

U. Albrecht 21-032 "Rootstock field trials - CRDF - Phase 3"

This contract provides for assistance from UF with the planning and establishment of rootstock field trials in different commercial groves, with evaluations to include tree size measurements, tree health/canopy assessments, and fruit quality and yield determination. As well, it will provide assistance with leaf sample collection for nutrient and CLas analysis and data interpretation.

U. Albrecht 22-001 "Directed research – Evaluation of different trunk injection devices and oxytetracycline formulations for efficacy against HLB, phytotoxicity, and feasibility" – 95% Completion of Objectives (June 2024 quarterly)

This project is to evaluate different trunk injection devices and oxytetracycline (OTC) formulations for efficacy against HLB, phototoxicity and feasibility. The project has three objectives and are as follows: 1) Test the efficacy of different injection devices, 2) Determine the most effective formulation of OTC, 3) Determine the best month of injection and most appropriate OTC concentration based on tree size. The project consists of 5 trial locations.
Trial 1: SW FL(Duda) - 8-year-old Valencia/Carrizo. (The grower collaborator stopped tree care after year 2 injections, and trees have been declining rapidly. We have therefore terminated our evaluations in this trial.)
Trial 2: SW FL (Graves Bros) - 8-year-old Valencia/Kuharske.
Trial 3: East coast (Graves Bros) - 9-year-old Valencia/sour orange.
Trial 4: East coast (Graves Bros) - 4-year-old Valencia/x639.
Trial 5: Central ridge (King Ranch) - 4-year-old OLL8/x630.

Harvests and fruit quality analyses were completed for trials 3 and 4. In trial 3, it found significant increases in yield and juice quality after OTC injection. Depending on the OTC dose and the method by which it was administered, yields were increased by 74-131% (control: 31 lbs. fruit/tree, injected: 54-72 lbs. fruit /tree) and Brix was increased by 11-20% (control: 9.7, injected: 10.8-11.6). Also improved by OTC injections were the juice color, acid, and the Brix/acid ratio.
Both methods (Chemjet injection + Arbor-OTC and Flexinjec injection + ReMedium) produced similar results. The best juice quality was obtained injecting ReMedium with the Flexinject injector at the high rate (1.1 g OTC/tree), but at the lower rate (0.55 g OTC/tree) two-sided injection using the chemjets produced slightly higher yields than one-sided Flexinject injections.

Results from trial 4 determined a significant difference among the different treatments. Overall, most treatments improved fruit yield and juice quality, with higher OTC doses producing better results. The highest dose (0.75 g/tree) resulted in 52 lbs. of fruit/tree compared to 20-22 lbs. for the non-injected and OTC - foliar spray controls.

OTC was delivered double-sided either using the Treecise system (dose: 0.18g and 0.38 g/tree) or the chemjet injectors (dose: 0.38 g and 0.75 g/tree). The best results for the juice quality were obtained when OTC was injected in August and at the high rate (0.75 g/tree), Brix was 11.3 compared to 8.7 for the controls. The same rate injected in May produced a Brix of only 9.6, but May injection resulted in larger fruit than August injection. The same trend was observed in the previous year.

Some of the data from this project were presented at the Citrus Institute, the Citrus OJ Break, the Florida Citrus Industry Annual Conference, and other venues.

Work anticipated during the next quarter will be the analyzation of data on OTC residue analyses (performed by the NSL lab) that is pending. PCR analyses will still need to be completed as will wounding evaluations. Data will be included for presentation at the Citrus Expo and in an upcoming Citrus Industry magazine article.
Spending on the budget is on track.

F. Alferez 22-003 “Determining best timing for Brassinosteroid (Brs) application to achieve maximum beneficial effects on citrus tree health and fruit yield and quality” – 75% Completion (July 2024 quarterly)

The objectives are: 1. To study the effect of Brs on priming immunity on young, newly planted trees. This will establish how long immune response will last after Br application, so the number of applications can be determined.
Summer flushes in the Br-treated tress are more synchronized than in unsprayed controls. Also, root growth is greater in trees treated with Brs. Canopy density and fruit growth are greater in Br-treated trees than in the controls. In general tree health continues to be improved significantly. Since flushes are more synchronized, Br sprays are targeted to the time of flushing in a set of trees, with the idea of limiting Br application to timing of maximum psyllid pressure.
2. To determine the best time of application (frequency) to achieve maximum protection against pests and disease in newly planted trees. After expression of genes related to SA pathway was determined to be maximum at 30 days, but still significant by 60 days, treatments were made every other month in young trees to see if this frequency of application is sufficient for protection.
3. To determine the effect of Br application on advancing fruit maturation in both Valencia and Hamlin.
A trial was initiated at CPI in Felda using trees that have been injected with OTC and showing signs of canopy recovery but not significant improvement in fruit quality to test if Br application can boost internal quality in the next season. This trial came about after seeing this year that the most effect of the Br effect on increasing Brix on trees was on trees with the best canopies (in plots not treated with OTCs).

Outreach: Susmita Gaire, Ute Albrecht, Ozgur Batuman, Mongi Zekri, Fernando Alferez (2024). Individual Protective Covers (IPCs) Improve Yield and Quality of Citrus Fruit under Endemic Huanglongbing (HLB). Preprints (accepted in Plants, in press) https://doi.org/10.20944/preprints202407.2288.v1 CRDF project 22-003 is acknowledged in the publication, since the use of Brs as a strategy to prolong tree health and improve quality is discussed in the paper.

During the next quarter we will continue to monitor response to Br of juice quality and gene expression as related not only to plant immunity ad also sugar and acid metabolism.
Trees with different response to OTC in terms of fruit quality will be treated with Brs in trials in collaboration with CPI.
Finally, for objectives 1 and 2, a new set of trials: i) concentrate Br treatments in young trees only at the time of flushing and ii) apply Br every other month.

Outreach: September 19. Alferez, F. Evaluating Brassinosteroids to improve citrus fruit quality and tree protection under HLB. On zoom.
This project was approved for 3rd year funding in July. The budget has been reduced to be more cost-efficient in this last year of the project.

T. Wood, CRAFT 22-010 Cycle IV – November, 2023 update

CRAFT received 130 applications representing more than 6,300 acres of potential new Florida citrus plantings. Solid set proposals accounted for 2,561 acres and reset proposals accounted for 3,747 acres. The geographic distribution represented 13 counties and all citrus-producing regions in Florida. Projects focusing on oranges, grapefruit, hybrid, and others were all proposed.
Fifty-seven CRAFT Participation Agreements were executed, providing for participation fees in the first two years of the contracts, which are intended to last for six years from the effective dates. Funds ($1,080-

$4,500 per acre, depending on whether the project is for re-sets or new plantings and the variety planted) will be disbursed with the first installment paid upon execution of the contracts with CRAFT, proof of deposit paid to the citrus nursery for project trees, and submission of the participant's claim form and invoice.
The second payment is payable upon completion of planting according to the contract design, execution of pre-audit and submission of claim form.
Payments three, four and five are payable upon submission of the first 3-month, 6-month and first year's data respectively and submission of claim form. The final payment is payable upon execution of cultivation requirements, submission of the first two years' data and submission of claim form.

