Citrus Research and Development Foundation, Inc.

CRDF-Funded Projects Summary Progress Report FY 2020-2021
Quarter Ending June 30, 2021

RESEARCH TOPICS COVERED IN THIS REPORT

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1. *CLAS* PATHOGEN INTERVENTION

a. Bactericides/antimicrobials

K. Pelz-Stelinski 18-018 "Disrupting transmission of Candidatus Liberibacter asiaticus with antimicrobial therapy" – 100% Completion of Objectives (May 2021 final reports)

Cumulatively, these data suggest that antimicrobials used for CLas management, particularly oxytetracycline, elicit lethal and sublethal effects in D. citri. We determined that antibiotic use has a negative impact on ACP, therefore there is utility of these applications for ACP management in addition to any effects on Las in trees. ACP feeding on bactericide treated trees could be less capable of transmitting CLas to healthy trees. This can be due to 1) trees treated with bactericides are more likely to have lower CLas titers for acquisition and 2) CLas in infected will be reduced or eliminated when ACP feeds on bactericides, and 3) trees treated with bactericides prior to ACP will be protected from CLas inoculation. Given the limited efficacy of antimicrobial treatments to eliminate CLas completely in infected trees, it appears that their greatest benefit may be due to their negative effects on the ACP vector and CLas transmission.

Major shortcomings, unfinished business:

The project was completed, with no portion of the objectives left unfinished.

Opportunities going forward:

These results indicate that there is a benefit to use of bactericides for preventing CLas transmission. This suggests that any improvements to antimicrobial therapies in citrus may concurrently benefit pest management programs. Furthermore, it indicates that development of antimicrobial strategies targeting the vector are important for reducing transmission of CLas. Going forward, it will be important to identify antimicrobial approaches that are specific to CLas to reduce both new infections, by targeting the vector, and existing infections, in order to disrupt transmission. Antimicrobial approaches that target the vector's internal bacterial communities as well as CLas will be particularly effective for HLB management.

Publications from this project:

This two-year research project was completed at the end of March. Following final analysis of data, results will be submitted for publication. Three publications are currently in preparation describing the work in each objective.

S. Santra 18-020 "Novel multi-metal systemic bactericide for HLB control" – 80% Completion of Objectives (May 2021 quarterly)

The effect of five multi-metal bactericides containing Cu as minor component, Cu-Mg (MM25C75M), Cu-Zn (MM25C75Z), Cu-ZnO (MM20C80Z), Cu-ZnS (MM17C83Z), Cu-Mg-Zn (MM17C17Mg66Zn) and four respective controls without Cu (i.e. coated Mg, Zn, ZnO, ZnS) was evaluated on seedlings to determine metallic uptake, movement and potential change in plant tissues. The rate of foliar application was 1600ppm for Zn and Mg and 400 ppm for Cu. Metal content in seedlings were determined 14hrs after treatment. Residues on the leaves were imaged using scanning electron microscopy. Seedlings were thoroughly washed following a UF-IFAS recommended published protocol and elemental analysis of tissue samples (leaf, root and stem) was performed. Preliminary results showed that Zn and Cu moved systemically to the root system 14 hrs post foliar application. It appears that the treatment moved through phloem vascular system. We have noticed that there is a difference in uptake and mobility of ZnO and ZnS particles within the plant vascular system. Further investigation is needed to understand the rate of uptake and metal movement. We could not reliably differentiate the movement of Mg due to the presence of large amount of Mg in untreated controls (background). Analysis of the infrared spectroscopic signature of the tissues suggested a slight change in composition for some of the formulations, but further investigation is needed to confirm. To determine the application frequency, we have initiated time-based studies. However, we experienced delay in data collection due to seedling unavailability from the CREC greenhouse facility. Additional characterization of all the above industry-grade formulations is being carried out including DLS, Zeta potential, infrared spectroscopy and nanoscale imaging. We have identified certain characterization limitations with the agri-grade material using these tools. We will report our finding in the next reporting period. Our industry collaborator on this project has provided a batch of agriculture-grade NEW Cu product containing 2% Cu for potential registration, which is being investigated within the scope of this project.

Z. He 18-040C "Evaluation of the spatiotemporal dynamics of bactericides within the citrus tree via different application methods" – 100% Completion of Objectives (May 2021 final reports)

The overall goal of this study is to maximize bactericide efficacy by the most efficient delivery methods, with the following objectives:
(1) to compare the delivery efficacy of bactericides with three application methods (foliar spraying, trunk injection, and root administration) based on the uptake and dynamic movement/distribution of the bactericide within the citrus tree.
(2) to clarify the systemic movement and transportation mechanisms of bactericides within the phloem of citrus tree,
(3) to investigate the effects of citrus variety and age on the delivery efficacy of bactericides.
Major accomplishments per objective:
Greenhouse and field trials had been completed during the period from December 2018 to May 2021 to investigate the spatiotemporal dynamics of two bactericides, oxytetracycline and streptomycin, within different plants of grapefruit and Valencia varieties via three different application methods, i.e. foliage spraying, tree trunk injection, and root administration.
The results are summarized as follows:
Foliage spraying provided the highest delivery efficiency of the two bactericides into the leaves, stems, roots, and fruits of the citrus plants for both grapefruit and Valencia varieties,

in the field. Tree trunk injection was the second effective method for delivering these two bactericides into the citrus plants in the field. Root administration was the poorest method for delivering oxytetracycline and streptomycin into citrus plants in the field. There was no oxytetracycline and streptomycin detected in all the leaf samples collected at day 7, day 15, and day 30 during the test.

Analysis of the results obtained from the tests showed that the uptake and dynamic movement/distribution of the two bactericides applied by all the three methods within the 3 trees were significantly affected by the total absorption-area/tissue-surface contacting bactericides during the application period, and the distance between the tissues that bactericides were transferred to and the tissues where bactericides were applied to

C. Vincent 19-023 "Which commercial adjuvants achieve systemic delivery of antimicrobials?" - 90% Completion of Objectives (Jan. 2021 quarterly)

The project has been delayed due to Covid related delay in receiving materials to complete the study. Supplies of oxytetracylcine and streptomycin have been received for the final two experiment and are now in process and should be completed in the coming weeks.

A no cost extension had been approved earlier and the project is now back on schedule to be completed prior to April, 2021. With the no cost extension the project should be able to be completed within the extension period.

U. Albrecht 19-027 "Large-scale testing of the endophytic bacterium Frateuria defendens, a potential biocontrol agent of HLB" – 75% Completion of Objectives (June 2021 quarterly)

The overall goal of the proposed research is to test the effectiveness of Frateuria defendens (Frd) in reducing HLB damage in FL citrus. The original proposal was revised for a 1-year feasibility study.

Objective 1. Determine the optimal application method of Frd to citrus trees.

A spray experiment that was initiated in Dec 2020 continues and horticultural and plant health assessment, CLas and Frd titer determination are being collected bimonthly. The last dataset collected (April) suggested a positive but weak effect of weekly foliar sprays of Frd on the number of leaves and internodes and a slight reduction of CLas titers compared to the plants sprayed biweekly and non-sprayed controls.

Experimentation continues regarding viability/survival of Frd in citrus tissues. Results indicate that an organisms or compound that is associated with CLas, but not CLas itself, inhibits Frd growth in CLas infected leaves.

Because of the growth promoting effects of Frd observed in the first experiment, a follow-up experiment was initiated to test the hypothesis that Frd influences the hormonal balance in citrus plants. For this experiment Frd was applied by leaf infiltration. Plant growth is evaluated on a bimonthly basis.

An experiment has also been initiated to assess Frd efficacy against CLas when delivered by stem injection. For this experiment Frd is compared against oxytetracycline.

Objective 2. Determine if Frd pre-treatment can prevent trees from getting infected with Candidatus Liberibacter asiaticus (CLas).

So far, efficacy of Frd applications (foliar or injected) in restoring health of HLB affected plants was not found to be strong enough to justify initiation of this experiment.

Plans for next quarter:

The foliar spray, leaf infiltration, and stem injection experiments will continue with collection of horticultural data, and CLas and Frd titer determination.

b. Diagnostics

M. Irey 19-001C "Continued Support for the Southern Gardens Diagnostic Laboratory"
127% Completion of Objectives, based on volume of testing (June 2021 final reports)

This report is the final report for project 19-001C which is for continued support of the Southern Gardens Diagnostic Laboratory that provides testing for researchers, growers, and homeowners. The current report summarizes the results from the last quarter of year 2 and an overall summary for the project. For the 4th quarter of year 2, a total of 8,648 samples were run. All samples were from plants (either leaves or roots). This brings the total for the project to date to 57,198 which is 12,198 samples over the 45,000 samples that were originally budgeted for the project (127.1%). As has been mentioned previously, there is a growing tendency for sample submitters to request copy number determination instead of just a Ct value (57%, 52%, and 59% for the quarters 2, 3, and 4 of year 2). It is expected that this trend will continue.

Next quarter: This is the final report for the project. However, the testing service project has been renewed as 21-002C. The new project is based on a sample volume of 28,750 a year (57,500 over 2 years). The increase in volume brings the expected sample volume up to the level from the previous project. It is expected that this high sample volume will continue due to the CRAFT project coming online as well as the CRDF rootstock and scion trials.

Budget status :
The original funding was based on 45,000 samples over 2 years. The actual number of samples run was 57,198 samples. SGC has requested additional funding of CRDF to cover the additional cost of expendables (of which SGC covers 1/3 of the costs) for the increased number of samples (12,198 samples over) over the budgeted amount of the project. No request is being made for salaries despite the increased number of samples run. This is the process that has been used in previous

funding cycles where the budget is reconciled either up or down based on comparison of actuals versus budgeted amount of samples.

2. ASIAN CITRUS PSYLLID VECTOR INTERVENTION

a. **Asian Citrus Psyllid management**

L. Stelinski 19-002 "Why spray if you don't need to? Putting the IPM back into citrus IPM by ground truthing spray thresholds" – 75% Completion of Objectives (May 2021 quarterly)

The objective of this study is to determine the effect of incorporating action thresholds for Asian citrus psyllid (ACP) on populations of natural enemies as well as secondary pests of citrus. ACP vector control is a basic component of HLB management even in situations where disease incidence is high. The use of action thresholds is a possible strategy to reduce unnecessary insecticide input to improve economic return and reduce potential for insecticide resistance. The idea is to spray only when one reaches a population level of ACP that cause economic damage rather than spraying for ACP all the time. It is suspected that an ancillary benefit of using action thresholds will be to reduce the negative impact of insecticides on populations of natural enemies and/or reduce secondary pest infestations because we spray less for ACP control. This may provide further benefit by improving activity of biological control. While the main goal of this investigation is to establish economic thresholds for ACP control to optimize returns on investment, we investigated this ancillary question of whether reducing insecticide input for ACP control may affect populations of beneficial natural enemies in citrus groves.

A year-long study was initiated on March 5, 2021, in an experimental sweet orange grove under standard cultural practices located in Lake Alfred and with an estimated initial HLB infection level of 100%. Prior to the start of the experiment, the entire site was sprayed with pyrethroid. Four replicates were established for each threshold treatment. Insecticide sprays for ACP were based on nominal thresholds of 0.2, 0.5, or 1.0 adult per stem tap and ACP treatment sprays were made of different insecticide modes of action in rotations. There were seven applications for the 0.2 adults per tap threshold treatment, five applications for the 0.5 adults per tap threshold treatment, and two applications for the 1.0 adult per tap threshold treatment. Also, a calendar based positive control treatment was established where ACP sprays were applied once per month, irrespective of ACP population density. ACP were monitored by calculating the mean number of adults per tap from ten randomly selected trees across all four plots for each treatment. Tap samples were made by holding a 22 × 28 cm plastic laminated white paper sheet horizontally underneath a randomly chosen branch, which was then struck sharply three times with a 40 cm length of PVC pipe. ACP adults falling on the sheet were quickly counted to obtain the number of ACP adults per stem-tap and tree. If the means reached or exceeded the target economic threshold, all replicate plots assigned to that treatment threshold were sprayed. ACP sampling occurred every 7 to 15 d.

Populations of ACP and natural enemies were assessed every week from March 19 to April 28, 2021, using the stem tap sample described above. Spiders (Araneae), arboreal ants (Hymnoptera: Formicidae), lady beetles (Coleoptera: Coccinellidae) and lacewings (Neuroptera) previously identified as key natural enemies of ACP or other important citrus pests were counted. Counts were made by visually inspecting randomly selected tree branches in each plot for 2 minutes per sampling period and counting all arthropods found during that period. Inspections were conducted weekly. Arthropods were identified to species level where possible. Ants were collected with pointed round paint brushes and preserved in 70% ethanol and identifications were made subsequently.

Cumulative numbers for ACP were 156, 129, 143 and 135 for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. There were 8, 11, 8 and 4 spiders found for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. We counted 25, 50, 41, and 51 arboreal ants for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. There were 3, 1, 0, and 0 lacewings for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. There were 10, 16, 16, and 22 ladybird beetles for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. Finally, there were 1, 4, 2, and 1 citrus root weevils for the 0.2, 0.5, and 1.0 psyllid per tap, and calendar treatments, respectively. This experiment will run throughout the remainder of the summer. At this early point in the experiment, we have not yet observed an effect of reducing sprays by employing treatment thresholds on populations of natural enemies as compared with plots treated with calendar sprays. However, we predict that differences may emerge as the investigation progresses, based on our previous experience. The possible improvement in the effect of biological control as a consequence of using treatment thresholds for timing sprays against ACP may prove to be a means of integrating insecticides with biological control for psyllid management.

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

3. CITRUS HOST INTERVENTION

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

K. Bowman 18-004 "Development of SuperSour and other outstanding rootstocks with tolerance to HLB" – 85% completion of objectives (April 2021 quarterly)

This project is involved in creating new rootstocks to be used in numerous trials to determine performance in Florida conditions and where HLB is present.

Create new candidate hybrids. This spring focused on growing out previous crosses, selecting among hybrids, establishing source trees, propagating trees for additional trials, and collecting data from existing trials. Additional crosses among the best parental material are planned for next spring, as resources are available.

Propagate and plant new field trials. Budwood increase trees of selected scions were grown, in preparation for budding trees for new rootstock trials. Trees for Stage 2 rootstock trials with Valencia and Hamlin on selected released rootstocks and the best of the next generation hybrids are being grown in the greenhouse in preparation for field planting in 2021. Nursery trees for two new Stage 1 trials with 60 new rootstocks and Valencia orange were propagated in the greenhouse in preparation for field planting in fall 2021. Some planned propagation for new trials was delayed because of USDA institutional Coronavirus shutdown.

Collect data from field trials. Extensive information on tree performance is collected from established field trials, and includes measurement of tree size, fruit crop, fruit quality, and pathogen titer, HLB symptoms, and assessments of tree health. Cropping data is collected during the time of scion harvest, and during this quarter data on yield and fruit quality from nine replicated trials with Valencia scion. Early fruit drop data was also collected from selected Valencia scion trials. Assessments of tree health and measurements of tree size were completed on 23 trials during this quarter. Progress continued in working through the backlog of brix, acid, and color for the fruit quality analysis of last season fruit quality assessments caused by institutional Coronavirus restrictions.

Evaluate effectiveness for seed propagation of new rootstocks and develop seed sources. Some of the newest hybrid rootstocks can be uniformly propagated by seed, but others cannot. As the best rootstocks are identified through testing, seed sources are established and used to determine trueness-to-type from seed. Studies were continued this quarter to evaluate seed propagation for 25 of the most promising SuperSour hybrid rootstocks (SSR). SSR analysis of progeny is progressing more slowly than planned because of institutional Coronavirus shutdown and restrictions. Cooperative work continues to compare field performance of rootstocks propagated by seed, cuttings, and tissue culture.

Posting field trial results for grower access. The USDA rootstock trials produce large amounts of information that is useful to identify the most promising of the new hybrids, as well as comparative information on the relative performance of many commercially available rootstocks. During this quarter, updated trial summaries were prepared for uploading to the website https://www.citrusrootstocks.org/.