A. Levy 22-017 "Improving the Systemic Uptake of Therapeutic Compounds by Trunk Injections" – 30% Completion of Objectives (February 2024 progress report)

Objective 1: Using callose inhibitors to improve systemic uptake and reduce HLB symptoms.
We repeated the field experiment on sweet orange ~8 years old (Citrus x sinensis) trees. We selected and labeled 48 trees according to 4 different treatments: (1) injected control (water), (2) Oxytetracycline (2 g per tree), (3) DDG (0.1mM) + Oxytetracycline (2 g per tree), (4) 3AB (0.1mM) + Oxytetracycline (2 g per tree). Then we located and designed the appropriate map for the experiment. Before applying treatments, baseline stomatal conductance data and data related to trunk diameter, canopy volume and canopy density was collected, and CLas titer and quantify callose level of each tree were measured on September 29th, 2023. The trunk injection was employed (as described in Vincent et al. 2022) on October 3rd, 2023. Following to the scheduled date, we collected eight mature leaf samples per tree to measure the CLas titer and quantify callose level of each tree at 10-day (October 13th, 2023), 1-month (November 1st, 2023) and 3-month (January 3rd, 2023) after treatments. Furthermore, we collected stomata conductance data to assess the treatment impact on leaf health, and also measured the canopy volume and density in pretreatment (April 20th, 2023) and 1st measurement (October 24th, 2023). In order to minimize the variation and difference on applying each treatment, the injection flow rates of each four treatment (see above) were also measured on another extra 24 trees with 4 directional sites (a) north, (b) west, (c) east, and (d) south on October 31st, 2023.

Objective 2: Maintaining water-saturated injection site: Sealed trunk injection ports that stay functional by avoiding wound responses. Field experiments for this objective are ongoing, testing the potential duration of single injection sites. We expect to have results next quarter.

Objective 3: Targeted root delivery. Experiment began in January and is ongoing.

Anticipated work for next quarter: We had a serious problem with the health of the trees we are using. Those trees, while on UF property, were not maintained well. We had some good preliminary results with the 3AB callose inhibitor, and we decided to make new injections in healthier trees, using 3AB and OTC. We will evaluate the trees for callose level and CLas titre bi-monthly starting in May 2024 (5-month after treatment) to evaluate the effect of the callose inhibitor on tree health and CLas infection. We will also measure the fruit drops via monthly data collection and the canopy volume and density via 6-month measurement to determine the effect of injections on tree health.

Potential commercialization products: We are still evaluating the effect of the callose inhibitor in the field trials
At 30% completion, 44% of the budget remains, underspent since it took some time to initiate the work properly.

U. Albrecht 23-002 "Directed Research: Evaluating different rates of oxytetracycline administered by trunk injection in mature sweet orange trees" – 35% Completion of Objectives – project start date 5/1/2023 (July 2024 quarterly report)

Progress this quarter: Objective 1: To compare different rates and annual use patterns of trunk-injected OTC on late-season (Valencia) and early-season (Hamlin) sweet orange tree and their effects on health, yield, and fruit quality over a period of 3 years in a commercial citrus production environment.

Year-2 injections were completed in May for the Hamlins and in June for the Valencias. Tree size and canopy volume data were collected. Leaf samples were collected for CLas analysis.

Objective 2: To compare spring with late summer injections in Valencia orange trees to determine if OTC effects on yield can be enhanced, and the harvest window extended, through timing of injections.

Valencia OTC fruit residue analyses were completed. Fruit from trees injected in June 2023 had residues below the allowed 10 ppb regardless of the rate. Fruit from trees injected in September had higher residues, suggesting that trees should not be injected in September. This year's late summer injections will therefore be conducted in August instead of September.

Next quarter: Preparation is underway for the Valencia August injections. Leaf sampling will be conducted for CLas analysis. Phytophthora trunk rot has been observed in this location. The rot does not seem to be associated with OTC injections; we will conduct measurements/ratings to document this.

Budget status: spending is as expected.

J. Curtis 23-009 "2. Use of Bactericide in Combination with GA and 2,4-D (Plant Growth Regulator)" –

The initial project was delayed due to application of product not in accordance with project protocol, requiring a new site selection. A new site in Avon Park was selected that followed the protocol; the trial was harvested without the knowledge of the PI so there was no resulting quality or yield date. No funds were paid under this project.

J. Curtis 23-011 "5. Alternative Insertion Sites for Bactericides" –

The PI has submitted incomplete fruit quality data on Valencias from the Hyatt field. Additional yield and fruit drop data is needed and has been requested to report on the project.

H. Yonce 23-012 "6. Bactericide Combined with Vismax TM" –

The EPA label for Vismax was approved in December 2023; the treatments have been applied according to label specifications. This Highlands County site was toured recently with the PI; there are no signs of phytotoxicity and the trees are in good shape.

H. Yonce 23-019 "Tank mix 3-day test of 2 products" –

The trial was set up to evaluate the capability of mixed and stored OTC solutions and whether the solution retained efficacy compared to freshly mixed OTC solutions. An additional scope of work was added for data collection at the Alico trial. Yield and fruit quality data was collected by the PI. There were no extraordinary results; the OTC treatments had better quality than the untreated controls. There were no significant differences found in yield, fruit drop, or pound solids per acre.

T. Wood, CRAFT 23-026 Cycle V – June, 2024 update

In the first three quarters of the agreement, the CRAFT Foundation accepted applications for Round Two of the Existing Tree Therapies program as well as Cycle Five of the traditional CRAFT new planting program. After review by CRAFT staff to validate the eligibility of applications, the CRAFT Board of Directors voted to approve all eligible Existing Tree Therapies applications submitted in Round Two, including those rolled over from the Round One waitlist. A total of approximately 920 projects were approved. Approximations are due to consolidation of applications, which may require some revision prior to final execution of agreements. These projects include acreage from all major citrus-growing regions in the state, as well as varieties including oranges, grapefruit, hybrids and others. While the majority of projects are focusing on the injection of oxytetracycline, treatments of gibberellic acid, 2,4-D and brassinosteroids are also included in the approved projects. The Round Two approved projects combine with those previously approved in Round One for a total of more than 1,100 Existing Tree Therapies projects representing approximately 74,000 acres. Participants from both Rounds One and Two have submitted their three-year historical data, first-year treatment data, and 2023-24 harvest data to CRAFT. Many participants have also submitted their second-year treatment data. This data will be added to the CRAFT Data Portal moving forward.

Cycle Five of the traditional new planting program received 175 new applications. An additional 46 applications rolled over from the Cycle Four waitlist for consideration in this cycle as well. A total of 211 applications representing more than 14,000 acres were reviewed and considered for approval by the CRAFT Technical Working Group and Board of Directors. Based upon the projects presented, applications were organized by solid-set and reset and further within subgroups of similar projects for replication and evaluation purposes. The Board of Directors approved a total of 71 new planting projects under CRDF funding for a total of 3,282 acres, including 360 acres utilizing Program for Expedited Propagation (PEP) Materials. These projects, along with approximately 60 others representing more than 6,000 additional acres approved by the CRAFT Board of Directors for Cycle Five under other, federal funding agreements will join the 226 projects representing more than 9,000 acres in Cycles One - Four.

All Participation Agreements were fully executed and returned to CRAFT prior to the June 30, 2024 deadline and planting of the Cycle Five projects has begun.

In addition to the development of projects and execution of contracts, CRAFT staff continued communications and outreach efforts including, a featured presentation at the Florida Citrus Industry Annual Conference, attendance at industry tradeshows and Expos, articles in multiple industry and general publications; updating of the CRAFT website (craftfdn.org); public meetings of the Technical Working Group and Board of Directors; and more. A full list of communications efforts is available upon request.

Existing Tree Therapies projects will continue to report treatments and harvest data for a term of three years to allow for full analysis of the impacts of approved therapies. Following completion of planting, pre-audits of each Cycle Five project will be used to confirm the planting of trees and compliance with contract. Growers will be expected to report production data in real time or at least quarterly in to the USDA-CRAFT Data Portal for a term of six years. The data portal will include a separate entry point for data/ measurements from third party partners such as FDACS and Aerobotics. Data entered by participants and third parties for each project will be visible initially by the growers for their individual projects, and at a later date, on public dashboards developed by CRAFT and USDA-ARS.

H. Yonce 23-048 "Yr. 2 of Bac.Trial.8: Yield Comparison Between Bactericide and Non-treated Control Blocks on Yield and Tree Health" –

There are two sites for this second-year trial: Hamlins in Frostproof, and Valencia at the Hancock groves. Year 2 is progressing as planned; Hamlin data will be collected in December 2024.

e. Alteration of hormonal response to reduce fruit drop, standardize color break, eliminate minor blooms

F. Alferez 21-007 "Reducing fruit drop by altering hormonal responses within the tree through nutritional and hormonal therapies: a mechanistic affordable approach" – 75% Completion of Objectives (July 2024 quarterly)

Objectives: 1. To determine the right timing for Zn and K treatments to minimize fruit drop.
Auxin content was analyzed in treated Valencia and Hamlin fruit. For Hamlin, there is a 3-fold increase in IAA content in both abscission zone and flavedo around October and November, when treatments of Zn+K are performed in late September-early October. This increase is transient but coincides with a decrease in fruit drop. In Valencia, the auxin response following Zn+K treatment is less clear. So far there is an IAA increase around late February.
2. To determine effects of GA3 and 2,4D applications on fruit retention when applied at different times during fruit development.
Now that harvesting is finished, plans are for a new set of treatments and selection of the plots.
3. To develop a strong and proactive outreach program.
-Alferez F. New tools for growing citrus under endemic HLB, Davie UF Extension, Master Gardener Talk Series, June 4, 2024.
-Otavio de Sousa, M., Alferez F. 2024. Fine-tuning zinc and potassium treatments to increase yield in sweet orange FSHS annual meeting, June 2024, Orlando.

During the next quarter: Treatment trees under objectives 1 and 2, will be monitored to characterize tree health, and continue auxin analysis.

3) Budget status: spending continues to be on track.

J. Curtis 22-006 "CRDF Study on Preharvest Fruit Drop Prevention Using Plant Growth Regulators (PGRs)" –

This study tested the hypothesis that Citrus FixTM and ProGibb®, when used alone or in combination, may significantly reduce or prevent preharvest fruit drop, with the products used in conjunction with quality horticultural care. Both products are commercially available and have industry acceptance. There were

three Hamlin sites and three Valencia sites. Nine foliar applied treatments were evaluated plus an untreated control (10 treatments). The 10 treatments were replicated five times with each plot containing seven trees. Evaluations were focused on the center five trees of the seven-tree plot. Treatments began after normal June drop. The project was funded for a second year to further assess six of the treatments and the untreated control after the second year. Brandon Page's summary report outlining the treatments and sites and containing Dr. Cutris's data can be found at the following link:
https://citrusrdf.org/wp-content/uploads/2024/07/Study-on-Preharvest-Fruit-Drop-Prevention-using-PGRs-Yr.-2-July-2024.pdf

OTHER CITRUS DISEASES

a. Lebbeck Mealybug

L. Diepenbrock 20-002C "Developing near and long-term management strategies for Lebbeck mealybug (Nipaecoccus viridis) in Florida citrus" – 50% Completion of Objectives (June 2024 quarterly – 2nd qtr of year 4)

The objective for this project is the development of monitoring tools to time management actions for the control of Lebbeck mealybug (N. viridis) in Florida citrus groves.

In previous reports it has been noted that captures of N. viridis in cardboard band traps (CCBT) are increased in areas of citrus trees that are mechanically injured as compared with similar but uninjured citrus branches. Research has subsequently identified volatiles (γ-terpinene, citronellal, citronellyl acetate, β-E-farnesene, α-humulene, and α-E-E-farnesene) that are upregulated in response to damage and confirmed attraction of N. viridis to these volatiles associated with mechanical damage of citrus. Recently, the project documented increased captures of various life stages of N. viridis in CCBTs baited with a 10 µg/µL concentration of farnesene: ocimene: sabinene blend (in 7:13:17 ratio), as well as those releasing either farnesene or ocimene alone at this same concentration, as compared with the mineral oil (diluent) negative control. These volatiles could be useful for the development of an effective monitoring trap for N. viridis, or other control tools. Current, a goal is to optimize dosage and blend ratio of volatile components to further increase mealybug captures. Studies have been attempting to evaluate traps baited with the farnesene: ocimene: sabinene 3-component blend as well as farnesene or ocimene alone at a series of loading concentrations ranging between the 10 µg/µL shown to be attractive previously as compared with higher concentrations of 100 and 1,000 µg/µL. The study hypothesis is that increasing the loading concentrations of volatiles used bait traps will increase captures of mealybugs on traps in the field. To better understand effectiveness, studies are being conducted under authentic field conditions because it has already been determined which volatiles are involved in attracting the mealybug under laboratory conditions and the investigation has moved toward development of practical tools in the field.