Release of superior new rootstocks for commercial use. Release of new USDA rootstocks is based on robust data from multiple trees in replicated field trials over multiple years, including information on tree survival and health, canopy size, fruit yield and fruit quality, and observations on tolerance of disease and other biotic and abiotic threats. Several of the 350 advanced SuperSour rootstock hybrids in field trials are exhibiting outstanding performance in comparison with the commercial standard rootstocks. Information on some of the most promising of the next-generation USDA hybrid rootstocks was provided to CRDF for consideration as candidates for the next set of CRDF Stage 3 trials. Performance data continues to be collected, but it is anticipated that 2-3 of the most outstanding of these will be officially released in 2022-23.prepared in the greenhouse for field planting in 2021.

F. Gmitter 18-011 "Part A - The UF/CREC Core Citrus Improvement Program"
75% Completion of Objectives (April 2021 quarterly)

This project deals multiple objectives related to the citrus improvement program located at UF/CREC in Lake Alfred.

1. Develop new rootstocks that impart HLB-tolerance to scion cultivars. Seeds were extracted from 27 candidate rootstock seed source trees, including several UFRs and other experimental rootstocks, that were found to be free of seed transmissible pathogens by DPI testing. We are authorized to distribute seeds to licensed nurseries for the UFRs, and to collaborative nurseries and other organizations within Florida, in the US, and globally. New trials will be established with these rootstocks in Australia (New South Wales and Queensland) and South Africa in the next year. A rootstock trial with previously exported rootstocks was propagated in Sicily this spring. These trials will provide information relating to performance under diverse soil and environmental conditions and can help development of recommendations for Florida conditions. More than 3,000 flowers were pollinated in twenty-one new rootstock cross combinations of 13 different parents, to recombine multiple useful traits for rootstock improvement. We updated and added new data to existing rootstock trial files and added new files to our website (https://crec.ifas.ufl.edu/citrus-research/rootstock-trials/); currently there is information from 26 locations. Seedlings are being selected from 2020 diploid and tetraploid rootstock crosses in calcareous, high pH soil inoculated with two species of Phytophthora, the first step of the `gauntlet' screening. More than 50 gauntlet rootstock candidates in citripots are growing off for grafting of replicate trees as necessary to meet the new CRDF guidelines for Stage 1 rootstock evaluations (approximately 500 liners).

2. Develop new HLB-tolerant scion cultivars from sweet orange germplasm, as well as other important fruit types such as grapefruit, mandarins, and acid fruit. Shoots generated from 37 interploid crosses made using selected HLB tolerant plants in 2020, including 10 crosses for red grapefruit improvement, 10 for sweet orange improvement, and 10 for mandarin improvement are in the process of micrografting on to 3 different rootstocks, and some have been already moved to the greenhouse. Seedlings from somaclone seedling-derived populations of early maturing (January), high soluble solids OLL selections were planted, to screen for even earlier maturing OLL types; seedling populations of a nearly seedless HLB-tolerant mandarin were also planted to get a completely seedless selection, and to extend the maturity season. A September-maturing pink grapefruit hybrid C2-53, with canker and HLB tolerance exceeding standard grapefruit, was approved for release by the IFAS cultivar release committee.

3. Screen our ever-growing germplasm collection for more tolerant types and evaluate fruit quality of candidate selections. We have continued monitoring a unique hybrid family of more than 400 individuals (with many of these planted as 3 tree replicates) from the cross of Clementine mandarin with a wild species reported to be nearly resistant to CLas attacks, by collecting detailed HLB

phenotypic data, including Ct values and other tree health measures as described previously. The frequency of CLas-negative trees remains unexpectedly high. We have revisited a replicated planting of sweet orange-Poncirus hybrids that was mapped for HLB tolerance QTLs (Huang et al. 2018) and are collecting new phenotypic data to study long term performance of these trees.

4. Conduct studies to unravel host responses to CLas and select targets for genetic manipulations leading to consumer-friendly new scion and rootstock cultivars. We selected ~ 450 mandarin hybrids for genome-wide association studies (GWAS), using the data referred to in Obj 3 above. DNA samples were prepared, each individual was genotyped using the citrus Axiom SNP array, and GWAS analysis is proceeding. This work will validate previously identified, or identify new genomic regions, associated with HLB tolerance or sensitivity. We continued seedling and callus line transformation experiments using the BG gene, and have plants coming from 3 orange, 1 grapefruit, and 2 rootstock lines.

T. McNellis 18-016 "Testing grapefruit trees expressing an anti-NodT antibody for resistance to HLB" 80% Completion of Objectives (April 2021 quarterly)

This report covers the period of March 1, 2021 - May 31, 2021. The objective of this project is to test transgenic 'Ducan' grapefruit trees expressing an anti-HLB antibody fused to the FT (Flowering Locus T) protein (FT-scFv protein). Several accomplishments were made during this reporting period. Graduate student Mr. Chad Vosburg was able to take a trip to Florida for several days in May, 2021 to assess trees again, take samples, and send samples for qPCR analysis at Southern Gardens for detection and quantification of 'Candidatus Liberibacter asiaticus' (CLas) within the tissues. The samples sent to Southern Gardens included those from a field trial natural inoculation, an Asian citrus psyllid (ACP) infection in the greenhouse, and a separate experiment of graft challenge of FT-scFv scions grafted to HLB-infected rough lemon rootstocks. Data from these samples are anticipated to be received fin July-August 2021. In addition, growth data were taken from FT-scFv scions on infected rough lemon. The transgenic scions appeared to be consistently growing faster and more robustly than the control non-transgenic scions but were not statistically different. The FT-scFv protein appears to provide a mild benefit to the grapefruit trees when inoculated by grafting, even if not statistically significant. The replicate grafting inoculation experiment begun in January, 2021, has additional replicates and may provide more statistically robust results. In March, 2021, we applied for a no-cost extension through November of 2021, and it was granted in May, 2021.

Additional qPCR data from the field, graft, and psyllid-transmission HLB challenge tests will be received from Southern Gardens in the summer of 2021. These data will be analyzed during the next reporting period. Additional sampling of field grown trees will be performed with help from collaborators at the USDA USHRL. These will be sent to Southern Gardens for qPCR CLas quantification for an additional infection time point.

Budget status: This project is running under budget thanks for wage support from Penn State for Chad Vosburg's work on the project, which resulted in some salary funds not being expended.

Z. Mou 18-017 "Establish early-stage field trials for new HLB-tolerant canker-resistant transgenic scions" – 50% Completion of Objectives (June 2021 quarterly)

The project has five objectives:
1) Remove the flowering-promoting CTV and the HLB bacterial pathogen in the transgenic plants, 2) Graft CTV- and HLB-free buds onto rootstocks, 3) Generate a large number of vigorous and healthy citrus trees, 4) Plant the citrus trees in the site secured for testing transgenic citrus for HLB responses, 5) Collect the field trial data

In this quarter, the following activities have been conducted:

1) A total of 85 plants including 65 transgenic plants and 20 non-transgenic control plants were planted into the field on May 20, 2021. These transgenic plants include replicates of three transgenic lines that have been shown to have robust tolerance to HLB in the greenhouse. Ten replicates of another transgenic line that expresses a different disease resistance gene were also included. This transgenic line has shown HLB tolerance in the greenhouse for more than eight years. In addition, we have eight transgenic Carrizo lines that express three different disease resistance genes. These lines have been replicated and grafted with Valencia. Three constructs were added onto the field trial permit. These plants will be transplanted into the field in the Fall of 2021. The transgenic plants that were transplanted in 2019 were examined. The plants grow well in the field and none of the plants has shown HLB symptoms. We plan to collect samples for CLas titer assay in this fall.

2) The citrus gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) was cloned and sequenced. The two conserved amino acids T177 and P181, which are responsible for glyphosate tolerance, were changed to isoleucine (I) and serine (S), respectively. The resulting citrus TIPS EPSPS gene was cloned into a T-DNA binary vector, which was introduced into Agrobacteria. To test if the citrus TIPS EPSPS gene can provide tolerance to glyphosate, we transformed it into Arabidopsis, since it will take shorter time to know the result in Arabidopsis. We will have plants for glyphosate tolerance test in the next quarter.

3) Transgenic citrus plants expressing the Arabidopsis nicotinamide adenine dinucleotide-binding receptor were inoculated with CLas using psyllids. We are waiting for the HLB symptom development. Meanwhile, we repeated nicotinamide adenine dinucleotide-binding experiment for the citrus lectin receptor kinase proteins using Monolith NT.115. We plan to use radiolabeled nicotinamide adenine dinucleotide to confirm the binding results in the next quarter.

E. Stover 18-022 "Delivery of Verified HLB-Resistant Transgenic Citrus Cultivars"
75% Completion of Objectives (May 2021 quarterly)

Objective 1, Mthionin Constructs: Assessment of the Mthionin transgenic lines is ongoing. As the most proven of the transgenics, they continue to be used as a reference in detached leaf assays, as well as studying them in established greenhouse and field trials. The first MThionin field trial (45 plants, WT or transgenic Carrizo on rough lemon scions) has shown transgenics maintaining higher average CLas CT values (2.5 CT higher @ 18 months), but with a high degree of variability. The larger second MThionin planting (205 total grafted plants of transgenic Hamlin scions, transgenic Carrizo rootstock, or WT/WT controls) is producing encouraging results; with the transgenic Hamlin on WT Carrizo having statistically greater trunk diameter, tree height and canopy volume compared to controls. Leaf samples have also been collected and are being processed for CLas quantification. The Mthionin construct has also been extensively transformed into additional varieties; with 10 confirmed transgenic lines of US-942 and 44 putative lines of Valencia and Ray Ruby now undergoing expression analysis.

Objective 2, Citrus Chimera Constructs: Detached leaf assays, with CLas+ ACP feeding, have been conducted to screen citrus lines expressing chimera constructs TPK, PKT, CT-CII, TBL, BLT, LBP/'74', '73', and '188' (as well as scFv-InvA, scFv-TolC, Topaz and Onyx). Testing of all 35s driven Carrizo lines is complete and the analysis of phloem specific and scion-types is well underway. This work has already identified numerous lines with significant effects on CLas transmission and increased ACP mortality (up to 95% from TBL and >70% from TPK). Endosymbiont analysis of high mortality causing transgenics (outlined in Objective 4) has also shown an accompanying reduction in Profftella titer that may indicate a mode of action. The best performing of these lines have been moved forward to greenhouse trials.

Initial ACP-inoculated greenhouse trials on 8 lines of citrus Thionin-LBP chimeras ('73', and '74') showed a statistically significant reduction (13x) in CLas titer for '74' transgenics vs WT. However, many plants of both treatments remained CLas negative due to low inoculation efficiency. In June, 150 plants representing the best performing 6 lines of `74' and 7 lines of `188' were no-choice caged ACP inoculated using a new protocol to improve transmission rates. At 3 months, control plants tested positive at twice the rate of the earlier inoculation; 6-12 month tissue samples are now collected, processed and ready for qPCR.

A larger greenhouse study is also underway to directly compare the best performing 3rd generation chimera (TPK and TBL) with the earlier 1st (Mthionin) and 2nd (`74' and `188') lines. A total of 420 grafted plants (all on WT Carrizo rootstock for uniformity) were made and bud inoculated with CLas+ RL to ensure high transmission. The first (3 month) growth assessment and leaf collection are complete, with samples awaiting qPCR analysis. An additional ~1200 rooted cuttings have been made from those same lines for paired ACP-inoculated greenhouse and field trials.

Field trials of 2nd generation chimeras (`74', and `188') with included MThionin plants are ongoing; with 165 plants (WT Hamlin and Ray Ruby on transgenic Carrizo) and 70 plants (WT Valencia on transgenic Carrizo) moved to the field in August 2020 and May 2021 respectively. An additional 200 plants (transgenic Hamlin on WT rootstocks) are being produced to complete the planting.

Eighteen new transformations, totaling over 6200 explants, have been completed to generate sufficient events of Valencia, Ray Ruby, US-942, and Hamlin lines expressing `74', `188', TBL, TPK and other advanced chimera constructs. From this effort, over 325 new lines from 74-Valencia, 74-Ray Ruby, 74-US-942, 74-Hamlin, 188-Ray Ruby, 188-Valencia, 188-US-942, TBL-US-942, TBL-Hamlin, TBL-Ray Ruby, TPK-Ray Ruby, TPK-US-942 and TPK-Hamlin are now in soil. Transgene expression analysis has confirmed the first 29 of these lines as positive with the remainder still being tested.

In addition to the use of the Mthionin and its chimeric variants, new strategies have been implemented in to develop HLB resistant citrus. These efforts include the expression of insecticidal peptides to control ACP (CLas vector) and the downregulation of the DMR6 genes to enhance defense responses against HLB disease. 54 independent transgenic lines of Carrizo, Hamlin and Ray Ruby expressing the insecticide peptide Topaz (a code name to protect IP), under constitutive and phloem specific (SCAmpP-3) promoters were evaluated by detached leaf assay. From these, 12 lines (4 events of each genotype) showed significant ACP mortality and were selected to move up in the screening pipeline for HLB/ACP tolerance. Also, 24 Carrizo transgenic events highly expressing Onyx (a code name to protect IP), a peptide with antimicrobial and insecticide activity, were evaluate by DLA. The 5 Onyx lines showing high ability to kill ACP (to 83% mortality) were selected for further evaluation. These strongly performing lines were replicated as rooted cuttings (103 Onyx and 189 Topaz plants) that will soon enter greenhouse trials. The available Onyx transgenic material is being expanded through production of additional constitutive (13 Valencia and 6 Ray Ruby) and phloem specific lines (25 Carrizo, 5 Hamlin, 5 Valencia, and 13 Ray Ruby).

Down regulated DMR6 Carrizo, either by stable expression of specific hairpin RNA or by specific Cas9-sgRNA were generated, cloned, and are being assessed. Since DMR6 is a broad immune suppressor, downregulated plants were first evaluated for Canker resistance as a quicker assay. After Xanthomonas challenge, both transgenic and gene edited DMR6 lines showed reduced bacterial titers and statistically significant reductions in Canker symptoms compared to controls; including some lines that developed no symptoms whatsoever. As an effort to accelerate development of non-transgenic HLB resistant plants through gene editing, early flower transgenic plants (carrying FT-SCFV gene) were transformed with the DMR6 targeting the CRISPR construct. The early flowering trait will greatly decrease the time needed to produce an edited but non-transgenic offspring. A set of 30 plants resulted from this gene stacking effort will be evaluated for the presence of both genes.

Objective 3, ScFv Constructs: ACP inoculated greenhouse studies on 5 scFv lines have been completed with transgenics showing significantly reduced CLas titer (up to 250x reduction) and a significantly higher incidence of no CLas rDNA amplification in roots and leaves compared to WT. These lines have been grafted with WT Ray Ruby scions and are undergoing field trials at Picos farm. The first assessment was completed in March with leaf tissue collected and awaiting CLas quantification. An additional 129 rooted cuttings are propagated for follow up plantings with grafted Hamlin scions. A second greenhouse trial testing new lines (150 plants from 12 lines) have been bud inoculated with HLB+ RL. A group of 370 plants for a third greenhouse trial has been propagated with the first 54 plants to reach a suitable size ACP-inoculated using the improved protocol. Plant tissue from both second and third (partial) greenhouse trials has been collected and processed; now awaiting qPCR analysis for CLas quantification.

Objective 4, Screening Development and Validation: A protocol using a high throughput ACP homogenate assay for selecting lytic peptides for activity against CLas is now in use. A manuscript on the protocol has been published in Plant Methods (DOI: 10.1186/s13007-019-0465-1) to make it available to the HLB research community. Transgenic Nicotiana benthamiana plants expressing His-6 tagged variants of the chimeras TBL, TPK, PKT and LBP have also been generated to produce sufficient protein extracts for use in exogenous applications.

The detached leaf ACP-feeding assay (DLA) has undergone several small revisions to improve sensitivity and maintain consistent inoculation, adjusting feeding period and ACP numbers. the analysis of ACP bodies has been expanded to include quantification of other major endosymbionts (Wolbachia, Carsonella and Profftella) to better investigate the activity of peptides causing CLas mortality.

An array of phloem specific citrus genes has been selected for investigation as potential reference genes to improve detached tissue and plant sampling techniques. Multiple sets of sequence specific qPCR primers for each gene have been synthesized and tested for efficiency. Six varieties of citrus have been propagated for endogene stability testing. A phloem specific endogene would allow normalizing to phloem cells, more accurately evaluating CLas titer relative to Citrus DNA and potential therapeutic effects.