The experiment employs CCBTs that are deployed around three branches and trunks of each replicate infested citrus tree. A designated volatile treatment is enclosed in a release capsule, which is then attached to each CCBT. Mealybug captures on traps are recorded over time to determine if volatile treatments affect captures of mealybugs as compared with unbaited controls. Unfortunately, during two previous attempts in the last quarter to conduct the experiment under field conditions, we were unable to collect reliable mealybug catch data because plots were over sprayed with insecticides for management purposes. These management sprays crashed mealybug populations and prevented us from collecting reliable data. Although studies will continue attempting to conduct the experiment at this same location, where mealybug populations have been historically high, a new site has been identified as a backup that is infested with N. viridis mealybugs and that will not be receiving sprays. Therefore, we are confident that we will be able to conduct the above-described experiment and finish this investigation in the next quarter by evaluating higher loading concentrations of our volatile baits.

Describe the feeding interactions of Lebbeck mealybug with citrus trees. Work is continuing to make progress in collecting data on these feeding interactions and are in the process of analyzing the baseline feeding interactions trials.

Measuring insecticide efficacy and residues on leaves from Citrus Under Protective Screen (CUPS) compared to open groves. For this project we are collaborating with two growers who managed CUPS: CUPS Chesire and CUPS Groveland. The objective of this study is to replicate insecticidal sprays made in CUPS in an open-air plot located at CREC and compare the residual activity of these sprays between the two systems as well as their efficacy in controlling the hibiscus mealybug. For each insecticide application, we followed the protocol below: Since May 2024, we were able to follow two sprays. Growers used their own materials to spray their CUPS while we used a hand-gun sprayer (PCO Skid Sprayer MCCI100K43HR1M, Chemical Containers) with pump pressure set at 200 psi delivering 99.64 gal/acre to spray the open-air plot. The tank was triple rinsed between applications. Leaf samples were collected the day before the spray as well as 1, 3, 5, 7 and 14 DAT after the spray. One set of leaves were frozen on the day of their collection at -20°C and processed for leaf residues through Ultra High-Performance Liquid Chromatography - Mass Spectrometry (UHPLC-MS). Samples were run against standards to construct a five-point linear curve in a concentration range of 0.5-50 ppm, and then against a five-point standard curve in the range of 5-300 ppb. The concentration represented by the curve was then converted back to µg/g leaf tissue using the exact sample weight. The second set of leaves was used to assess mealybug mortality. Leaf petioles were put in a 1.5 ml Eppendorf tube containing deionized water and secured with parafilm. Leaves were placed into individual Petri dishes and ten 2nd to 3rd instar mealybugs were transferred onto the upper surface of the leaves using a camel hair brush. Petri dishes were sealed with Parafilm and held in a growth chamber under controlled conditions (28 ± 2°C; 70 -80% RH and 12:12 (L:D) h photoperiod). Mortality was assessed under a stereomicroscope by gently probing mealybugs with a fine brush on days 1, 3, 5, 7, and 10 after samples were taken from the field. Only leaf samples from spray #1 have been processed for pesticide residues so far. Small concentrations of spirotetramat were detected before the application was done in CUPS probably due to a previous spray of Movento on March 21st. Spirotetramat was not detected in the open-air grove before the spray. In CUPS, concentrations of spirotetramat reached 7 µg/g leaf tissue 1 and 3 days after the application and decreased to 1.7 µg/g leaf tissue 14 days post-treatment. In the open grove, spirotetramat concentration reached 4.1 one day after treatment but almost nothing was detected 3 days after treatment. 14 days after application spirotetramat concentration was 0.76 µg/g leaf tissue. We noticed that spirotetramat concentration is systematically lower in open grove than in CUPS. Further sprays need to be evaluated to confirm this trend.

Survey of natural enemies for N. viridis. The first half of this year's samples for this objective have been collected. So far, zero parasitoids have emerged from sentinel hosts. 628 Hymenoptera specimens (excluding ants) were found in the yellow pan traps and 272 specimens (43%) were identified as potential parasitoids. Chalcidoidea was the second most frequently detected superfamily. This superfamily contains many potential mealybug parasitoids. The specimens will be identified molecularly and morphologically over the coming months. This experiment will be repeated and expanded in the fall.
Rearing of parasitoids of N. viridis. This experiment is still ongoing. We will report our findings in the next quarterly update.

Work anticipated during the upcoming quarter will continue to work on all objectives outlined in this report from the previous quarter.
Spending on the budget status is on track with our allocated budget.
From work conducted within this project, the potential for commercialization of lures may be a reliable indicator of mealybug activity allowing growers to better target populations earlier than using other field survey methods. Potential commercialization products resulting from this research could occur if we are able to develop an attractive lure, that would be a product that can be commercialized but that end point will be outside the timeline of the funding of this project.

b. Phytophthora

M. Dewdney (previously E. Johnson) 19-010 "Determining new cost-benefit guided Phytophthora propagule treatment thresholds for HLB-affected citrus" – 100% Completion

Objective 1. Determine if labelled Phytophthora management maintains efficacy in the field on HLB-affected trees for reducing fibrous root loss and improving yield.
Objective 2. Determine benefit-cost thresholds for Phytophthora treatment on HLB-affected trees.
We evaluated 6 or 7 phytophthora programs (depending on site) in mature, HLB-affected trees located in either southwest Florida (SWF) or south central Florida (SCF). We looked at Hamlin, Valencia, and a mix of Early varieties among the four experiment locations. The programs were were 1) untreated control, 2) three applications potassium phosphite (4.7 liters/ha; ProPhyt, 54% a.i.), 3) oxathiapiprolin (0.7 liters/ha; Orondis, 18.4% a.i.), 4) mefenoxam (0.6 liters/ha; Ridomil Gold, 45.3% a.i.), 5) fluopicolide (0.3 liters/ha; Presidio, 39.5% a.i.), 6) program of mefenoxam, fluopicolide, phosphite, and oxathiapiprolin in that order (same rates as above, referred to as the combination treatment), and 7) oxathiapiproline and mandipropamid (0.6 liters/ha; Orondis Ultra, 2.77 and 23.1%a.i.). Treatment 7 was only applied in the SCF location. The applications were made in April, early June, July, and September to be timed with the root flushes. All treatments were soil applied through irrigation except for the foliar phosphite applications, which were applied via handgun. In each location, root density, phytophthora propagule count, yield, and fruit quality measurements were collected. There was no significant treatment ($P > 0.05$) effect on root density or phytophthora propagule counts in the SWF Valencia or the SCF Early oranges or Valencia. There were significant improvements ($P < 0.05$) in root density and propagule counts compared to the control in the SWF Hamlin. Unfortunately, this did not translate to improved yield in any year. There were no yield improvements or differences among the treatments in the other site either. Furthermore, all the sites were affected by Hurricane Ian and the trees struggled to recover from the damage. There were no significant differences ($P > 0.05$) for Brix, Brix/acid, acid, juice content, or juice color. The exception was the juice content from the fruit of SWF Hamlin, where the untreated control had significantly more juice ($P < 0.05$) than the foliar phosphite applications and the four-product treatment program. These results did not correlate with the yield responses. There were no significant differences in fruit volume from the SWF sites, but the SCF sites both had significant differences ($P < 0.05$). However, the trends were not consistent between the Early oranges and Valencia and did not correlate with the yield results. Based on the results in this study, no consistent measurable effect of treating mature HLB-affected citrus trees could be discerned. This may be because Hurricane Ian damaged the trees and masked any positive outcomes after year 2 in SWF and less than one year in SCF.