The best performing lines of Mthionin, chimeras `74',`188', TPK, TBL and scFv transgenics have been submitted to Florida Department of Plant Industry for shoot-tip graft cleanup in preparation for future field studies. Hamlin/Mthionin transgenics (3 lines), Carrizo/Mthionin (2 lines) and Carrizo/'74' (1 line) have been returned certified clean.

Objective 5, Transgenic Product Characterization: Experiments are also underway track the movement and distribution of transgene products using antibodies and affinity tagged protein variants. CLas+ RL have been grafted as scions onto MThionin expressing Carrizo as a platform to test peptide movement and effects across the graft union. Transgenic Carrizo lines expressing His6 and/or Flag tagged variants of chimeric proteins TBL (15 lines), BLT (15 lines), TPK (17 lines), PKT (20 lines), scFv-InvA (22 lines) and scFv-TolC (18 lines) have been generated and expression confirmed by RT-qPCR. Total protein samples have been extracted from His-tagged transgenic lines and sent to our CRADA partner for testing.

U. Albrecht 18-028C "Comparison of field performance of citrus trees on rootstocks propagated by seedlings, cuttings, and tissue culture" – 68% Completion of Objectives (June 2021 quarterly)

Work conducted during this quarter on the two projective objectives are as follows.

Objective 1. Investigate effects of rootstock propagation method and the interaction with rootstock on root structure, root growth, and tree performance during the first 3 years of growth in the field.
- Bimonthly root growth measurements with the mini rhizotron imaging system continued in all trials.
- For trials 2 and 3, third-year tree size data collection was completed.
- Fruit quality analysis was completed for trial 3.
- Currently working on completing the analysis of the year 3 data of trials 2 and 3 that will be included in a publication of all three years of data.

- A Citrus Industry magazine article summarizing findings of this project was published (July issue).
- A virtual seminar was presented on June 21 that included information on this project.

Objective 2. Investigate if trees on rootstocks propagated by tissue culture or cuttings differ in susceptibility to Phytophthora-induced decline or wind-induced blow-over compared with trees on rootstocks propagated by seed.
- Monthly root growth measurements with the rhizotron imaging system continued and working on the PCR and ELISA assays.

2. Please state what work is anticipated for next quarter: We will continue with our mini rhizotron root imaging analysis along with the statistical analysis and interpretation of all data collected.

3. Please state budget status (underspend or overspend, and why): Approximately 67% of funds have been spent, which is somewhat underspent due to Covid-19 related complications that affected research, travel, and hiring of personnel.

U. Albrecht 18-029C "Evaluation of citrus rootstock response to HLB in large-scale existing field trials using conventional and automated procedures" – 75% Completion of Objectives (June 2021 quarterly)

Project objectives and work completed during this quarter are as follows.

Objective 1. Investigate rootstock effects on horticultural performance of Valencia and Hamlin trees commercially grown under HLB-endemic conditions using standardized field data collection procedures.

- Fruit collection and fruit quality analysis for the Valencia trials was completed.

- Harvest was completed for both Valencia trials.

- Tree/canopy size and trunk diameter measurements were completed for this year.

- We are still working on the leaf CLas analysis for trees in the Valencia trials.

- The statistical analysis of all data is nearly completed and is being prepared for publication.

Objective 2. Develop outreach to transfer information to growers and other industry clientele.

- A one-hour virtual seminar on rootstocks, including a summary of results from this project, was presented on June 21.

Anticipated work for next quarter:

- Continue with the data analysis.

- Data are being prepared for a presentation at the Citrus Expo in August.

Budget status (underspend or overspend, and why):

Approximately 70% of funds have been spent, which is somewhat underspent due to Covid-19 related complications that affected research, travel, and hiring of personnel.

R. Ferrarezi 18-037C "Performance of newly released grapefruit cultivars and rootstocks in the Indian River Citrus District" – 61% Completion of Objectives (May 2021 quarterly)

The project has two objectives: (i) Assess performance of new grapefruit cultivars with certain rootstocks under HLB endemic conditions in the IR district and (ii) Evaluate the influence of UFR and other recent rootstocks on grapefruit, navel, and mandarin in the IR in comparison to legacy/standard rootstocks.

Trials tested: T1) grapefruit cultivars on three rootstocks, T2) 36 rootstocks with Ray Ruby grapefruit as the scion, T3) 30 rootstocks with Glenn 56-11 Navel orange, and T4) 30 rootstocks with UF-950 mandarin.

Planting of 3,400 trees occurred in Sep/2019, 1,100 trees in Aug/2020, and 400 trees on Jun/2021. Grapefruit trees on UFR-8 are scheduled to be planted in Fall 2021.

Tree height, tree width in two positions (E-W/N-S), and trunk diameter were measured in three central trees from each experimental plot in Feb, July and Nov/2020, and Feb/2021. Canopy volume was calculated, and the following summary reflects the best and worst treatments in each trial during the first 18 months of growth. In Trial 1, Pummelette UF-5-1-99-2 grapefruit on US-942 was 6x larger (4.7 m3) than US 1-83-179 grapefruit hybrid on sour orange (0.8 m3) (P<0.0001). In Trial 2, Ray Ruby grapefruit on US-812 was ~3x larger (4.1 m3) than on Orange 16 (1.3 m3) (P<0.0001). In Trial 3, Glenn F-56-11 Navel orange on C-22 was ~2x larger (2.9 m3) than on Willits (1.2 m3) (P<0.0001). In Trial 4, UF-950 mandarin on US-942 was ~2x larger (2.5 m3) than on WGFT+50-7 (1.2 m3)(P<0.0001). Cultivars grafted on different rootstocks show no differences in tree phenological stage. HLB is spreading in the field and individual trees are getting infected as visual HLB symptoms are seen on some treatments in each trial. Leaf samples for determining HLB incidence were taken from a pool of trees from each experimental plot in May and Sept/2020, and Feb/2021 and sent to the Southern Gardens lab for analysis; on average, all samples tested negative (no trees with Ct values <32) but there are several positive trees with visible symptoms. Leaf samples were taken in Mar/21 for HLB diagnostic and bacterial titer quantification.

Asian citrus psyllids, Diaprepes root weevils, whiteflies, and other insects are less abundant in the field, except for leaf miners, which caused severe damage due to the excessive rainfall and wind gusts that made pesticide application challenging during this quarter. Nonetheless, tree growth has not been significantly affected by these pests.

Fruit phenology, pests and diseases have been monitored monthly. Canopy thickness, canopy color and HLB incidence have been measured quarterly in all experimental plots.

J. Grosser 18-039C "Part B - The UF/CREC Citrus Improvement Program's Field Trial Evaluations" – 85% Completion of Objectives (July 2021 quarterly)

The trial block containing the OLL somaclone seedling population is in jeopardy of being sold for development due the death of the property owner. Efforts to rescue the most promising clones from this trial are underway. Permission was obtained from DPI (Ben Rosson) to propagate the rescued trees at the CREC. Clones being rescued include those showing earlier maturity, higher soluble solids, and better HLB tolerance.

Potential HLB resistance from 'gauntlet' rootstock candidates: qPCR was completed on root samples from 45 additional promising gauntlet rootstock candidates. 18 rootstock candidates had ct values above 30, including 6 that had ct values of 40 (no bacterial detected). Among these, 5 rootstock candidates showed suppression of CLas in the Valencia scion; including 3 vigorous pummelo x latipes hybrids (13-76, 13-53 and 13-43), and two [Amblycarpa+HBPummelo] x [sour orange + rangpur] hybrids (13-15 and 13-12). Both C. latipes and the sour orange+rangpur are highly HLB tolerant parents. Pathogen-free material of top gauntlet rootstocks have been sent to TC labs for micropropagation include potentially HLB resistant S10xS15-12-25, S11x50-7-16-12, S11x50-7-16-6, and A+HBPxCH+50-7-12-11; and potentially HLB tolerant LB8-9(SugarBelle)xS13-15-16, LB8-9(SugarBelle)xS10-15-9, S10xS15-12-34, and A+HBPxCH+50-7-12-39. S10, S11, S13 & S15 are all salt tolerant pummelo/mandarin parents.

Molecular marker analysis of the promising super-root mutant UFR-1 clone#28 (Fast 28, discovered by Beth Lamb at the Rucks TC lab) showed that it is a deletion mutant of UFR-1; this vigorous rootstock continues to look promising in the field with sweet orange and grapefruit scions. Additional liners are being propagated at the Rucks TC lab for advanced trials. Identification of probable zygotic rootstocks at St. Helena showing good HLB tolerance (mentioned in previous report): 6 trees on apparent zygotic rootstocks were identified producing 3 to 3-5 boxes of Vernia/Valquarius fruit per tree with 12 to 12.5 brix. One is a tetraploid from Orange 12 (Nova+HBP-derived), one from Purple 2 (Nova+HBPxCleo+SO), two from HBPummelo x Shekwasha, and two from HBPummelo (open pollination). Since the staked-up scaffold roots did not sprout, we cut the tops (scion) off the trees as necessary to recover the rootstock genotypes. Two of the trunks have begun to sprout. We plan to generate pathogen-free material of these selections, followed by TC micropropagation to generate material for advanced trials. We also identified a truly stellar Valquarius tree on rough lemon; we are conducting a molecular marker test on the roots to see if the rootstock is a unique zygotic. If so, it will be treated as the above rootstocks.

St. Helena: The entire trial was assessed tree by tree, and all under-performing trees were marked for removal. This has created approximately 2,000 rotational spaces, and new rootstock candidates along with new early-mid season sweet orange candidates will be planted. Tree removal and replanting are expected to get underway this quarter.

Field Trial Data Collection: Tree size data was collected from the following trials: Bryan Paul, Smoak, Greene River Citrus (lemon), Tom Hammond, Post Office and Peace River. Tree health assessment data was collected from the Mislevy trial.

Data analysis and entry onto the Rootstock Data Website: annual updates included: Heller Bros., Peace River, Bryan Paul Doe Hill, Smoak, Post Office, and Tom Hammond. Trial data being uploaded and analyzed included data from the following trials: St. Helena, Premier Citrus, Greene River, Lee Family Groves, IMG, Banack, Cutrale and Wayne Simmons.

E. Stover 18-058C "Fort Pierce Field Test Site for Validating HLB and/or ACP Resistance" – 90% Completion of Objectives (June 2021 quarterly)

Update for this quarter:

A significant USDA-funded infrastructure project at the Picos Farm location is now complete. Improvements included a full renovation of the water management system; with drainage channels re-dug and cleared of vegetation, culverts inspected and replaced as needed, and new irrigation controls with remote access capability added. This will significantly improve storm and flood protection. Site management and field trials are progressing well. Four new transgenic trials were planted this quarter; two antimicrobial peptide expressing designs from the Stover team and two enhanced disease resistance designs from UF researchers. With these trials, the transgenic site is now operating at full capacity (some UF and other non-transgenics have been managed under this grant in adjoining blocks, exceeding 10 acres total for three years). The UC Riverside-led trifoliate and trifoliate hybrid trial has concluded, a manuscript regarding identified HLB-tolerance is in preparation, and trees will be removed to make space available for future plantings. The primary BRS permit has been amended to include a new UF gene construct that has shown a reduction in CLas titer in greenhouse trials. All regulatory protocols are being observed and the annual site review from APHIS/BRS has been conducted.

Availability of the test site for planting continues to be announced to researchers.

N. Wang 18-064C "Evaluation of the control effect of bactericides against citrus Huanglongbing via trunk injection" – 82% Completion of Objectives (July 2021 quarterly)

Objective 1. To illustrate whether application of bactericides via trunk injection could efficiently manage citrus HLB and how bactericides via trunk injection affects Las and HLB diseased trees.
1.1. Determine minimum bactericidal concentrations (MBCs) of bactericides against Las
This has been completed for both streptomycin and oxytetracycline against Las. A manuscript entitled: "Residue dynamics of streptomycin in citrus delivered by foliar spray and trunk injection and effect on Candidatus Liberibacter asiaticus titer" was accepted for publication by Phytopathology.
1.2. Effect of bactericides via trunk injection on citrus HLB disease progression, tree health, yield and fruit quality in different aged trees with a different disease severity

The field experiments were performed at four different groves on different aged trees with a different disease severity. They are one located in Avon Park, FL, 3-year old Valencia trees; one in Bartow, FL, 2-year old W. Murrcot trees; and one in Auburndale, FL, 7-year old Hamlin trees (planted in 02/2012). The last one is in CREC-, Lake Alfred, FL, 20-year old Hamlin trees. The HLB disease severity and tree size (canopy volume and trunk diameter) in the four groves were estimated immediately prior to treatment application. For the field tests, the experiment design is a randomized complete block design (RCBD) for 9 treatments, including 6 injection treatments (3 different doses for OTC or STR), 2 spray treatments (OTC or STR spraying), and one No Treatment as a negative control. Each injection treatment consisted of 9 or 15 trees divided into 3 blocks of 3 or 5 trees each. Each spray treatment consisted of 30 trees divided into 3 blocks of 10 trees each. For all the four field trials, the injection treatment applications were completed by the end of April 2019. The 1st applications of spray treatments were completed during spring flushing in February or March 2019, the 2nd applications were conducted in late June to early July 2019, and the 3rd applications were conducted in early to middle October 2019. Leaf samples have been collected from the treated trees at time points: 0 (pre- injection), 7, 14, 28 days, 2, 4, 6, 8, 10 and 12 months after treatment (MPT). The estimation of Las titers in these leaf samples are ongoing with qPCR assays. The first estimations of HLB disease severity and growth performance (height, trunk diameter, and canopy volume) of immature trees after treatment were performed in May 2019 (three months after the injection) and continued at 3-months intervals. Fruit yield and quality data were collected for the Bartow trial (W. Murrcot), Auburndale trial (Hamlin), and CREC trial (Hamlin) in January 2021.

We investigated the effect of 10 adjuvants on oxytetracycline absorption via foliar spray. Four adjuvants including Flame slightly increased the antimicrobial effect of OTC on Las.

Objective 2. To examine the dynamics and residues of bactericide injected into citrus and systemic movement within the vascular system of trees and characterize the degradation metabolites of bactericides in citrus.

Leaf and root samples have been collected from OTC or STR treated trees in the Avon Park grove at time points:0 (pre- injection), 2, 4, 7, 14, 28 days, 2, 4, 6, 8, 10, and 12 months after injection. The samples have been processed for OTC or STR extraction, and the concentrations of OTC and STR in these samples were determined by HPLC assays.

Fruit samples were collected for the Bartow trial (W. Murrcot), Auburndale trial (Hamlin), and CREC trial (Hamlin) during harvest in January 2020, and for the Avon Park trial (Valencia) in April 2020. The samples were processed for OTC or STR extraction, and the concentrations of OTC and STR in these samples were determined by HPLC assays. We have collected data for 60 and 360 days post treatment.

We have analyzed the residues of OTC and STR for fruit samples harvested in January 2021.

Objective 3. To determine whether trunk injection of bactericides could decrease Las acquisition by Asian citrus psyllids (ACP).

Twenty 1.5-year old citrus (Valencia sweet orange) plants were graft-inoculated with Las carrying buds in February 2020. These plants are being tested for *Las* infection and 4 plants were confirmed positive with *Las* infection (Ct values are between 34.0 and 35.0) at 4 months after grafting. They will be subjected to OTC or STR treatment by trunk injection and ACP acquisition access for 7 to 14 days. We have determined the time points to test OTC and STR treatment on ACP acquisition of Las. We have conducted the test regarding how trunk injection of OTC affects ACP acquisition of Las. OTC significantly reduces ACP acquisition of Las.

Objective 4. To monitor resistance development in Las against bactericides and evaluate potential side effects of trunk injection of bactericides
Monitoring resistance development in Las against bactericides. Leaf samples for this test have been collected from 5 trees injected with OTC and 5 trees injected with STR at the highest doses in each of the three groves at 6 and 9 months after the injection, respectively. PCR-sequencing analysis on Las 16SrRNA gene showed there was no mutation compared with the reported sequence. We are further confirming the results.
Evaluation of potential side effects of trunk injection of bactericides have been completed. We have collected another set of samples to monitor *Las* resistance against OTC and STR.
PCR-sequencing analysis on Las 16SrRNA gene showed there was no mutation compared to the reported sequence, indicating no major changes in bacterial resistance against antimicrobials.
Leaf samples were collected from OTC or STR injected trees in the Avon Park grove at two and four months after treatment for the analysis of the degradation metabolites of the bactericides. The extraction of the degradation metabolites was completed and will be subjected to HPLC assays with the three OTC metabolites: 4-epi-oxytetracycline, α-apo-oxytetracycline, and β-apo-oxytetracycline as standards.

E. Stover 18-065C "High -Throughput Inoculation of Transgenic Citrus for HLB Resistance" – 83% Completion of Objectives (June 2021 quarterly)

Update for this quarter:
This quarter the colony of CLas infected psyllids supplied a total of 5,045 ACPs used for (1) evaluation of rootstock breeding material, (2) transgenic events evaluation, (3) applied research for CLas control in citrus performed by USDA and University researchers; and (4) monitoring the colony quality.
The Bowman lab continues the experiments with grafted Valencia trees on groups of rootstocks which were inoculated with ACP during the previous quarter (January to March 2021). Each experiment compares replications on nine different rootstocks. This quarter 63 trees were inoculated with 1,280 ACP from the positive ACP colonies. Periodic evaluation of tree health, growth, and CLas titer via PCR were conducted on trees from rootstock experiments that were inoculated during the previous months following a set schedule. Periodic colony checks were conducted by PCR to maintain CLas positive colonies.

The Stover lab conducted weekly detached leaf assays (DLAs) challenging transgenic citrus with CLas inoculated by infected ACP in the lab, which is used to identify best performing transgenic events (transgenics varying by position of transgene insertion etc.) expressing antimicrobial peptides and defensive proteins targeting CLas, as well as natural insecticide peptides to control ACP. Six detached leaf assays experiments, involving 240 individual leaves, were inoculated using 2,400 CLas infected ACPs. Transgenic material tested in DLAs were Carrizo and Hamlin plants expressing ONYX peptide under constitutive and phloem specific promoter. A total of 48 independent events were tested alongside WT controls. The leaves (midribs) and ACPs are being processed and submitted to qPCR for CLas titer after each DLA to better understand the effect of the transgenic peptide in bacteria control and transmission. These trials have been very useful in terms of providing information that allow selection of the best transgenic events (ones causing high ACP mortality and/or low CLas transmission to plant) for propagation and further evaluation at greenhouse environment. Substantial ACP mortality was observed from feeding on CLas-killing transgenic leaves.

Research involving evaluation of the microbiome of ACPs fed on transgenic causing high insect mortality was conducted this quarter using 230 ACPs fed on a set of 22 transgenic leaves. In addition, 775 CLas+ ACP were provided to researcher collaborators (600 ACPs for Dr. YongPing Duan of USDA and 175 for Florida International University, for Jessica Dominguez, a Ph.D. student, who is developing a thesis in alternative compounds to control CLas bacteria). Also, to monitor colony quality, 360 ACPs were used for Clas detection by qPCR.

In this quarter, seven new colony cages (with 250 ACPs/cage) were set up to renew and support the demand of infected ACPs for future use.

E. Johnson #19-009 "Whole tree vs. rootstock or scion tolerance to HLB" – 50% Completion of Objectives (April 2021 quarterly)

CLas inoculation of experimental trees has occurred and sampling at 2 and 7 weeks for RNAseq and metabolomics has taken place. Soil samples were also collected from around the sampled roots for metabolomic analysis for root leachate.

The amount of information in progress report is rather brief and makes it difficult to fully understand if the project is meeting the outlined objectives.

In the previous quarterly report, the PI indicated that the propagation of experimental trees has proven more difficult than anticipated. His report indicated he thought he had sufficient number of trees for the experiment but evidently did not and has contacted nurseries to see if they could provide trees for the second experiment.
However, this quarterly report did not mention if sufficient trees are now available to conduct the second experiment. Therefore, one could question if this project is going to be able to meet the stated objectives.

N. Killiny 19-015 "Evaluation of the tolerance of newly developed citrus cultivars, on different rootstocks, to Huanglongbing" – 70% Completion of Objectives (April 2021 quarterly)

The objectives of this project are to evaluate new scions and rootstocks for their tolerance to HLB by studying the metabolite content by GC-MS and by challenging new varieties with psyllids and HLB.

Progress on Objectives:
Objective 1. To understand the mechanism behind the tolerance of different varieties toward HLB. The comparison between the varietal responses will allow for the determination of the mechanism

of tolerance to CLas. This quarter study was focused in three areas: 1) Marathon Mandarin analyses; 2) CUPS Mandarins; and 3) new Valencia varieties.

a. For the evaluation of the new mandarin hybrid “Marathon”, leaf samples were analyzed for volatiles and polar metabolites and run on the GC-MS and integrated. The analysis found eucalyptol in Marathon, which we have not detected before in other citrus varieties. However, overall profile strongly suggests that Marathon is unfortunately susceptible and does not behave like Sugar Belle. Challenging with CLas by grafting has confirmed CLas susceptibility.

b. For the leaf samples from inside the CUPS taken in March 2021, samples were collected from all Mandarins or mandarin hybrids that were available from inside the CUPS structure. The VOC analysis has been completed.

Findings: From the VOC analysis, results show two distinct chemotaxonomic groups - those that produce thymol and those that do not. Previously, it was found that leaves of Sugar Belle, a mandarin hybrid considered “tolerant” to HLB, contained thymol and its methyl ester, and leaves of most sweet orange (considered HLB susceptible) do not. The group that contained thymol and/or its methyl ester included Sugar Belle, Dancy tangerine (a parent of Sugar Belle), Minneola (also a parent of Sugar Belle), BB4-8-20, and UF711. We associated thymol with tolerance to HLB based on its well-known antimicrobial properties.

The thymol non-producers were UF950 Clementine, Murcott, Bingo, Early Pride, UF411, and Kinnow. These thymol non-producers also did not produce gamma-terpinene and had high levels of sabinene. Studies will be undertaken to look at the ratios of thymol and other blends of VOCs to detect any trends that might indicate HLB tolerance. It is expected that robust data analysis will help separate the varietal differences and identify key volatiles involved in ACP deterrence or action against CLas bacteria. It is hoped that in the future to be able to predict or screen for HLB tolerance based on the chemometric profiles of the new varieties if we can develop an accurate model.

c. For “Lucky” and its parents Sugar Belle and Nova × Osceola, we repeated this experiment in June using CLas-infected ACPs. We will measure the response to ACP infestation and the acquisition of CLas over time.

New work:

d. Selection has been obtained for Valencia and two newer varieties from Southern Citrus (5 each of Valencia, Valquarius, and Vernia) for a small study. Thus far, VOC profile of the three varieties is nearly identical. Additional studies will look at non-volatile metabolites next and perform some ACP challenges.

Objective 2. To understand the role of rootstocks in citrus tolerance to HLB. The comparison between rootstock metabolites will allow us to determine the best scion/rootstock combinations for tolerating CLas.

a. The rootstock seeds from the USDA selections (US-802, 812, 897, 942, 1283, 1284, 1516) for metabolite profiling and HLB/nematode screening are about three months old and ready to be moved outside to encourage growth.

b. The continued evaluation of previous rootstocks for growth habits and HLB tolerance is underway.

The PI greatly appreciates the Schumann Lab for granting access to the CUPS at CREC for sampling for parts of this study. Trees inside CUPS are presently one of the few sources of healthy mature citrus trees that are HLB free.

Y. Wang 19-024 "Near-term approaches of using alternative HLB-tolerant cultivars for increased production and improved juice quality" – 70% Completion of Objectives (April 2021 quarterly)

There are two objectives in this project.

Objective 1. Evaluation of blended juice using released HLB tolerant sweet orange & mandarin cultivars via analyses of sensory and consumer acceptance. A sensory and consumer study for Hamlin blended with Sugar Belle was conducted in late February. The study contained 79 consumers of which 56% was female and 44% was male. To fully evaluate the market and understand consumer behavior, the ethnicity, income, education status as well as weekly food expenditure and OJ consumption were investigated. Consumers were asked to rate overall appearance, overall liking, overall flavor liking, sweetness intensity, sourness intensity, bitterness intensity and overall flavor intensity for 5 different products (e.g. 100% Sugar Belle juice, 100% Hamlin juice, 90/10 Hamlin blended with Sugar Belle, 50/50 Hamlin blended with Sugar Belle, and a commercial product purchased from grocery store). In addition, in order to provide more information on the economic value of Sugar Belle blended juice, consumers were asked to give the price they intent to pay. As a result, 50/50 Hamlin blended with Sugar Belle juice showed the highest overall liking and best overall flavor. The commercial product showed the highest bitterness intensity among the 5 products. Averagely, consumer would be willing to pay ~50 cents more for the 50/50 Hamlin blended with Sugar Belle juice, compared with the commercial product. The results showed in this year were quite consistent with that from last year. Therefore, it was concluded that Sugar Belle blended with early cultivar Hamlin could largely improve juice quality and increase consumer liking compared to the current commercial products in the market. More important, consumer would be willing to pay more for the Sugar Belle blended juice, which would benefit our growers. In addition to conducting the sensory study and performing data analysis, we harvested late season Valencia in April. The fruits were washed, processed, and pasteurized in the same month. Sensory study has been scheduled on May 26th. The results will be reported in the next quarter.

Objective 2. Identify more tolerant cultivars resembling the quality of Valencia for the juice market and identify a chemistry definition of consumer accepted orange flavor. In this quarter, we focus on analytical work: identifying the chemistry definition of consumer accepted orange flavor and Valencia orange flavor. Flavor mean aroma and taste, which are compounds perceived by our nose and tongue, respectively. In the previous quarters, we have used gas chromatography-mass spectrometry to identify aroma compounds. In this quarter, we completed qualifying and quantitating taste compounds by using liquid chromatography-mass spectrometry. These taste compounds include sweet, sour, bitter and astringent compounds in sweet orange, mandarin and sweet-orange-like mandarin. In total of 56 compounds were analyzed in this quarter including: 10 sugars, 14 organic acids, 18 amino acids and 14 flavonoids. Studies were conducted for these analyses for 14 cultivars. We also performed principle component analyses (PCA) and Pearson correlation to correlate sensory mapping data and flavor analytical data. From the results, we could clearly conclude the major chemical difference between sweet orange flavor and mandarin flavor. For example, more aldehydes were correlated with sweet orange flavor but more terpenoids were correlated with mandarin flavor. A manuscript about this study is in the preparation and can be completed by the next quarter.

U. Albrecht 19-030C "Use of compost and interaction with low- and high-vigor rootstocks to accelerate young sweet orange tree establishment and enhance productivity" – 40% Completion of Objectives (April 2021 quarterly)

Objective: To determine the influence of compost during the first three years of tree establishment on growth, productivity, and root and soil health of citrus trees on rootstocks with different vigor-inducing capacity.

Monitoring tree performance and organizing and analyzing the data continue. Several additional activities in the trial were started including regular soil moisture measurements, flush ratings, and root health assessments to compare among compost/no compost treatments and rootstock cultivars.

Work anticipated for next quarter: Sample processing and data analysis will continue as well as the next compost application.

M. Dutt 20-014 "Understanding the role of systemic acquired resistance (SAR) in enhancing tolerance to HLB in the Parson Brown sweet orange" – 50% Completion of Objectives (June 2021 quarterly)

The project objectives are:
1) Determine the effect of systemic acquired resistance (SAR) in enhanced tolerance to HLB;
2) Monitor tree health and HLB levels;
3) Conduct juice analysis and evaluate quality parameters from selected Parson Brown trees.

Leaf samples were collected from 8 Parson Brown groves. Groves are in all the major citrus growing regions: 3 groves in Polk Co., 2 groves in Highlands Co., 1 grove in St. Lucie Co., 1 grove in Glades Co. and 1 grove in Collier Co. Samples were collected for both RNA and DNA extractions. Most of the trees are from the PB 56-2 clone but some groves have trees of the older PB 1-2-3 clone. All growers reported little to no fruit drop on the Parson brown irrespective of rootstock used. This is in comparison with Hamlin which had severe fruit drop this year. Hamlin leaf samples from the same block or neighboring blocks were also collected for analysis.

In the next quarter, leaf samples from the same groves will be collected, HLB levels will be measured using qPCR and the genetic differences between the samples will be evaluated.
We are on target with the spending for this project.

b. Gene technology, e.g., deploying resistance genes, antimicrobial peptides

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" – (June 2021)

Update on Citrus Greening project lead by Bayer Crop Science, now USDA **CAP** project, titled Collaborative approach between academics, growers and agrochemical industry to discover, develop and commercialize therapies for citrus huanglongbing (HLB)

This collaborative project has had a change in funding since the update provided in 2020. The first three years of the project was funded by CRDF and during a two month gap while applying for a USDA CAP grant, funding was provided by CRB and CRDF. With the successful grant application, this is now a USDA funded project for which funding began on September 1, 2020 and was approved through August 2023. The final year of funding will depend on review of progress and adequate funding available from the USDA NIFA program, but funding through August 2022 is confirmed.

The objectives of the grant are:

	Project Objectives	Responsible
1.	Optimizing a lead class of synthetic plant defense inducers	Bayer Lyon France
2.	Developing promising microbial strains into viable product candidates	Bayer Sacramento US
3.	Determining relevance of hairy root plant assay in predicting activity on HLB	Texas A&M
4.	Using greenhouse citrus assays to determine best conditions for field testing leads	University of Florida
5.	Field testing of leads to determine efficacy against HLB development	Florida Agco
6.	Investigating metabolomics as an early detection method for HLB in field conditions and monitoring tree health in response to therapy	University of California Davis
7.	Defining a registration pathway for moving leads towards commercialization	Bayer Global
8.	Developing an effective extension and outreach program	All PI's

Progress on the synthetic Plant Defense Inducers included synthesis of hundreds of additional analogs of the H class and screening of efficacy according to greenhouse activity, initial Human Safety screening and phytotoxicity. Analogs were also assessed for induction of plant defense in citrus and systemic movement in plants. From these efforts, an additional seven compounds have been identified for testing in Florida field trials. Several of these have shown a 3 month delay in HLB development in greenhouse citrus with collaborator Ozgur Batuman at the University of Florida. Ongoing screening of analogs to obtain the best candidates that can pass toxicology testing for registration is continuing.

Microbial strains identified as candidates from the screening process are being further tested in the Hairy Root assay in collaborator Kranthi Mandadi's lab at Texas A&M AgriLife research. Evaluation in greenhouse plant tests, including Clavibacter in tomatoes in Bayer Woodland as well as UF's HLB greenhouse test is underway.

External leads are also being evaluated including an endophytic organism from a research group in Israel a the Volcani Institute. The strain has been imported to Bayer's West Sacramento research facility is and currently being fermented in preparation for further testing. Any promising external leads will be considered for screening in the platform developed for this project.

Trials initiated in 2019 with first candidates have been concluded. While none of the early leads were able to prevent HLB infection, important knowledge was gained on best methods for applying and maintaining trials. In addition, one of these trials included metabolomics sampling of leaf samples which were also sent for qPCR analysis. It was determined that disease prediction by metabolomics could be determined approximately 6 months before positive results were confirmed in qPCR. No visual symptoms were seen at these timepoints. A second set of trials was initiated in 2020 and is ongoing and sampling is being made by both qPCR and metabolomics. Two new trials were also started in 2021 with the latest candidates from the PDI program in testing. Phytotoxicity trials of these candidates with and without adjuvants are also being set up. A fall trial will test the most advanced microbial candidates as well as the endophyte, depending on obtaining permits needed.

An initial call was held with CRB and UC Riverside to discuss a trial set up in California and planning continues on this aspect of the project.