The economist was unable to recruit personnel for this project, so the funds were returned and the economic analysis did not occur. No commercialization products resulted from this research project.

Opportunities going forward

If oxytetracycline injections are as truly promising in improving root mass as has been recently alluded to from circumstantial and correlative data, it will be important to learn how to best protect those roots, so they are not lost due to phytophthora diseases just as the trees are starting to recover. We know that roots on trees infected by *Candidatus* Liberibacter asiaticus are more attractive to *Phytophthora* spp. than those of uninfected trees. How does oxytetracycline affect this equation? No one knows. This is a complicated system with two organisms that cause high variability, so teasing out the answer will be challenging. Unfortunately, this project was not able to give me the information I was hoping for to make better recommendations to growers about treating for phytophthora root diseases. While we did not see significant effects on highly declined trees, there is still the concern that if roots were badly affected by phytophthora root rot in high inoculum groves, trees will decline even faster. The case for protecting young trees, particularly under IPCs is much clearer. If there are
sufficient populations of phytophthora propagules, growers should treat because the root systems of the IPC covered trees are much healthier and the trees should be able to respond better to treatment. This was not tested directly in this project, but preliminary data from other projects show that there would be merit in studying the tree establishment and long-term tree health effects of protecting the young trees from damaging levels of phytophthora.

c. Nematodes

L. Duncan 21-013 "Integrated management of sting nematode in newly planted citrus trees" – 75% Completion of Objectives (February 2024 quarterly)

The third mass screen of germplasm for sting nematode tolerance in large sand-filled tanks was completed in January 2024. Sixteen rootstocks were compared by planting four replicates of each in each of 4 tanks (256 seedlings total) - two tanks infested with sting nematode and 2 that were not infested. The trial began in May and ran for 7 months, two months longer than the previous trials, to enhance the nematode pressure. The trial is noteworthy for comparing rootstocks created at USDA and UF to each other and to historical industry standards. Most of the rootstocks have been available for propagation for several years; however, only the "historical industry standards" were available for comparison the last time sting nematode tolerance was evaluated 4 decades ago. An initial trial in this project evaluated UF rootstocks, whereas USDA rootstocks were screened in the second trial. Here, differences among the rootstocks were evaluated for consistency with the previous trials. Nematode populations were evaluated, prior to plant recovery from tanks, finding more than 200 sting nematodes of all stages per 250 cm3 soil in each infested tank, and no nematodes were detected in non-infested tanks. Plants were carefully dug from tanks and photographed after soil was rinsed from roots. They were then separated (top, tap roots, fibrous roots), dried for 72 h at 70oC, and weighed. Roots appeared abnormally thickened when damaged heavily by the nematode which may mask differences if measured exclusively by mass. A rating system (1-10) was used to assign a score to each plant within a rootstock, relative to the best plant(s) (given 10) in the non-treated group of that rootstock. Each rootstock was assigned a tolerance index T, where T= mean rank of 8 infested plants/mean rank of 8 non-infested plants. Three investigators rated the plants independently, the T rankings were highly significantly correlated, and were averaged. Tolerance of the rootstocks was surprisingly similar within 1) those used historically, or 2) produced by USDA or 3) by UF. The four historical standard rootstocks had low T values measured visually or by mass suggesting that most newer releases confer some tolerance to the nematode. While USDA rootstocks tended to have higher Tsubj values than historical standards, this was not the case for Twt. UF rootstocks tended to have the most tolerant lines according to both measures. The results of the trial were consistent with previous results and field observations. Kuharske which is widely used for burrowing nematode control was heavily damaged by sting nematode in a 3-year trial to evaluate cover crops and nematicides. Swingle citrumelo was rated as susceptible to sting nematode in previous surveys. In this and our previous tank trial using

USDA rootstocks, US812 and US942 and US1516 outperformed sweet orange or sour orange. Similarly, in this and the previous trial using UF rootstocks, UF1, UF5 and Orange 16 had the highest Tsubj and Twt scores. There were discrepancies, however, such as LB8-9xS13-15-16 which performed poorly in the first compared to this trial. The very small plant size of LB8-9xS13-15-16 compared to the better performing rootstocks may make relative differences between infested and non-infested plants more difficult to measure accurately.

The rootstock screening trials have provided consistent evidence that newer rootstocks are somewhat more tolerant under these conditions than the previous generation of commonly used rootstocks. The widespread occurrence of sting nematode in Florida may be a factor driving grower adoption of newer rootstocks. This is somewhat encouraging; however, there is no evidence in these trials that an acceptably tolerant citrus cultivar has been identified. We shall continue screening new cultivars with Kuharske and UF5 as standards for intolerance and tolerance, respectively.

d. Greasy Spot

M. Dewdney 21-012 "Evaluating the role of greasy spot and peel disorders in the greasy green defect on citrus fruit" – 69% Completion of Objectives (June 2024 quarterly)

Progress: Objective 1: To determine whether the flush cycle and infection period for Z. citri-griseum have changed due to the influence of HLB on citrus physiology and other factors such as the changing environment, a site located in Fort Pierce was selected based on feedback from growers. In 2022, two blocks with different grapefruit varieties were selected at the site for monitoring. Within each block, two groups of twenty mature grapefruit trees with similar canopy health status were selected and ten flushes per tree were tagged. As was anticipated, there was significant shoot and fruit growth over time in both seasons and for both varieties. In the 2022 season, new shoot production fluctuated greatly, with peaks observed in June and August for white grapefruit and in June and September for red grapefruit. In the 2023 season, new shoots were mostly observed in June, July, and September for both varieties. Fruit assessment revealed that in the 2022 season, fruit size for both varieties started small in May, with white fruit being slightly smaller than the red variety. Both varieties showed a significant increase in size from May to July, maintaining similar sizes with slight variations, and reaching around 8 cm by December. In 2023, a similar increase in fruit size was observed from May to July, with both varieties reaching around 8 cm by October. Our later season data points were not able to be collected because the grower harvested without prior communication in mid-November. They also hedged the red grapefruit trees in the spring, removing a significant proportion of the fruit and foliage.