M. Leslie 20-015C – "Vismax™: A novel peptide-based therapeutic for mitigation of citrus diseases, including HLB" - 15% Completion (April quarterly)

Objective: Determine whether Vismax treatment promotes resistance to other major citrus diseases, specifically citrus canker and phytophthora root rot in greenhouse assays with Dr. Evan Johnson

Progress (Year1 Q1; 3/4 - 6/11/2021)

a. Canker trial #1: Status = Complete. Trial included foliar and soil application methods for Vismax, 7 days prior to inoculation of sweet orange with X. citri. Rate testing of foliar applications (1X, 2X, and 4X concentration) was performed. Trial was successful with controls performing as expected. Raw data and preliminary report received.

b. Phytophthora trial #1 (traditional method; potted trees): Status = in progress, propagule counts. Trial includes foliar and soil applications of Vismax, and Ridomil Gold control applications.

c. Phytophthora trial #2 (traditional method; Rhizotron): Status = in progress, imaged weekly for root growth. Trial includes foliar and soil applications of Vismax, and Ridomil Gold control applications.

d. Trials were visited by EE personnel in May 2021 (no cost to grant)

Work is anticipated for next quarter:

a. Completion of Phytophthora trials #1 and #2, including data analysis
b. Second canker trials to be repeated to confirm the results of Canker trial #1
c. Disease-free seedlings on order for repeat of Phytophthora trials.

3. Please state budget status (underspend or overspend, and why):

Status = on budget ($14,462 of $30,600 budgeted in Year 1 - March 1, 2021- Feb 28, 2022)

This was the report for the first quarter of the grant. Work was done in Evan Johnson's lab.

1. Two canker trials were done.
2. The Phytophthora trials are underway.

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

N. Wang 18-026 "Control citrus Huanglongbing by exploiting the interactions between Candidatus Liberibacter asiaticus and citrus" – 85% Completion of Objectives (July 2021 quarterly)

The goal is to understand how citrus interacts with Candidatus Liberibacter asiaticus (Las) infection and develop improved and long term HLB management strategies.

Objective 1. Identification of the receptors for Las PAMPs in susceptible and tolerant citrus varieties

Potential PAMPs from Las (either homologous to known PAMPs or pilin genes) LasFlaA (flagellin), LasEF-Tu, LasCSP (cold shock protein), LasSSBP (single strand binding protein) and pilin assembly genes were cloned under 35S promoter and the Arabidopsis phloem specific promoter SUC2 and introduced into Agrobacterium. Their receptors have been tested in tobacco and citrus. Specifically, the receptors are being identified in HLB susceptible Valencia, HLB resistant Poncirus and HLB tolerant Sugar Belle. Multiple receptors for the aforementioned PAMPs have been identified and are in the process of confirmation using pull-down assay or co-immunoprecipitation assays.

Las outer membrane proteins are hypothesized to directly induce plant immune response in the phloem sieve elements because Las lives in the phloem. 21 outer membrane proteins have been cloned and the putative targets in citrus are being identified using Yeast 2 hybrid (Y2H) system and surface plasmon resonance (SPR) assay. Two outer membrane proteins showed positive interactions with citrus proteins based on Y2H assays. These interactions are being further confirmed using GST pull-down assays

In addition, multiple Las PAMPs have been tested for their effects in inducing plant defense against Las in the greenhouse and at least four different Las PAMPs showed significant effect in inducing plant immunity. Those Las PAMPs are being tested for whether they can inhibit Las titers after foliar spray in the greenhouse.

RNA-seq analyses of Poncirus and sweet orange have been conducted and the data currently being analyzed.

The effects of different PAMPs have been evaluated for control of HLB. Three PAMPs showed strong activity in inducing plant defenses.

trials in the greenhouse have been completed. different PAMP products are being evaluated in field trials by spraying on newly planted young sweet orange trees. Significant control effect has been observed for certain PAMP products against HLB.

Objective 2. Generate transgenic/cisgenic citrus expressing PAMP receptors recognizing Las putative receptors or targets (identified in Poncirus) of Las PAMPs are being transgenically expressed in Valencia sweet orange or Duncan grapefruit. They are driven by 35S promoter and phloem specific promoter AtSuc2. *Las* inoculation will be conducted via grafting or psyllid transmission once the transgenic plants are about one year old.

For those identified receptors or targets, the promoter regions in Valencia, Sugar Belle, and Poncirus to are being sequenced to compare their differences. If the native promoter of Poncirus is strong enough, Poncirus promoter will be used to drive the expression of PAMP receptors or other target genes to avoid concerns about 35S promoter or AtSUC2 promoter. Expression of one defense inducing gene is also being driven using a pathogen-inducing promoter. Several plants expressing the constructs were generated. Testing of those plants showed that they responded to canker. They will next be tested for whether are resistant to HLB. More plants are currently being propagated for testing.
Six constructs to express PAMP receptors individually or in combinations have been made in sweet orange. Three overexpression lines have been generated.

Objective 3. Investigate the roles of effectors in HLB disease development

Screening of 30 putative *Las* effectors have been completed and 4 of them repressed plant defense. another 20 putative Las effectors and 3 more effectors that suppress plant defense are being screened.

Y2H assays of the four defense-suppressing effectors have been completed and their targets in Valencia identified. Confirmation of the targets is ongoing using coimmunoprecipitation and BiFC assays. Meanwhile, CTV-mediated gene silencing of 15 putative HLB susceptibility genes has been conducted in collaboration with the Dawson lab. Sweet orange plants carrying the CTV constructs were inoculated with Las via grafting. Interestingly, gene silencing of one of the putative HLB susceptible genes led to significant HLB tolerance. The plants showed mild HLB symptoms, similar growth as non-inoculated plants whereas the growth of control plants was significantly reduced and showed severe HLB symptoms. The putative mechanism of the HLB S gene is being characterized. genome editing of the identified HLB S gene of Valencia sweet orange and Duncan grapefruit id being conducted to generate HLB resistant or tolerant citrus. In addition, the HLB S gene in Valencia is being overexpressed to further understand the mechanism. Plants will be inoculated with Las once they are one year old.

Other targets of putative effector genes will continue to be tested.

In addition, the effectors in Poncirus and Sugar Belle are hypothesized to induce plant defense. Y2H assays are being conducted to identify putative targets of effectors in Poncirus and Sugar Belle. RNA-seq analyses of Sugar Belle have been completed. Data analyses identified some interesting leads regarding chemicals to control HLB. These results are being summarized for publication. One manuscript entitled Citrus CsACD2 is a target of Candidatus Liberibacter asiaticus in Huanglongbing disease has been published by Plant Physiology.

The binding sites of CsACD2 with SDE15 have been identified. Y2H assays have been conducted for identification of the targets of CLas effector genes. In total, six promising HLB susceptibility genes have been identified. One line that silenced one of the target gene remains healthy despite being infected with CLas for over a year.

V. Orbovic 18-066C "Support role of the Citrus Core Transformation Facility remains crucial for research" – 83% Completion of Objectives (June 2021 quarterly)

Juvenile Tissue Citrus Transformation Facility (JTCTF) completed second quarter of operation as an EBA unit. The lab is open for customers who need transgenic citrus material and are ready to pay for services based on the EBA price list. At the same time, JTCTF still work based on the research grants that were received in previous years.

In the first half of the 2021, JTCTF has charged clients for the work it performed as the EBA unit and multiple payments were successfully processed. The Business Office at CREC helped make this process work. As more orders are received, the support from the business office in processing of payments will be important.

Between April and July, JTCTF accepted five new orders. Clients are still mostly interested in transgenic Duncan grapefruit and Valencia sweet orange. Three of five placed orders were associated with the existing USDA grant, while two orders came from clients through EBA. The facility is still expecting the influx of orders that were going to be placed already based on JTCTF as the designated subcontractor on grants. Those orders have been delayed because progress of those projects was delayed by COVID-19.

Output of the JTCTF in the last 90 days was eight transgenic plants. Six plants were Duncan grapefruit, one was Hamlin orange, and the last one was Valencia orange. The number of transgenic shoots JTCTF produced within the last six months was substantial. However, those experiments were done for the proof of concept and transgenic shoots were not grafted and grown into plants. The output of the facility would have much been better if these plants were developed.

J. Zale 18-067C "Continued Funding for the Mature Citrus Facility to Produce Disease Tolerant, Transgenic Citrus" – 85% Completion of Objectives (Mar. 2021 quarterly)

Since this CRDF proposal was written in 2018 and the external review occurred, the status of Mature Citrus Facility (MCF) has changed at UF. In the future, less research will be conducted and focus will be more on service. In any event, the objectives of this project were to produce disease resistant, commercially and agronomically desirable, mature citrus transgenics, cisgenics, and intragenics that will flower and fruit naturally using Agrobacterium and biolistics. The research focus of this project is to improve Agrobacterium and biolistic transformation efficiency of mature citrus, so that the mature citrus protocols become more productive, decrease prices for scientists, and contribute more to financial self-sufficiency of the lab. Great strides were made in increasing Agro-mediated transformation efficiency of some cultivars in Year 2 (after testing virB5 and plant hormones). A completely unrelated method that improves transformation efficiency was developed. A methods manuscript was submitted for review. An all-citrus selectable marker that functions well in transformation, as an alternative to nptII, was also elucidated in Year 2. A manuscript is being written describing the new selection system. The objective of research into DNA-free gene editing will be continued only in collaboration with a customer(s).

From one scientist, four vectors, were received to perform five transformations. None of these vectors worked and no transgenics were produced. This scientist is using a new vector backbone that doesn't function in mature citrus scions or rootstocks. The process of troubleshooting this issue is ongoing. In addition, the Agro strain being used, EHA105, tends to rearrange the vectors and the genes of interest can be lost. Some of the vectors were transformed into the AGL1 strain RecA that doesn't rearrange as often. From now on, a control transformation with a reliable vector will be included that consistently gives positive results.

Another scientist gave us three vectors to transform into mature citrus and 50 transgenic shoots were produced. He did not want these shoots grafted onto rootstock, but high quality photographs were taken for him. The scientist wrote a manuscript with the photographs of the transgenics and included MCF as co-authors. For another order, ~82 transgenic shoots were produced in one rootstock transformation, more than 40 were micrografted. It is too soon to tell how many will survive.

Several vectors have been transformed into mature citrus and results are pending. In addition, more orders for Agro-mediated transgenics have been received, so mature citrus are being budded for these transformations. In addition, two potential orders for biolistics are pending, however the efficiency of biolistics is still being increased by testing different osmotic treatments and cultivars. Budding of Kuharske, Carrizo, US942, Hamlin, Valencia, & EV1 was performed for biolistic transformation in order to collect stem tissue explants more appropriate for bombardments. In addition, Dr. Manjul Dutt is currently making genetic constructs that will be tested for one biolistic order. additional orders may be forthcoming once the outcomes of grant competitions are announced.

CRDF funding is sufficient, however there will probably overspend of the Director's account since time and funds were lost doing five transformations that didn't work.

d. Horticultural Practices

E. Triplett 18-024 "Foliar phosphate fertilization: a simple, inexpensive, and unregulated approach to control HLB" – 88% Completion of Objectives (May 2021 quarterly)

Our field trials to test foliar fertilization with potassium phosphate to alleviate HLB symptoms and increase yield of citrus continue with the most recent fertilization done a month ago. More sampling is being done within a couple of weeks.

Foliar phosphate fertilization field trial (Hamilton and Immokalee)
Phosphates have been applied every three months since July 2019 (Hamilton) and September 2019 (Immokalee). We have currently extracted DNA and analyzed CLas copy number through TerC and 16S qPCR. The copy number of CLas declined in the 2mM phosphate treatment group after a year of spraying but that decline has now leveled off. We are currently collecting data on the leaf area index of the tree to determine if leaf density has improved with the spring flush. Fruit yield data will also be available soon.

Foliar phosphate fertilization on CLas negative Valencia trees (Greenhouse)
Our greenhouse experiment continues to test whether foliar application of soluble potassium phosphate can reduce citrate level in the phloem of citrus trees. Treatments consist of foliar application of 6mM and 2mM potassium phosphate and/or 6mM insoluble calcium phosphate applied to the medium. Each treatment has six replicate trees and control trees are treated with 6mM potassium sulfate to standardize potassium application. All treatments receive a base fertilization with all macro and micro nutrients without phosphate. Every three months three leaves are collected from each tree, midribs are excised and sent off to Dr. Killiny's laboratory in UF CREC for quantification of citrate and phosphorus levels. Citrate levels have declined significantly in the foliar fertilized plants but it is not yet clear whether that decline will be significant enough to stop CLas infection.

Foliar phosphate fertilization on graft-infected Valencia (Greenhouse)
The greenhouse experiment hosted by Nabil Killiny's group continues to show measurable differences of CLas titer between treatments. These trees are given a potassium phosphate foliar spray, a calcium phosphate soil drench (to mimic FL grove soils), or no added phosphate other than what is in the typical fertilizer mix. We continue to visually observe the disappearance of HLB symptomology in the treated saplings. Titer has declined but we need more sampling to determine if these is sustainable over a longer period. In the lab, qPCR continued from leaf samples to assess HLB titer in both greenhouse and field plants. Organic acid measurements were made in each experiment above. K-phosphate treatments reduced phloem citrate levels by 50%.

Anticipated work for next quarter:
Field trials continue. A manuscript is being written now on our results to date. Yield and canopy measures expected next quarter. q PCR analysis on first 24 months of samples will be done and analyzed.

F. Alferez 18-032C "Preventing young trees from psyllids and infection with CLas through use of protective netting" – 80% Completion of Objectives (May 2021 quarterly)

Objective 1. Assessing tree growth and absence of psyllids and HLB disease symptoms (including CLas bacteria titer) under protective covering (i.e., IPC). The most striking finding in this objective during the last quarter has been the finding that fruit retention was significantly improved in trees that were covered by IPCs, resulting in larger yields and improved internal quality. As a reminder, we removed covers in August 2020. These trees bloomed and set fruit for the first time in February 2020; therefore, this represented the first crop. Fruit drop was non-existent in trees that had been protected by the IPCs, whereas non-covered trees showed 60% fruit drop. Fruit quality was also dramatically improved by use of IPCs: Brix was 10.9 in IPC trees whereas in non-covered trees Brix was 7.5.

Objective 2. Assessment of alternative netting approaches involved in 'targeted', 'alternated' or 'patterned' setup of IPC in groves for more cost-effective protection. We have started analyzing samples to determine HLB incidence in the different layouts and if layout dictates different psyllid colonization.

Objective 3. Monitoring the transition from vegetative to reproductive stage in the covered trees as compared to the non-covered trees. In the new plots, after having a good bloom in all three varieties (SugarBelle, Early Pride and Tango) covered by IPCs, we assessed fruit set and did not find significant differences compared to non-covered trees. This is promising, since it shows that these varieties do not need to be exposed to pollinators to set fruit, and IPC protection could potentially be prolonged to get the trees well into the productive age, producing high quality fruit, as we have shown in Objective 1. By applying brassinosteroids we expect to prolong further tree health and produce a commercial-size crop of good quality fruit.

Objective 4. Comparing IPC with CUPS-like systems. We have collected data on bloom and fruit set on deficit irrigation plots for the second year, and we were able to confirm more bloom and fruit set in protected trees. We installed an automated irrigation system that is helping us to perform these treatments consistently. We have also installed several moisture probes to fine tune the treatments.

Outreach for this quarter:
-Alferez, F. Citrus Institute 2021. Virtual. April 6.Individual Protective Covers (IPCs) influence on tree performance, fruit production, pests, and diseases.
-Alferez, F. Invited presentation at CRDF BOD meeting. April 26, Arcadia.
-Alferez, F, Batuman, O, Gaire, S, Albrecht, U, Qureshi, J. Assessing spatial patterns of IPCs deployment in young citrus. Submitted to Citrus Industry
-Batuman, Alferez, Qureshi: Assessing spatial patterns of IPCs deployment in young citrus. CRAFT TWG meetings and one-on-one grower meetings.

Anticipated for next quarter:
Objective 1. We will perform regular work on horticultural/pathology parameters in all plots.

Objective 2. We will continue collecting data on psyllid populations and HLB incidence in the different netting layouts. Our idea here is to find any seasonal differences that can be affected by the different patterns.
Objectives 3 and 4.We will continue collecting data on fruit set and fruit growth and maturation for this second season of deficit irrigation treatments. We will also monitor early fruit drop, if it occurs.