Preliminary results of epiphytic growth on leaves in the 2022 season showed initiation in June, with peaks in late August and early October. Spore count fluctuated throughout the season, with peaks around August and mid-September, and declined at the end of the season in November. In the 2023 season, epiphytic growth started in May, declined sharply in late July, and peaked again in August and September. The spore count started in May and increased sharply in June, with significant peaks in early August. Another peak was observed in early September, followed by a slight decline in October and November. Overall, there was a close relationship between spore count and epiphytic growth, with peaks in spore count often aligning with increases in epiphytic growth. The 2023 season showed more variability and higher peaks in both spore count and epiphytic growth compared to the 2022 season. These results reflect a subset of samples taken. Data collection on the leaf disk samples began but there are still over 50,000 samples to observe. Trends may change based on what is observed with the full data set.

In the 2022 season, epiphytic growth on fruit was observed in early September, with several peaks: one in mid-September, another significant rise in October, and again in November. Spore counts fluctuated over time, starting in September and peaking around mid-September. In the 2023 season, epiphytic growth was observed in September, with slight increases in mid-September and early October. Overall, the results show more variability in both spore counts and epiphytic growth in 2022, while the 2023 season exhibits more stable trends. Data collection for a third year is ongoing to determine the exact period of epiphytic growth on fruit. Design of specific primers is underway to detect the pathogen on fruit and leaves.

DNA sanger sequencing of the transcription elongation factor (TEF1), long ribosomal subunit (LSU), and the internal transcribed spacer (ITS) regions was performed for a set of putative fungal isolates collected in Florida from leaf litter from grapefruit trees exhibiting greasy spot symptoms. We successfully identified Z. citri-griseum with a high sequence identity percentage (>95%) from a blast search against the NR database at NCBI. From the blast results, a few isolates (6 in total) were identified that corresponded to *Cercospora loranthi*, a co-occurring fungus which is not considered a citrus pathogen (Huang et al., 2015). This work has allowed design of candidate species specific primers. Some primers have been tested but were not adequately specific. More primers are under design and testing.

Objective 2: Efforts continue to meet with growers to compare programs from greasy-green affected and non- or less-affected blocks. The response is that there is not much difference between blocks. Some have indicated a willingness to meet, but the actual dates for those meetings are still pending. The Co-PI has indicated that it has been very challenging to get grower participation, which is unfortunate. The responses would be very helpful in pinpointing what has caused the greasy green challenges.

Objective 3: Fruit were collected for a second season of degreening trials. Because the grower harvested fruit from trial locations where observations of the epiphytic growth on fruit were being collected, the opportunity for further data was lost from these blocks. However, there was a fungicide trial for greasy green that was not funded by this project in the same grove operation. The opportunity to collect some fruit from this trial enabled an evaluation of whether fungicide treatment would change the greasy green outcome. There were no field treatment effects, only between degreened and non-degreened regarding fruit health, but NOT color (except chroma with interaction). Very interesting about no difference in color from the degreening treatment. Color and decay were evaluated 11 days after receiving the fruit and starting the ethylene treatment. The degreening treatment was at 50F for 3 days because of room availability and the fruit didn't appear to warrant degreening at 85F over the weekend. Afterwards, the fruit was at ambient room temperature (~75F). Interesting that the ethylene treatment was sufficient to impact shelf life, but not color development. Take home message is do NOT try degreening greasy green grapefruit at 50F.

During the next quarter we will continue the observation of the epiphytic growth on leaves and fruit. Work on data analysis. Primer design. Continue to collect new fruit for epiphytic growth observations. Look at the relationship between earlier than normal leaf symptoms and current state of epiphytic growth in symptomatic grove.

The budget is underspent since the graduate student was awarded a fellowship that pays for half of the stipend (this project only covered 75% of the plant pathology mandatory stipend) and half tuition.

e. Diaprepes

L. Diepenbrock 22-013 "Getting to the root of the problem: Managing Diaprepes root weevil on trees with HLB" – 50% Completion of Objectives (June 2024 quarterly progress report)

Progress this quarter: Objective 1: Evaluate currently available registered insecticides in Florida citrus against Diaprepes root weevil (DRW). Evaluation continued for the use of Btt against Diaprepes larvae. A laboratory experiment was conducted in preparation for field experiments to determine the effects of concomitant infection of insects by Btt and entomopathogenic nematodes. To optimize conditions, DRW larvae of two ages (6 or 12 wk old) were fed on artificial diet inoculated with either of three different concentrations of Bacillus thuringiensis subsp. tenebrionis (Novodor 3% [AI], [30 mg spores and δ-endotoxin crystals per ml product]) (0, 300, or 3000 ppm) for 10 or 21 days. After Btt feeding exposure, beetles were transferred individually to soil, which was inoculated with the entomopathogenic nematode (EPN), Steinernema riobrave, at a rate of 15 injective juveniles (IJs) /cm2. The mortality of 6- or 12-week-old DRW larvae was recorded after three days of exposure to the EPNs based on symptoms of infection. Cadavers of dead DRW larvae were transferred individually to White traps to confirm infection with EPN IJs by detecting emergence of nematodes from killed larvae.

No mortality of DRW larvae was observed in the control. Mortality of DRW larvae that were pre-exposed to Btt at both concentrations evaluated (75-87%) was higher than that of larvae exposed to diet free of Bt (54%). There was a trend suggesting that larvae exposed at the higher rate of Bt exhibited more specific symptoms of nematode infection than larvae exposed to the lower rate of Bt. EPN infection of DRW larvae exposed to the higher rate of Btt was higher after 10 (89%) than 21 (72%) days of exposure to Btt. Six-week-old larvae were much more susceptible to EPN than 12-week-old larvae, but larvae are difficult to evaluate under in field conditions due to their small size. Therefore, based on the results of this laboratory investigation, 12-week-old DRW larvae will be exposed to Btt at a concentration of 3000 ppm concentration for 10 days in upcoming field trials.

Also, nematode symptoms and the presence of infective juveniles in the White traps indicated that larvae infected with Btt provided better conditions for nematode population growth compared to control insects that were not exposed to Btt. More IJs were produced in larvae exposed to 300 ppm of Btt than 3000 ppm Bt. Overall, the results indicate that exposure of DRW larvae to Btt renders the beetle larvae more susceptible to EPN infection. These results suggest that combining Btt with EPN may be more effective for EPM management than either factor alone. Furthermore, the results suggest that the use of Btt against DRW larvae may render beetle larvae more susceptible to naturally occurring EPN in citrus groves.

Previous research identified a potential trophic cascade affecting the abundance of DRW in groves. Elevated soil pH is necessary for the adherence of bacterial endospores of Paenibacillus sp. JF317562 to the cuticle of the native entomopathogenic nematode Steinernema diaprepesi (El-Bori et al., 2005). Large numbers of spores on its cuticle inhibit the ability of the nematode to move through soil and infect DRW larvae. Therefore, the hypothesis is that reducing the soil pH can reduce the number of spores attached to the nematode and thereby increase the infection of weevil larvae resulting in less damage to trees. To study this possibility, preliminary trials using several Paenibacillus species showed that molecular primer-probe sets provided a means of measuring the Paenibacillus sp. JF317562 and S. diaprepesi in soil samples. However, the specificity of the molecular tools remained unproven for species that were not initially tested. In our present survey 126 amplicon sequence variants (ASV) ascribed to at least 55 species of Paenibacillus were detected in the grove, including Paenibacillus sp. JF317562. Also, more than 50 species of nematodes were detected. The spatial distribution of the Paenibacillus sp. JF317562 ASV was significantly correlated only with those of S. diaprepesi and one other nematode symbiont of gall forming insects. None of the other Paenibacillus ASV were associated with S. diaprepesi. The results confirm that 1) the molecular tool is highly specific for Paenibacillus sp. JF317562 and 2) Paenibacillus sp. JF317562 attaches almost exclusively to S. diaprepesi or perhaps other nematode species associated with insects.

Objective 2. Determine the source of DRW infestation and how their dispersal affects management decisions. DRW adults became active in late June 2024, therefore, data from these studies will be in the next report.

During the next quarter Btt formulations will continue to be evaluated in the lab and in field trials to determine field efficacy. Mark-recapture studies have begun that will be reported on in the next quarterly report.

The budget is underspent in year 1 due to unforeseen challenges with implementing planned work due to delayed DRW field activity. Expenditure of second year funding is expected to be on track.

f. Bulimulus bonariensis snails

L. Diepenbrock 22-014 "Developing management for Bulimulus bonariensis snails in Florida citrus" – 50% Completion of Objectives (June 2024 quarterly progress report)

This project has four stated objectives related to the management of B. bonariensis snail and each objective is individually discussed below.

Objective 1: Document lab and field biology of B. bonariensis in Florida citrus.
Laboratory: We have tested both the protocol in our proposal provided by FDACS-DPI and additional protocols to support development of laboratory populations, however none have proved successful. In most designs, snails will lay eggs, but the juveniles do not survive through development. This suggests that either our assays require significant redesign, snails produce far more offspring than survive in the field, or both.
Field: Field monitoring continues to be performed in 3 commercial sites in central Florida. Peaks in the populations at the sites in both Lake Alfred and Lake Wales can been seen in June 2023 and April in 2024.

Objective 2: Determine factors that influence snail movement/dispersal.
a. Influence of habitat on snail movement. The population in our Polk City site, via trapping, is always far lower than in our other locations, despite snails being present. One noticeable difference between these sites that may explain patterns is the difference in ground cover between sites. In our Polk City location, the grower has tall grasses and flowering forbs throughout the row middles and snails can be found readily in this tall, often moist vegetation. On our other sites, the ground cover is dominated by sand, with the only refuge available for snails to escape the hot ground surface being the tree trunks, irrigation jets, or to burrow into the sand.
b. Influence of physical damage on trees to B. bonariensis attraction. Trials are ongoing to better document the activity of B. bonariensis in relation to physical damage. For these studies, two young Valencia seedlings are potted into the same bin and soil added up to the soil line from the potting media. The trees are then placed into a pop-up mesh cage inside of a tan mesh tent. For the current trial, treatments include (a) no damage to either tree, (b) both trees receive 30 seconds of damage from a metal file, and (c) one tree is damaged as described and the other not damaged. Soil and tree canopies are misted twice daily, and snail location recorded daily for 7 days. Upon initial release, snails immediately move to locations with damage. After a few days, though, more snails were found on the sides of the pop-up cages than on plants or on soil. This suggests that the snails are likely climbing the closest substrate to them as temperatures increase, whether that is a potential food or water source, or just simply a physical barrier. Additional studies will expand upon our current findings to decipher triggers for snail movement.

Objective 3: Field evaluation of baits and exclusion. Baits and insecticides continue to be screened for efficacy against B. bonariensis. At present, the products with 90% or greater mortality include Agri-Mek SC and the baits Deadline GT, Deadline MP, and Slugger Ultra. Agri-Flex induced 70% mortality, while Actara, Admire Pro, and malathion had less than 10% mortality and the water treated control had less than 5% mortality. While copper was previously found to not be effective for excluding B. bonariensis, other deterrents are under evaluation to determine if they could be used to reduce snail activity on unwanted surfaces, like microjets. Initial findings suggest that applications of pepper oil, copper II sulfate, and DEET to surfaces may deter snail movement. This effect persisted over the entirety of the 90-hour observation period.

Objective 4: Determine if B. bonariensis predators exist in groves.
Camera trap data collection for this experiment has been completed. We have recorded approximately 600 hours of footage and will continue to review the videos over the coming weeks. So far, we have not identified any predation events on camera, but it is still early. We have also collected 50 pitfall trap samples. We have been processing and identifying the hundreds of insects contained in the samples to identify potential natural enemies that may be present in citrus groves. These insects are being identified as specifically as possible.

Anticipated work during the next quarter: Objective 1: Document lab and field biology of B. bonariensis in Florida citrus. We are redesigning the laboratory rearing methods and will continue working on these methods. Field: Aboveground populations monitoring will continue until we complete a minimum of 2 years of seasonal pattern data. Additionally, it is believed that this species lays eggs in the fall, therefore we will begin seeking egg clutches from groves in early October using the methods described in our proposal.

Objective 2: Determine factors that influence snail movement/dispersal. a. Influence of habitat on snail movement. During the next quarter, we will perform mark-recapture studies with snails in a minimum of 3 sites with varying ground cover similar to the trapping sites in Objective 1. For these tests, 100 snails will be marked with nail polish (this has no impact on their health or survival and has been used in previous trials). Snails will then be released in one central location within each field. We will observe where snails are at varying timepoints over the course of a week to determine if their movement patterns are based on local abiotic conditions. b. Influence of physical damage on trees to B. bonariensis attraction. The trend described in this report was based on preliminary trials. Wethell will continue to evaluate attraction patterns as snail availability continues.

Objective 3: Field evaluation of baits and exclusion. We are continuing to evaluate available pesticides for activity against B. bonariensis. These assays will continue as snail activity permits. We will continue evaluating deterrents, experimenting with concentrations and other potential test materials, as well as to study the persistence of any deterrent effects under field conditions.

Objective 4: Determine if B. bonariensis predators exist in groves. We are continuing to identify potential snail predators from the pitfall traps. We will analyze the contents of their stomachs to determine whether they have eaten snails. Additionally, we collected 100 snails from the same citrus groves to analyze their stomach contents and determine whether they have consumed citrus, weeds, or other plant material. The snail gut content analysis is scheduled to be completed in the Fall.