Outreach:
-Gaire, S, Alferez, F, Albrecht, U. Horticultural attributes of SugarBelle, Tango and Early Pride mandarin trees grafted on two different rootstocks grown with and without individual protective covers (IPCs). ASHS Annual meeting. August 5-9, 2021, Denver CO

Budget status :
We are on track with activities and spending. Budgeted amounts for salaries and student stipend and tuition are being spent as predicted. We expect to spend more this quarter, as we will be paying publication fees for our first paper accepted and registration for the ASHS annual meeting in Denver, CO.

L. Duncan 18-036C "Cover crops and nematicides: comprehensive nematode IPM across the grove landscape" – 83% Completion of Objectives (May 2021 quarterly)

Nematicide treatments for the final spring season were applied. Sting nematode populations were monitored monthly in row middle treatments of cover crop or native vegetation. Results to date were reported at the Florida Citrus Show in May and in the June issue of Citrus Industry. The primary information conveyed to both outlets is that 1) during two years, perennial peanut reduced the number of sting nematodes in row middles by more than 90%, which may be beneficial as the trees grow and root systems expand, 2) to the extent that the different nematicides reduced sting nematode numbers, the trees responded with increased vegetative (fibrous roots and trunks) growth, 3) during the first two years of production, tree growth rate was inversely related to number of fruit, 4) preliminary observation of tree growth in groves heavily damaged by sting nematode indicates that tolerance of sting nematode herbivory is likely conferred by blocking HLB occurrence via use of individual protective covers.

E. Johnson 18-041C "Characterizing HLB-pH interaction to improve management of root function and tree health" – 95% Completion of Objectives (Jan. 2021 quarterly)

The objectives of this study are to identify optimal pH range for root function and minimize root turnover on HLB-affected rootstocks and how uneven pH levels in the root zone (e.g. irrigated vs. row middle portions of root system) affect the overall health of the tree. This is being done in a split root system in the greenhouse where pH of different parts of the root system can be controlled and maintained.

The following progress reported is based on an October 31st report date.

The breakdown of the 2nd experiment testing different irrigation pH's on each half of the split root system (to mimic irrigated and non-irrigated root zones) is complete. Data are being analyzed and samples for RNAseq are being prepared. The 2nd repetition of the 1st experiment is underway to confirm the results that US942 becomes highly sensitive to low pH (5.5) when infected with HLB (root damage and excessive root leakage causing irrigation leachate pH to increase. We also expect to partially confirm this finding in the ongoing data analysis of the 2nd experiment.

This project terminated April 30, 2021 – The PI has requested additional time to collect and compile the data and analyze in his final report.

D. Kadyampakeni 18-042C "Development of Root Nutrient and Fertilization Guidelines for Huanglongbing (HLB)-Affected Orange and Grapefruit" – 95% Completion of Objectives (July 2021 quarterly)

The purpose of the project is to develop new guidelines for restoring root health and improving overall tree nutrition for Florida oranges and grapefruits. The objectives of the project are to:
1. Determine optimal nutrient concentrations in roots and leaves for multiple grapefruit and orange varieties.
2. Compare and contrast fertigation, soil, and foliar fertilization to identify best application method for uptake of nutrients into both underground and aboveground components.
3. Investigate the relationship between root and leaf nutrient contents to tree health, yield, and fruit quality as well as bacteria titer.
4. Generate updated and new guidelines for optimal nutrient contents for roots and leaves for HLB-affected trees.

Progress to date:
The project is being conducted at three sites: Citrus Research and Education Center (CREC), Southern Gardens Citrus near Clewiston, FL and Indian River Research and Education Center (IRREC). Data collection continued during this quarter on root scanning, canopy size determinations and soil sampling on the central Ridge and southwest Flatwoods along with fertilizer treatment applications. All sampling for the third quarter of the third year of the project are complete.

The aim of the study at the UF/IFAS IRREC in Fort Pierce, FL was to relate nutrient concentrations in grapefruit leaves and roots to indicators of tree health and root growth. The research was conducted on flatwoods soils in a randomized complete block design field study on 'Ruby Red' grapefruit. Micronutrients (B, Fe, Mn and Zn) were applied using three different concentrations (1x, 2x, and 4x current UF/IFAS guidelines) in the form of either dry granular water-soluble fertilizer, controlled-release fertilizer, or liquid fertilizer. A total of 600 trees divided in 40 experimental units were employed. We collected leaf and root nutrient concentrations, canopy volume and tree height twice a year. Mini-rhizotrons were installed at the beginning of the experiment and root images were taken four times a year. Results showed increased micronutrient concentrations in the leaves among all treatments but there were no significant treatment differences in tree height, canopy volume, root length, and root diameter. Yield and fruit quality data were collected in February 2021 and are being analyzed.

Graduate student Lukas Hallman completed his MS program under the supervision of Dr. Rossi. Portions of his work will be published in peer reviewed journals. PI Kadyampakeni and graduate student Tanyaradzwa Chinyukwi published a review article in the Journal of Plant Nutrition.

Plans for Next Quarter

The team will continue with fertilizer treatments and data collection including HLB rating assessments, canopy size and root growth measurements and reporting on the progress of the project. Former graduate student Tanyaradzwa Chinyukwi and graduate student Lukas Hallman will present their works at the Florida State Horticultural Society Meeting in Daytona Beach, FL in September 2021. Lukas will also present his work at the American Society of Horticultural Science in Denver, CO in August 2021.

Dr. Ferrarezi departed from IRREC this quarter and his tasks will be completed by co-PI Dr. Alan Wright to make sure all project milestones are completed as planned.

R. Niedz 18-050C "The effect of the ionization state of iron and citric acid on the health of HLB-infected trees" – 100% Completion of Objectives (Apr. 2021 final report)

Objective 1 - Determine the effect of the ratio and concentration of Fe2+ + organic acid on hydroxyl radical production and stability. Objective complete.
Objective 2 - Determine the phytotoxic levels of Fe2+ + organic acid solutions on citrus. Complete.
Objective 3 - Determine the effect of Fe2+ + organic acid solutions on HLB titer using a rapid greenhouse, HLB-infected citron, rooted shoot bud assay. Continuing work to develop a rapid greenhouse screening system. Current systems are not yet ready for screening methods to cure or manage HLB. *No CRDF funds being used for this research.*
Objective 4 - Requires screening system - see objective 3 discussion.
Objective 5 - Requires screening system - see objective 3 discussion.
Objectives 6 and 8 (Note: there is no 7) - These are the field tests for the various ferrous iron (Fe2+) and citric acid treatments on HLB status and horticultural measures for both mature (HLB symptomatic) and nonbearing (non-symptomatic) trees. Throughout this quarter (Jan 1 - Mar 31) as well as the project timeline, conventional pesticide spray applications for non-bearing grapefruit trees were applied to all the treatments in the trial and were based on scouting and were in accordance with IFAS guidelines. Similarly, irrigation events were made based on tree and field conditions as determined by soil feel and appearance, tensiometer readings, water table observation well measurements and visual assessment of tree canopy. Fertilizer applications were made via fertigation and were `spoon fed' with frequent small applications bi-weekly. No dry fertilizer was used. All 7 experimental treatments were applied to the trial block as per the protocol.
There was one cold front that impacted the trial location in Indian River County on 02/04/2021 with a low temperature documented at 35.97 °F. Only very minor leaf drop occurred 5 days

after the cold weather. Rainfall over this quarter was 5.79 in. with on 0.33 in. recorded in March 2021 requiring frequent irrigation events. Visual assessments of tree condition were made on 01/14/21 using a numerical rating of 1-4 for each tree quadrant and were averaged for each tree. Other field measurements included fruit counts (01/28/2021) and trunk caliper measurements (03/31/2021). Leaf sampling for PCR analysis was performed on 01/27/2021. As in prior quarters, a ground application of herbicide was made, and plant material was removed in preparation for a 03/06/2021 UAV flight to collect aerial imagery for canopy area determinations.
Aerial images were taken with a UAV equipped with a 20 MP digital camera on 3/6/2021. A set of overlapping images were taken at two altitudes - 75 and 150 feet. Images taken at 75 feet are for photo-documentation (0.27 inch/pixel) of the experiment and will be made available at the completion of the project. Images taken at 150 feet (0.53 inch/pixel) were processed by segmenting the image into trees and background using machine learning image analysis. Once the trees were segmented (identified and separated from the background), various measures were made/calculated including canopy area, density, perimeter, convex hull area and perimeter, and minimum and maximum caliper diameters. These measures, as well as the ground measures, will be correlated (statistical analysis) with the iron treatments to determine their effects.

J. Qureshi 18-052C "Sustainable Management of Asian citrus psyllid (ACP) and Citrus Production" – 80% Completion of Objectives (June 2021 quarterly)

This project is focused on conducting research in four Integrated Pest Management (IPM) programs and biological control only program for ACP. The programs are :

1. conventional and organic insecticides plus biological control,
2. organic insecticides, and Horticultural Mineral Oil (HMO) plus biological control,
3. conventional insecticides plus biological control
4. HMO plus biological control.
5. biological control only.

Sampling for ACP was conducted on biweekly basis. On April 19, the ACP population reached the treatment threshold of 0.1 adults per tap sample in all the programs and therefore programs 1-4 were treated with foliar sprays of insecticides. Program 1 and 3 were sprayed with Apta and Program 2 with Entrust. These sprays also included 435 oil at 1% of the spray volume. Program 4 which depends on the use of horticultural mineral oil received 435 oil at 3% of the spray volume. These treatments kept psyllids below treatment threshold until the first week of June, when adult numbers exceeded 0.1 per tap sample in programs 2 and 4 and therefore 435 oil was used at 2% of the spray volume in these programs.

Parasitoid Tamarixia radiata releases were made after the April sprays. However, nymphal populations were not very high. We observed 6% parasitism in program 5 and 4% in programs 2 and 4 but no nymphs were parasitized in the samples collected in programs 1 and 3. The programs 1 and 3 include the use of conventional insecticides and in this case Apta, suggesting the negative effects on the beneficial organisms. In the laboratory experiments, we also tested imidacloprid, dimethoate, and fenpropathrin to determine LD50 against T. radiata using topical exposure method. The LD50 of imidacloprid was 1.16 mg, dimethoate 9.65 mg, and fenpropathrin 11.48 mg. We also initiated experiments to determine the lethal and sublethal effects of a number of compounds under field conditions. The experiment to determine the release rate of the predatory mite Amblyseius swirskii against ACP tested three different release rates, 1:2, 1:1, and 2:1 (predatory mite: ACP egg). The proportion of 2:1 was the most efficient in reducing the number of ACP. Two commercially available predators 1) Brown lacewing Sympherobius barberi and 2) Rhyzobius lophanthae ladybeetle were tested against ACP. The adult of each species was evaluated against ACP at an average of 80 eggs or 24 nymphs per shoot of Murraya paniculata. A consumption of 12% eggs with S. barberi and 28% with Rhyzobius and 60% in the combined treatment using both predators was observed after 24 h exposure. For nymphs, 40% were consumed S. barberi, 36% by Rhyzobius and 54% by both in the combined treatment. These findings suggested that these species are effective predators of ACP eggs and nymphs and ladybeetle were more voracious against eggs. Both predators were also released in the field in all programs at the rate of 200 adults per plot for a total of 4000 individuals of each species.

J. Qureshi 18-055C "Optimizing Benefits of UV Reflective Mulch in Solid Block Citrus Plantings" – 60% Completion of Objectives (June 2021 quarterly)

Treatment application and sampling were performed at three young tree experimental blocks of citrus planted at 1) Southwest Florida Research and Education Center (SWFREC), Immokalee, FL, 2) Citrus Research and Education Center (CREC), Lake Alfred, FL, and 3) Florida Research Center for Agricultural Sustainability, Vero Beach, FL.

Trees on UV reflective mulch and bare ground at all three locations received 1) soil-applied neonicotinoids interspersed with sprays of a different mode of action insecticides on a calendar basis, and 2) rotation of insecticide modes of action sprayed twice on each major flush. The full irrigation and deficit irrigation treatments using drip and the deficit treatment aimed to synchronize flush to target spray applications on major flushes, were also functional at the SWFREC and CREC locations in both mulch and bare ground plots. At CREC, an additional irrigation treatment using a micro-sprinkler was also implemented in the bare ground plots.

In previous reports, we have documented the effects of mulch vs. bare ground treatments and flush spray vs rotation of soil-applied neonicotinoids interspersed with spray treatment on the ACP. In this report, findings from soil nutrient and moisture analysis, tree size analysis, and grower survey are reported. Soil analysis from SWFREC Immokalee location showed that except for Mg, K, and B, all the other nutrient concentrations were higher in the mulch plots. For instance, the concentration of Ca, P, Zn, and Mn was at 36.5, 108, 41, and 52 mg/kg, respectively. For some nutrients concentration was high in the bare ground plots for instance Mg (19.8mg/kg) and K (30mg/kg). At CREC Lake Alfred location, most nutrients were at high concentration in mulch plots except for K which was high in the treatments of full irrigation using drip and micro-sprinkler in bare ground.

Between the full and deficit irrigation in mulch plots, full irrigation plots contained 20%, 25% and,

10% Ca, Mg and P, respectively, while deficit irrigation treatments had a higher concentration of K, Zn and, B at 31, 39 and, 12.5 mg/kg, respectively. The levels for Cu (12.7 mg/kg), K (36 mg/kg), and Zn (32 mg/kg) were high on the bare ground.
At SWFREC, trunk cross-sectional area and canopy volume were 38.8% and 44.2% larger, for trees on mulch than bare ground, respectively. At CREC, mulch treatment resulted in trees with 21.6% and 34.8% larger trunk cross-sectional area for plots with the deficit and full irrigation, than bare ground with full irrigation, respectively. The canopy volume was 52.7% and 65.4% larger for trees in mulch plots with the deficit and full irrigation treatments than bare ground with full irrigation, respectively. However, there was no significant difference for these variables between deficit and full irrigation treatments in mulch-planted trees. Data from April 2021 showed higher soil moisture averages from mulch treatment at all layers (8, 15 and, 45 cm). For instance, the highest difference was at 15 cm depth for the CREC site (37%) followed by the Immokalee site at depth 45cm (30.2%) and 8cm (25.3%).

The economics team began data collection via the survey instrument. The survey solicited information on grower's use of reflective mulch, willingness to pay to use reflective mulch, and ranked how they valued different soil and pest attributes. The survey instrument was administered online only by Qualtrics as well as emailed by extension agents to their mailing lists, to the Indian River Citrus League members, and to growers who attended the 2021 Citrus show (whose email addresses were collected by the student on the team). This effort resulted in 308 responses of which 89 surveys are usable: 44 from Florida and Georgia, 17 from Texas, and 6 from California. These responses do not constitute a representative sample, so data collection efforts continue. For Florida and Georgia, the preliminary sample includes 99,874 acres and the average farm has 2,323 acres of citrus. Reflective mulch is used by 23% of growers (27% of acres) and 70% are aware of reflective mulch use in citrus production. Those who use reflective mulch rent 36% of their farmland and have 22 years of farming experience, on average. Early results indicate that Florida and Georgia's growers are willing to pay $467 to implement reflective mulch.

The data collected does not constitute a representative sample. The economics team will continue to collect data and explore data techniques that will reduce the bias in the sample. The team will also collect auxiliary data to conduct a more robust data analysis and use partial budgeting analysis to estimate costs associated with using the practice.

The start of this project was delayed significantly due to the logistics involved in setting up the trials in three regions. Therefore, we will need one more year after the end date of December 2021 for the successful completion of this project.

S. Strauss 18-059C "Citrus row middle management to improve soil and root health" – 85% Completion of Objectives (April 2021 quarterly)

Objective 1: Determine how different cover crop mixtures impact soil and root health and weed cover in established commercial citrus groves.

Cover crops were last planted at the end of Nov 2020 and included sunnhemp, Austrian winter pea, daikon radish, oats, and winter rye. While the harvest in March did terminate some of the cover crops, there is still growth in the row middles. Further analysis of the soil nutrient and microbial samples collected in August 2020 found that the types of microbes performing denitrification (part of the nitrogen cycle) are completely different under the legume+non-legume cover crops compared to the other treatments. Soil organic matter has also significantly increased under cover crop treatments. Analysis of the weed data indicates cover crop planting impacts will have a significantly longer-term effect on weed suppression, which could be potentially attributed to the substantial reduction in the addition of weed seeds to the soil seed bank in the cover-cropped areas. The germination and establishment of cover crops in the second location appear to be improving over time, as noted from the comparisons of cover crop density in planted row-middles between 2019 and 2020.