The budget is within the FY 2025 projections.
Regarding commercialization of products resulting from this research, it is yet to early to determine but the traps may be useful for timing management actions.

g. Citrus Blight

R. Brlansky 23-021 "Improved Diagnostics and Determination of Triggers for Citrus Blight" – 75% Completion of Objectives (June 2024 quarterly progress report)

Objective 1: The development of an antibody based diagnostic assays. They have developed and used a polyclonal antibody that was made to the sequence of the viral moment protein (MP) to detect CBaPRV. It has been used successfully in dot blot immune assays and Western blot assays but some assays using these methods were unsuccessful. To overcome this problem, an antigen concentration method is under testing before an ELISA can become be a viable test. This this is also a necessary step before a usable lateral flow assay test can be considered.

Objective 2: The goal of this objective is to determine how prevalent inserted copies of viral DNA are in commercial citrus. For objective 2, PCR is used to screen DNA from 42 citrus varieties including 6 different species (orange, lemon, lime, grapefruit, mandarin, tangerine, and tangelo) and including 13 different varieties of rootstock. All tested citrus varieties have inserted CBaPRV DNA, meaning that they are capable of being infected if the proper triggering conditions occur.

Next quarter: Objective 1: To overcome the limit of detection of movement protein, they will use a bead base sample to concentrate the target protein before using the antibody to detect. They have also begun the process of creating a monoclonal antibody to increase the specificity. This will increase the capacity to develop a grower-friendly diagnostic assay to detect CBaPRV.

Objective 2: With the presence of the viral DNA sequence in all citrus chromosomes, it suggests that a CRISPR system is needed for the prevention of viral activation give that it is prevalent in all germplasm tested.
Budget is on track.

h. Citrus Root Weevil

B. Bonning 23-040 "Optimal combination of Bt toxins and gene silencing RNAs for management of citrus root weevil" – 5% of Completion (July 2024 quarterly)

Objective: 1) Screen Bt toxins for activity against Diaprepes root weevil (DRW); 2) Identify the most effective dsRNA constructs against DRW; 3) Assess the combined action of dsRNA and Bt toxins; 4) Assess four Bt transgenic citrus lines for DRW resistance.
Objective 1.

Bacterial Pesticidal Proteins: 11 of some 15 bacteria-derived pesticidal proteins drawn from four different structural groups were expressed in Bonning's lab by use of either E. coli- or Bacillus thuringiensis-based expression systems. Pesticidal proteins were harvested from E. coli or following Bt sporulation, purified and solubilized as required using standard procedures. Cry proteins were trypsin activated. These proteins are now ready for testing in initial bioassays against DRW.

DRW Colony: In Stelinski's lab, a new colony of DRW has been established to generate insects for this project. The culture was initiated from adult weevils collected from citrus groves in central Florida in 2023. Larvae are reared on an artificial diet developed by Beavers using procedures described by Lapointe and Shapiro.

DRW Bioassay: To establish a reliable bioassay protocol for testing of bacterial pesticidal proteins against DRW larvae, soil-column, seedling, and meridic diet bioassay methods were compared using a proxy formulation of B. thuringiensis subsp. tenebrionis (Btt; CX-2330 85% [AI]). Bioassays were conducted to evaluate survival of DRW neonates and 5-week-old larvae after exposure to bacterial suspensions of Btt. While all bioassays indicated activity of the Btt treatment as compared with the control (particularly against neonate larvae), the meridic diet method produced the most consistent results with the least mortality observed in untreated control treatments. Moreover, this method was the easiest to establish, and the least expensive in terms of material costs and time investment. While certain experiments may require use of other bioassay methods or variations thereof, these initial results indicate that the meridic diet bioassay will serve the needs for testing of bacterial pesticidal proteins.

Objective 2. Gene Silencing RNAs: the gene silencing RNAs (dsRNAs) and primers have been designed for all DRW target genes by Killiny, and reagents necessary for dsRNA synthesis are on order.

Plans for the next quarter: Objective 1: Initiate screening of bacterial pesticidal proteins for toxicity against DRW

Objective 4: Conduct bioassays to assess the survival of DRW on transgenic plants that express bacterial pesticidal proteins.

Budget status: Hiring of the postdoctoral researcher for work on Objective 2 in Killiny's lab has been delayed. Otherwise, the project is on track.

L. Duncan 23-041 "Breaking the Diaprepes Life Cycle with Physical Barriers" – 8% of Completion (July 2024 quarterly report)

The trial employs a factorial combination of 8 treatments (all combinations of Valencia trees on Phytophthora-tolerant (US-942) and susceptible (X639) rootstock, fabric mulch or no mulch, covered with IPCs or uncovered) in 8-tree plots and 8 replications, arranged in a randomized complete block design. The rootstocks will demonstrate whether there may be a need for Phytophthora management in addition to weevil control, and the IPCs will reveal the effect of mulch compared to no weevil control (no mulch, no IPC) and complete weevil control (IPC) since IPCs preclude egg-laying in the plots.

The trial was established near Zolfo Springs Florida. The soils across the site are three types of fine sand classified as Adamsville, Tavares, and Ona-Ona. Depth to groundwater is 6-18 inches in the Ona-Ona soil and greater than 18 inches in the other two soils. The site was formerly a citrus grove, heavily infested with Diaprepes root weevil, in which management was discontinued in 2023, and most trees had died by Spring 2024. In April 2024, 11 rows of trees were pushed to accommodate the trial. All examined trees across the site exhibited weevil damage to the roots (Fig. 1). A machine to install the fabric was ordered (Kenco, Ruskin, FL) on 2 May and delivered to the site on 17 June. Soil in the pushed row was disked repeatedly and root debris cleared before plots were measured and fabric installed on the first plot on 7 July (Fig. 2 show machine and rough fabric). An issue of uneven fabric surface was then addressed by repeated rototilling and roller-compacting (Fig. 3) before installing fabric on the remaining plots on 30 July. Tree spacing was measured and marked and an electric cattle brand was used to burn 10-inch-diameter planting holes in the fabric, while a hot-knife was used to make holes for the irrigation stakes (Fig. 4). Trees were planted during 5 days beginning 27 August. The setup will be completed in September by installing fabric covers over the tree planting holes to prevent weed growth, and installing soil moisture sensors to begin documenting variation between covered and uncovered soil. The soil moisture variation will then be minimized between the treatments by switching the emitters in the uncovered plots to deliver more water.
The project concept was introduced to growers in a presentation at the 2024 Citrus Expo.

NOTE: The full progress reports for these projects have been added to the Progress Report Search function of the citrusrdf.org web page.