Objective 2: Examine the impact of eco-mowing in conjunction with cover crops on soil and root health and weed cover in established commercial citrus groves. Eco-mowing occurred at the end of November 2020 with the planting of the next round of cover crops. Data from Year 2 (collected in Aug 2020) is still being analyzed. Visual root growth assessments show continued root growth under cover cropping and eco-mowing, but analysis is ongoing. Soil moisture appears to be similar across all treatments, possibly due to the presence of a high water table at both sites. Quantitative data on root growth (volume, length and area), root dieback, and recent soil moisture dynamics by treatments will be presented in the next quarter and is being finalized now. Preliminary evaluation shows that conducting eco-mowing in the row-middles has unremarkable effects on weed emergence and coverage in the tree-rows.

Objective 3: Quantify the effect of cover crops and eco-mowing on tree growth and production. Yield data for the second year of cover crops was collected in March 2021. Preliminary analysis of yield data indicates little change in with treatments in one location, and a slight increase with cover crops at the second site. Analysis of fruit quality, canopy volume, and trunk size is ongoing. Canopy and trunk size measurements, and leaf nutrient status will continue, and quantitative differences will be reported in the next quarter. However, the marginal changes are not unexpected, as trees of this age could take at least three years to show responses to treatments. We will continue to assess canopy volume and trunk size.

Objective 4: Identify the economic benefits of using cover crops
The cover crop survey is being administered via Qualtrics. Low response rates in Florida necessitated including other citrus producing states (Texas and California). To date, we identified about 60 usable surveys with respondents from each state. We attended the Citrus Show in May 2021 to encourage more Florida citrus growers to take the survey. Preliminary responses suggest that cover crops are more widely used by CA citrus growers than FL growers.

Objective 5: Communicate results to growers using field days and extension materials
Preliminary results are being presented at the Soil Science Society of America/American Society of Agronomy meeting in November. Observations on the impacts of cover cropping on weed control were presented at Weed Science Society meetings during Feb/March 2021. Information on cover crops was provided as a Tip of the Weed for the Citrus Industry magazine in May 2021. Cover crop

information and preliminary data were also presented at two regional grower extension talks in March and April 2021. Discussions are underway about how to host a field day, or a virtual field day, in the summer or fall of 2021.

Anticipated for next quarter:
Analysis of soil inorganic N fractions (ammonium and nitrate) for samples collected in August 2020 continues and updates on other soil/leaf nutrient variables will be provided in the next quarter. Half-yearly weed data analysis will be scheduled in the upcoming quarter. Canopy and trunk size measurements and leaf nutrient status along with root image collections and soil moisture monitoring will continue. Some soil moisture data loggers were disconnected due to wild animals and/or field equipment and data will be recovered this month and reported in the next quarter. The next set of cover crops are scheduled to be planted in June 2021. The annual soil sample collection will occur in August 2021. The composition of the mixes is still being discussed. The economics team will begin survey data analysis. They will also construct the framework for partial budgeting and assessing the cost of cover crop use and continue to collect data. Partial budgeting analysis will continue, and a report will be drafted.

Budget status :
We are mostly on track with our planned budget spending, however delays in hiring and limited travel resulted in the economics team underspending.

T. Vashisth 18-061C "Evaluating sustainability of yield and fruit quality of sweet oranges with use of controlled release fertilizer and micronutrients" – 85% Completion of Objectives (May 2021 quarterly)

This project is a continuation of an objective of existing CRDF funded project (# 00124558 ; ended in March 2019, final report submitted to CRDF) with some added treatments to be evaluated in comparison to control (dry conventional fertilizer with foliar micronutrients). Objective 1 which is the continuation of # 00124558 included 10 treatments.
Altogether currently there are 25 treatments of citrus nutrition that are being compared to control. Within this quarter we have successfully harvested the trial. The Mn and B treatment is still the best performing treatment across all the treatments in Fort Meade. Where treatment with CRF and tiger micronutrients (6-6-3-1) is consistent good performer at both the sites. Altogether our data so far suggests that use CRF and soil applied micronutrients with slightly acidic pH is beneficial for the trees.
No significant differences have been observed in new added treatments, it is speculated that two years is not long enough duration to statistically impact the yield. We anticipate that we should be able to see effect of treatments in upcoming year.
Unfortunately, due to Covid-19 related restriction we could not perform sensory analysis, however we collected juice for flavor analysis in order to assess the effect of nutritional treatments on sensory aspect of juice. Currently, we are in process of data organization and analysis of field data and flavor analysis of juice samples.

Within this quarter we successfully collected data on tree health, leaf and soil nutrition. We continue to analyze the data.

R. Ferrarezi 20-003 "Nitrogen fertilization of 4 to 7-year-old trees planted in high density" – 11% Completion of Objectives (June 2021 quarterly - initial)

The purpose of this project is to assess nitrogen (N) fertilization rates for high-density plantings of different scion and rootstock combinations in four commercial citrus growers. The overall approach is to 1) determine the appropriate N rates needed to support tree growth and productivity, 2) compare data obtained with existing UF/IFAS N recommendations, and 3) revise best management practices (BMPs) for N application in young trees planted in high-density plantings affected by Huanglongbing (HLB).

Field locations for the study are IMG Citrus Vero Beach, Graves Brothers Ft. Pierce, Peace River Citrus Ft. Meade, and Agromillora Florida/ Lost Lake Groves Lake Placid.

Yara has donated fertilizer for the first year (20 tons) for the study. Delivery was delayed due to logistics of finding a local distributor, blending the four different formulations, and delivering in Fort Pierce.

Fertilizer treatments were applied in all locations during this quarter and should complete the second fertilization for the season on 07/16/2021. AeroBotics will analyze images collected from drones to determine tree size and other required trial data.

E. Johnson 20-004 " Organic acids compared to conventional acidification for improved nutrient uptake and root physiology" – 18% Completion of Objectives (June 2021 quarterly - initial)

Objective 1: Determine effects of lowered soil pH on CLas populations and root physiology including internal root apoplast and vascular tissue pH.

Due to a collapse in our inoculum trees, we are psyllid-inoculating trees for these experiments
Objective 2: Field test multiple acidification materials including organic acids for tree response CLas suppression, nutrient uptake, and root and vascular pH changes.

We have marked out plots for our experiments at two sites on 15-year old and 14-year-old trees. We collected soil and leaf tissue samples for preliminary analysis. We have also purchased all necessary tools for evaluation include materials for lowering pH such as elemental sulfur, sulfuric acid, fulvic acid and humic acid and will apply these products in August 2021. We are currently determining canopy and trunk size and PCR measurements to establish baseline. We have also recruited postdoctoral associate to help with data collection and documentation of the results.

In the next quarter we will wait for psyllid-inoculated trees to develop initial symptoms before testing for CLas for objective 1.
Perform initial August treatment at the field sites
Additional instruments for measurements will be purchased in the next quarter.

Budget status :
Underspent because much of the equipment, treatment, and sampling costs were not spent until field sites were confirmed and plot design completed.

T. Vashisth 20-011 "Right Leaf Sampling-The first and most critical step to good nutrition program"
– 5% Completion of Objectives (Mar. 2021 quarterly - initial)

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 nutrients) levels change in the tree throughout the year.
4. To evaluate how the leaf age affects the leaf nutrient status.

The project started this quarter. The main goal for this quarter was to identify sites, mark trees, and collect baseline data.
We surveyed 10+ sites to finalize the 4 sites for this trial, two sites are on Ridge and 2 are in southwest.. In each site, more than 100 trees were manually screed for canopy density and disease index rating to identify trees as Mild and Severe. The final data trees were tagged and tree health data such as canopy volume, leaf area index, leaf chlorophyll, leaf size and weight, root density has been collected. Leaf samples for starch quantification and leaf, root and soil for nutrient analysis has been collected and are being processed. The spring flush has been tagged on the trees.

Anticipated for next quarter:
1. Data analysis and interpretation
2. Processing samples for starch
3. Tagging summer flush
4. Collecting samples for nutrient analysis
5. Applying fertilizer treatments based on leaf results

Budget status :
Currently, the project is still in the set-up phase.
The budget is spent as per the plan where major funds have been used for nutrient analysis. We have found dedicated personnel to work on this project starting this quarter.

Agromillora 21-001 – Contract signed; 50% deposit, balance on delivery of rootstock trees, expected summer/fall 2022.

4. OTHER CITRUS DISEASES

a. Post-Bloom Fruit Drop

M. Dewdney 18-034C "Improved postbloom fruit drop management and exploring PFD spread in Florida" – 100% Completion of Objectives (June 2021 final reports)

The objectives for this proposal are to 1) Conduct field trials of new products and fungicide programs for PFD management as well as validation trials for the Citrus Advisory System (CAS); 2) Investigate the reasons for the movement of Postbloom fruit drop (PFD) to new areas and recent major outbreaks; 3) Evaluate methods for initial inoculum reduction on leaves so that early fungicide applications could be more effective and identify the constituents of the flower extracts using "omics" techniques.

We conducted 7 field trials to evaluate how well the Citrus Advisory System (CAS) predicted PFD outbreaks from 2017 to 2021. During this period there were very few PFD outbreaks. In all four years, only one fungicide application was recommended by CAS. This was in 2019 in Fort Meade. In most seasons, there was no significant difference among the treatments, applications recommended by CAS, the older model PFD-FAD, Weekly applications and an untreated control. This indicates that the recommendation of no fungicide application was correct. We also looked at the economic savings of using CAS over the other fungicide application recommendation methods and found there were considerable savings. We also conducted five fungicide trials from 2018 to 2021. Unfortunately, we were not able to make solid conclusions about fungicide efficacy from these trials because the disease was not at high enough levels to statistically separate the treatments reliably.

We investigated how far the conidia of Colletotrichum acutatum (syn. C. abscissum) can travel under different wind speed conditions: 5, 10, and 20 m/s (~11 to 44 mph) with and without rain in a laminar flow wind tunnel. We also used a turbulent wind tunnel at 5 and 7 m/s with and without rain. We found that the secondary conidia formed on leaves could travel at least 15 m at the 15 and 20 m/s winds (~33 and 44 mph)with and without rain. This has not been observed before. We were unable to test primary conidia from flowers in the laminar flow tunnel because of COVID-19 and a mechanical breakdown. In the turbulent wind tunnel, the conidia from the leaves did not travel as far but could still move further than if from simple splash with and without rain.

We determined that the high polarity fraction of the sugars from citrus (flowers and leaves) can stimulate the germination of C. acutatum conidia nearly as well as the raw floral and leaf extracts. We did not expect leaf extracts to stimulate or stimulate as much as floral extracts, but surprisingly they did. We have tried most of the constituents of the high polarity sugar blend but no single constituent seems to work as well as the original extract. We tested the stimulation of conidia production on leaves with and without fungicide. Ferbam completely inhibits the production of conidia and Headline partially inhibited it. We are trying to figure out why the production of conidia are not stimulated by leaves in the field. Ultimately, we would like to test whether we could suppress inoculum by stimulating it and suppressing it with fungicide before it could infect flowers.

This is the final report and the budget is closed so no further spending will occur.
Dr. Dewdney will be invited to present these findings to the CRDF Board.

b. Citrus Black Spot

M. Dewdney 18-006 "Understanding the underlying biology of citrus black spot for improved disease management" – 82% Completion of Objectives (May 2021 quarterly)

Objective 1: Evaluate the optimal spray timing for Florida and investigate if tree skirting or alternative products improves fungicidal control of citrus black spot (CBS).

Objective 3: A MAT-1-1 isolate may enter Florida and allow for the production of ascospores. The industry needs to know if this happens, as it will affect management practices. Additionally, the existing asexual population may be more diverse than currently measured. If multiple clonal linages exist, then there may be different sensitivities to fungicides or other phenotypic traits. We also need to determine whether P. paracitricarpa or P. paracapitalensis are present in Florida for regulatory concerns due to misidentification. We plan to survey for the MAT-1-1 mating type, unique clonal lineages, and two closely related Phyllosticta spp.

We collected data from the large spray timing and skirting trial in March. We evaluated 50 fruit each for disease severity on approximately 125 trees in 32 rows. We made significant progress on the incidence analysis of the data and are close to finalizing the analysis. We found that fungicide program significantly reduced the black spot incidence compared to the control. The greatest reduction was from the Florida standard timing with applications from May to September. Skirting had no significant effect on the disease incidence but the interaction between fungicide timing and skirting did have a significant effect. The Florida standard timing with skirting was the best performing treatment. For the late fungicide timing (May to October), there was a slight decrease in incidence with the addition of skirting. It should be noted that the 2019-2020 season was light for black spot incidence and severity. The minor plots were re-randomized within the main plots and we were able to get the trial re-flagged just in time for the early spray (delayed by 2 weeks but no rain occurred from the first of April until after the early application). All applications were made on time in the spray trial. Data was collected from the trial and has been entered into the computer (~188,000 entries) and is being verified for accuracy. As noted previously, there was far more disease this year than 2020. We are examining the weather data from both seasons to see what factors rainfall and temperature may have played.

Pre-treatment data were collected at the end of March for the CBS spray trial. We have 15 treatments included in the trial. One application has been completed of a planned 6 applications with the 2nd scheduled for this week.

The second season of trials in which different fungicide products are tested for their efficacy to protect citrus fruit from CBS infection, is currently underway in South Africa. Products being evaluated include Enable (Indar) and Luna Experience sprayed on their own as well as Copper hydroxide sprayed in alternation with either Amistar Top or Headline (Cabrio). Fungicides are applied every 4 weeks from October 2020 until March 2021. The fungicides are being tested in a 'Valencia' orange orchard with a history of CBS. The trials will be evaluated at the end of August 2021.

An additional 8 South African and 8 global (from Argentina and Swaziland) isolates have been sequenced. As the next-generation sequencing data becomes available, the analysis and results are continuously updated to include the new data. DNA from 16 isolates (Argentina, Australia, Brazil and China) passed QC and is in the process of being sequenced. More isolates from Brazil and China are being cultured for DNA extraction and sequencing. Sixty-five P. citricarpa isolates (36 from South Africa, representing the five provinces where CBS is found, and 29 from other countries, Argentina, Australia, Brazil, China, Eswatini and USA) have been sequenced. Sequence data for 6 isolates, 1 from China, 1 from Brazil and 4 from USA are in the process of being generated. The sequencing data for the 65 isolates were analysed in the same manner as previously described, namely read mapping and variant calling, and in silico genotyping. Both analyses approaches showed the same patterns in terms of genetic distribution of isolates. Isolates from China are the most genetically distinct, while there is different degrees of genetic connectivity between isolates from Argentina, Australia, Brazil, Eswatini, USA and those form South Africa, and corresponds with previously published results (Carstens et al., 2017).

Objective 3 (Survey for the MAT-1-1 mating type and two closely related Phyllosticta spp.). Studies to determine what Phyllosticta spp. associated with citrus are present in Florida are on going. From 125 screened isolates using DNA sequencing of the translation elongation factor-alpha (tef-1α) locus, three isolates grew distinctly from species previously reported in Florida. Within the three new isolates identified, only two were screened using the internal transcribed spacer (ITS) and the actin (ACT). The result demonstrated that both isolates (Gc-6 and Gc-7) are from distant lineage from P. citricarpa and P. capitalensis and grouped with P. hymenocallidicola, a Phyllosticta species not previously associated with citrus. An assay was conducted on citrus fruit (Valencia and Meyer lemon) using isolates Gc-6, Gc-7, Gm33 (P. capitalensis), Gc-12 (P. citricarpa), and water (control) to determine if both isolates are pathogenic to citrus or not. The result showed that only isolate Gc-12 produces symptoms on citrus fruits, demonstrating that P. citricarpa appears to be the only plant pathogenic species associated with citrus in Florida and isolates Gc-6 and Gc-7 are non-pathogenic species in citrus. Moreover, a pathogenetic test was performed in Hymenocallis littoralis leaves because P. hymenocallidicola was originally described from this host. Leaves were inoculated using two methods (fungal plug and spore suspension), and similar symptoms (brown to reddish spots) were observed as in the only documented report. Koch's postulates for this disease had never been done. The screening and further characterization of these new species is continuing to obtain robust information on the diversity of Phyllosticta species and determine the presence of cryptic species in Florida.

Samples (fruit, twigs, leaves) were collected in the spring of 2021 from groves for mating-type screening using the conventional PCR primers described in Wang et al. (2016) to determine if previously undetected MAT1-1 idiomorph is present or not. Isolates are being purified so the study is ongoing to determine if MAT1-1 is still absent in the Floridian population or if it has entered Florida and the pathogen is reproducing sexually. Thus far, no isolate has been identified with MAT1-1.

To determine the phenology of fruit susceptibility inoculation studies of citrus fruit (Meyer lemon) were performed in a quarantine greenhouse at the Florida Department of Agriculture and Consumer Services (DPI) in Gainesville. A total of 97 fruit were used in this experiment. Of these 97 fruits, 25 served as controls and 72 were inoculated with the Gc-12 isolate of P. citricarpa. Disease assessments were performed weekly for a full calendar year. A total of 50 fruit produced symptoms in this period. All 50 were from fruit inoculated with the Gc-12 isolate, and no symptoms were observed in the control treatment. Therefore, from all inoculated citrus fruit, 69.4% produced symptoms, and 30.5% remained asymptomatic. The indications from this one experiment suggest that citrus fruit are susceptible at all stages of their development regardless of their maturation time. A second trial will be conducted in 2021 to determine the period of fruit susceptibility to P. citricarpa.

c. Citrus Canker

J. Jones 18-013 "Using a Multipronged Approach to Engineer Citrus for Canker Resistance" – 65% Completion of Objectives (May 2021 quarterly)

This project has focused on transforming Duncan grapefruit with genes that express EFR or a gene construct designated ProBs314EBE:avrGf2 that is activated by citrus canker bacteria virulence factors. Citrus transformed to modify the bs5 gene is being tested to characterize resistance to the citrus canker bacterium.

Objective 1. To determine if Bs3-generated transgenic grapefruit plants are resistant to diverse strains of the citrus canker bacterium in greenhouse experiments and to the citrus canker bacterium in field experiments in Fort Pierce. Characterizing different TAL effector variants to activate the transgene will determine if variants of TAL effectors are effectively targeted. To do this TAL variants are being inoculated into leaves of the transgenic plants. JJ5 and 5'-RACE will be used to identify transcription starts. The goal is to determine if several unique TAL effectors can activate transcription. As for developing a transgenic with ProBs314EBE:avrGf2, the construct has been placed in a different vector that is acceptable for future transgenic purposes. The previous constructs contain an additional selectable marker that allowed for identification of putative transgenics with a higher success rate. Given that there was concern about the additional marker, the new construct contains only NPT as a selectable marker. However, there were issues with the construct. Therefore, a new construct was recently sent to Vladimir Orbovic who is in the process of creating additional transformants.

Objective 2. To determine if EFR-generated transgenic grapefruit plants are resistant to the citrus canker bacterium in field experiments in Fort Pierce. The two most promising EFR transgenic plants (based on ROS activity) have been grafted onto two rootstocks (US 812 and sour orange) and planted in the field at Fort Pierce in collaboration Dr. Ed Stover. These plants will be rated in the next few weeks. Further greenhouse assays of ROS activity will be conducted to determine if other EFR transgenics may have more potential for resistance to citrus canker than the two currently in the field.

Objective 3. To determine if bs5-generated transgenic Carrizo plants are resistant to X. citri and to generate transgenic grapefruit carrying the pepper bs5. Budwood from UC Berkeley from two transgenic events was recently received and a third was from a tree that was run through the transformation process, but that was negative for the gene. The latter serves as the negative control as it had undergone the transformation process. Buds have been grafted and several have developed into branches. In the past few months neither of the branches that were created by CRISPR were actively growing. Recently, branches from both of the transgenic trees have begun growing and bacterial population experiments will be conducted to confirm the level of canker resistance.

d. Lebbeck Mealybug

L. Diepenbrock 20-002C "Developing near and long-term management strategies for Lebbeck mealybug (Nipaecoccus viridis) in Florida citrus" – 45% Completion of Objectives (June 2021 quarterly)

Work done this quarter on each of the project areas is as follows:
(1a) Develop monitoring methods to time management actions: The PI and team have developed methods for following the seasonal populations of lebbeck mealybugs and are working with 7 growers throughout central Florida to monitor populations. Based on data collection through the end of May 2021, there is some consistency in the onset of population development, with mealybug populations increasing in March and April concurrent with early fruit development. Lebbeck mealybugs appear to be highly attracted to developing fruit and establish populations around the calyx at fruit set. In our study and in a fruit drop study by a colleague, we are seeing high levels of fruit drop related to the mealybugs not related to physiological fruit drop. While we are not quantifying it in our phenology study, Dr. Vashisth has added this quantification to her fruit drop trials.

Along with population monitoring, the team is evaluating the potential use of virgin females as a lure for males with the intention of using these data to develop a targeted lure in the future to help reduce field populations and detect cryptic populations before they increase to damaging populations. No data yet, this method is in the preliminary stage.

(1b) Adjuvant screening: Preliminary trials have been conducted with adjuvants alone to determine their lethality to lebbeck mealybug. A total of 10 adjuvants, including 4 from Helena Agri Enterprises were mixed with DI water at label rates and sprayed until dripping on Volk lemon leaves with mealybugs attached. 9 of the 10 adjuvants resulted in significantly greater mealybug mortality over a 7-day period compared to a DI water control.

(1c) Evaluate promising materials in open grove setting: A field trial has begun at a 10-acre commercial citrus planting that was heavily infested in 2020. First insecticide applications occurred as a pre-bloom prophylactic spray of a systemic material, with a second spray in the end of April.

(1d) Ant Management: Testing a variety of methods to remove red imported fire ants (Solenopsis invicta) from citrus groves. Four experimental treatments were tested and compared to an untreated control: Chlorpyrifos drench, Clinch ant bait (Abamectin), Extinguish ant bait (S-methoprene), and spot treating ant colonies with hot water. Fire ant abundance was assessed by counting the number of surviving colonies, and by determining presence/absence of foragers with pecan sandie baits. Natural enemy abundance was assessed by dissecting mealybug clusters for predator larvae. Sampling for each of these methods took place every 2 weeks. Currently, all treatments have been applied and we are conducting follow-up surveys to determine their efficacy. Preliminary data shows that both insecticidal baits and hot water treatments significantly reduced fire ant colony abundance, while the chlorpyrifos drench had no effect on colony abundance. Impacts on mealybug predator population development is currently being quantified now that populations are starting to establish.

(1e) Evaluate management options for IPCs Entomopathenogenic Fungi (EPF) data shared last quarter. More work to come.

(2a) Predator assessments, Predators who directly consume prey: The commercially available predators Cryptolaemus montrouzieri, Orius insidiosus, Adalia bipuctata, Hippodamia convergens, and Chrysoperla carnea have been screened using no-choice assays to determine if they will feed on larval lebbeck mealybugs, and on mealybug ovisacs. Additionally, several wild-caught and lab reared predators have also been screened, including Harmonia axyridis, larva of the genus Ceraeochrysa (colloquially called trash bugs), Diomus austrinus, and Coccinella septempunctata. Both adult and larval C. montrouzieri readily feed on lebbeck mealybug larvae and ovisacs, as do larval Ceraeochrysa. Larval Chrysoperla carnea and adult Diomus austrinus consistently consume mealybug larvae, but do not consume ovisacs. Adult Orius insidiosus, early instar Adalia bipunctata, and adult and larval Harmonia axyridis do not feed on lebbeck mealybug larvae or ovisacs. Adult Hippodamia convergens, Coccinella septempunctata, and Adalia bipunctata do feed on some lebbeck mealybug larvae but appear to attack and kill the larvae more than fully consuming them. Neither feed on ovisacs. From study results, only Cryptolaemus montrouzieri shows promise as a commercially available biological control agent for lebbeck mealybug, although Ceraeochrysa larvae may act as natural biological control in the grove. Chrysoperla carnea may be useful for controlling mealybug larvae, but not reproductive adult females.

Parasitoids: Limited numbers of Anagyrus pseudococci, a commonly used parasitoid for mealybugs in greenhouses, were tested to determine if they would parasitize lebbeck mealybug adults and ovisacs. A. pseudococci adults were placed in arenas with adult lebbeck mealybugs, or ovisacs and left for 40-48 hrs. Surviving parasitoids were then transferred to adult citrus mealybugs and given an opportunity to parasitize them, to act as a positive control. Mealybugs were checked for parasitism after 16 days. The majority of parasitoids died in arenas with lebbeck mealybugs, and none parasitized adults or ovisacs. However, half of those that survived went on to parasitize citrus mealybug adults. These preliminary results suggest that A. pseudococci will not readily parasitize lebbeck mealybug, however further trials with more individuals and life stages of mealybugs are needed.

Gut content assays of field-collected potential predators: Species-specific primers for lebbeck mealybugs and a DNA extraction protocol have been established and used in feeding bioassays with the predator mealybug destroyers (Cryptolaemus montrouzieri). Using DNA extracted from the predators after they fed on the mealybug in PCR, we were able to model the length of time that the mealybug DNA fragment associated with our primers is detectable in mealybug destroyers. We found that the lebbeck mealybugs amplicon was detectable in 100% of mealybug destroyers for 4 hours after feeding, in 40% of mealybug destroyers up to 56 hours after feeding and was no longer detectable in mealybug destroyers 60 hours after feeding.

Field collections were executed in mealybug infested groves August 2019 through December 2020. Species identified as potential predators of lebbeck mealybugs were selected for DNA extraction and PCR using the lebbeck mealybugs primers. So far, we have found that green lacewings have the lowest abundance but the highest rate of positive detection of lebbeck mealybugs DNA. Field-collected mealybug destroyers has the next lowest abundance and the next highest rate of positive detection of lebbeck mealybugs DNA. The most abundant predators are spiders, but which have a far lower rate of positive detection of N. lebbeck mealybugs DNA. The spider which tests positive most frequently is the jumping spider, Hentzia palmarum. Predatory flies in the family Dolichopodidae will also be screened for lebbeck mealybugs DNA using our primers.

(2b) Determine how to implement mealybug management concurrent with other pest management programs: Starting in July 2021 for field efficacy then planning to test programs in Spring 2022.

(2c) Determine what insecticide chemistries inhibit feeding: We have completed EPG documentation of lebbeck mealybug to determine wavelength correlations on one host plant and will be starting on a second host plant once plants are voided of insecticides (3-4 month holding process in the greenhouse). Baseline feeding interaction data are necessary for a minimum of 3 host plants prior to including insecticides to look at feeding inhibition and the ability to kill the adult female in her ovisac.

(2d) Develop tools to minimize spread, killing crawlers with isopropyl alcohol: We tested different concentrations of isopropyl alcohol to determine how lethal they are to 1st instar lebbeck mealybugs. 50%, 70%, and 90% solutions of isopropanol were sprayed onto mealybug crawlers placed on cloth swatches and compared to a DI water control. Both 1 spray at each concentration, and 2 sprays at each concentration were tested. Mortality was assessed after 5, 10, and 15 minutes. The test was repeated, this time assessing mortality at 30 min, 1hr, and 2hrs. For all concentrations of isopropanol, 1 spray resulted in significantly greater mealybug mortality compared to the control. However, several mealybug crawlers remained alive and active after a single spray for all concentrations. 2 sprays of each concentration resulted in almost 100% mortality or incapacitation at all time points.

Using steam to kill adult mealybugs and ovisacs: Adult mealybugs and ovisacs may be accidentally transferred from infested groves on tools and equipment. Steam treatments may be an effective method of sanitizing equipment and killing both adults and ovisacs. Using a steam cabinet on-station, we steam treated adults and ovisacs at 100-, 120-, and 130-degrees Fahrenheit for 5, 10, and 15 minutes to determine mortality. Previous preliminary experiments showed steam treatments at 130 degrees Fahrenheit for 15 and 30 minutes resulted in 100% mortality. Mortality was assessed at 0, 3, and 5 days after treatment, and ovisacs were held for an additional 2 weeks to see if crawlers emerged. At 100 degrees F for all times points, adult mealybug and ovisac mortality was not significantly different than the control. At 120 degrees F for 5 minutes, mortality was also functionally 0%. However, mortality rose to 100% at 120 degrees F for 10 and 15 minutes. At 130 degrees F, mortality was 100% for all time points. This week we have begun testing ovisacs embedded in IPCs, which are insulating the ovisacs, requiring higher temperatures and longer time points.

2. Please state what work is anticipated for next quarter: (1a) Seasonal population monitoring will occur at 7 groves for one calendar year. Virgin female lure traps will be deployed once seasonal rains end. (1b) Adjuvant screening will continue to determine optimal adjuvants to work in synergism with insecticide sprays. We plan to take the most effective adjuvants and add them to an insecticide with good, but not great, efficacy on its own. (1c) We will continue treating and scouting the grove we have started a management comparison trial. (1d) The ant management project will continue throughout the upcoming quarter with the addition of monitoring for predatory insect establishment. (1e) IPC management trials will continue, incorporating conventional materials for management and spray penetration by tractor mounted sprayers (this was delayed from our work plan last quarter by unexpected challenges in establishing infestation). (2a) Working with FDACS, we have permission to deploy sentinel infested materials to screen more broadly for predators that may be present in the system which we missed with haphazard rearing from infested grove sites. We plan to do this starting in late summer 2021, once higher populations have established, which would attract a higher abundance and diversity of predatory insects. (2c) Feeding mechanisms will continue to be worked out using EPG with the future plan to determine if we can interfere with their feeding via chemical intervention leading to reduced offspring production. (2d) We will continue evaluating sanitation options and are planning to look at vehicle & equipment sanitation in the next quarter.

3. Please state budget status (underspend or overspend, and why): on target

e. Phytophthora

E. Johnson 19-010 "Determining new cost-benefit guided Phytophthora propagule treatment thresholds for HLB-affected citrus" – 45% Completion of Objectives (April 2021 quarterly)

The goal of this project is to develop new soil propagule density management thresholds and recommendations for chemical management of Phytophthora root rot based on economic analysis of yield responses in different soil conditions.

Yield was collected on the Valencia plots in the last quarter. Irrigation systems for chemigation treatments has been installed and the spring treatment has been put out. In all but one treatment the first treatment of the year is phosphite.

HLB inoculations of Phytophthora positive rootstocks have begun for a greenhouse experiment to determine if the newly labeled chemistries have the same limitation on HLB-affected plants as fosetyl-Al and mefenoxam have shown. Many of the new chemistries are directly effective against Phytophthora in the soil rather than acting after uptake by the roots, so this is likely to reduce the limitations of Phytophthora management in HLB-affected groves and test the hypothesis.

f. Nematodes

L. Duncan 19-016 "How do subterranean pests and diseases affect root health of trees with and without HLB?" – 65% Completion of Objectives (April 2021 quarterly)

Citrus Nematode Exp 1. The effects of Las and citrus nematode treatments on plant size and on nematode populations were described in the previous report. Subsequently, the titer of Las in the leaf midrib from samples taken from each plant just prior to termination of the trial was determined. The presence of nematodes increased the amount of Las in the plants. In both rootstocks, the bacteria populations were higher ($P<0.05$) in plants first treated with nematodes than in plants not treated with nematodes. In both rootstocks, plants treated with nematodes following inoculation with Las had bacteria titers intermediate between those of Las controls and Las following nematodes. In the resistant Swingle plants, there was an inverse relationship (-0.30, $P=0.02$, $n=60$) between the bacteria titer and the nematode progeny per gram root. Thus, the experiment demonstrated that nematodes increase the virulence of Las in citrus plants, whereas Las-infected plants are less suited to nematode population growth than non-infected plants. However, the net effects of the two pathogens on the plants were obscured in this trial due to contamination by Phytophthora. The experiment will be repeated with greater attention to preventing contamination.

Burrowing nematode Exp 2. Soil cores taken from the plants revealed a low population density of the nematode. Burrowing nematodes reared on carrots in the laboratory were inoculated again at rates of 7700 nematodes per plant.

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