CRDF Citrus Research and Development Foundation, Inc.

CRDF-Funded Projects Summary Progress Report FY 2020-2021

Quarter Ending March 31, 2021 (includes April reports received to date)

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" – 90% Completion of Objectives (Feb. 2021 quarterly)

Objective 1 Initiated final replicate of development time and fecundity assays and complete data analysis.
Objective 2. Determine the effect of antimicrobials on Las transmission.
Hypothesis: ACP will be less capable of transmitting CLas after feeding on antimicrobials because trees treated with antimicrobials are more likely to have lower CLas titers for acquisition.
This experiment was replicated in mature, infected citrus trees located in a research grove located at Lake Alfred, FL to determine whether field applications of foliar antimicrobials were capable of suppressing the acquisition of CLas. Eight-year-old CLas-infected citrus trees received foliar applications (of streptomycin, oxytetracycline, or receive no antimicrobials (Control). All trees were treated with monthly insecticide sprays. One day after the application, ten CLas-free insects (5 females and 5 males) per plant from a CLas-negative laboratory colony were caged on young leaves (flush) of treatment and control trees to analyze ACP survival, CLas-acquisition in ACP parents (P1) and progeny (F1), the total trees sampled consisted of 5 individual trees per treatment. Survival of ACP adults was monitored the day after inoculation. After one-week, P1 adults were collected in microcentrifuge tubes containing 1 mL of 80% ethanol, ACP adults were collected individually and then stored at -20°C for subsequent CLas detection using real-time PCR. Egg clutches remained on trees enclosed in mesh sleeves after parental removal. After the nymphs reached the adult stage (approximately 2 weeks after ACP inoculation), adult psyllids were collected for analysis.
Results
ACP adults are considered CLas-infected (positives) when CT values are below 35. The highest CT values were observed on P1 ACP feeding on bactericide treated trees when compared with an insecticide only treated trees in which the lowest CT values were observed. Overall, parental ACPs feeding on bactericide treated trees had lower CLas titers than P1 ACP feeding on insecticide-treated trees, suggesting that bactericide treatment reduced the acquisition of CLas by ACP adults. In this study, the F1 progeny was affected by predators and environmental conditions, resulting in lower numbers collected in comparison to P1 ACP. In the recent replicate, ACP feeding on monthly bactericide applications had significantly lower CLas titers as compared with ACP feeding on quarterly bactericide treated trees and insecticide only.
Conclusion: Overall, the data suggest that P1 ACP and progeny feeding on bactericide treated trees were less likely to acquire CLas from infected trees. ACP parents and progeny feeding on monthly bactericide treated trees showed the highest CT values and lowest CLas titers. Therefore, 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,

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and 3) trees treated with bactericides prior to ACP will receive some protected from CLas inoculation.

Anticipated wok\: Objective 1. Pending work on this project includes completing PCR on samples from bioassays and completing data analysis.
Objective 1. Complete data analysis.
Objective 3. Determine the effect of antimicrobials on plant response and associated ACP behavior. Pending work on this project includes completing PCR on samples from bioassays and completing data analysis.

Budget status: Our budget spent is in line with anticipated spending for the project during the past quarter.

S. Santra 18-020 "Novel multi-metal systemic bactericide for HLB control" – 70% Completion of Objectives (Feb. 2021 quarterly)

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) were included 2020 field trial. 8 year-old 'Ray Ruby' grapefruit block with windbreaks consisting of approx. 25 ft. tall Corymbia torelliana was selected in Vero Beach area. Foliar application rate included one gallon per tree (equivalent to 145 trees per acre, a total of eight foliar applications) starting June 1 and ending October 26, 2020. Early spring applications were missed because of COVID-19 work restrictions preventing delivery of materials. Disease evaluation assessed the incidence of fruit with canker lesions for 100 fruit per treatment from the middle 3 trees in each plot. Melanose and scab were also assessed at time of canker rating. Any phytotoxicity (peel burn) was noted and recorded at harvest. Yield was measured as lbs fruit per tree for the middle 3 trees in each plot. A subset of fruit from each plot was sized and tested for juice quality at the CREC packing house/pilot plant facility.

No monthly rainfall was detected in March 2020 when initial infection of the spring leaf flush would be expected. Monthly rainfall was about average from April to June and then dropped well below average from July through September. The lack of rainfall during the spring flush probably reduced inoculum buildup in the leaves and early fruit infection.

Despite the low rainfall early in the season, the incidence of fruit canker lesions increased slightly to 12.4% from 8.2% the previous year in the UTC. All experimental treatments performed well and comparable to the standard Cu, Kocide 3000. Although not significant, there was an apparent dose effect of ZnO with the lower rate having the highest incidence of canker other than the UTC. Most products performed numerically better than Kocide 3000, but this could be due to rate differences as the only product tested at multiple rates showed a rate response trend.

COVID-19 prevented early season treatments, so the full effect of the products on yield cannot be determined; however there was a significant retention of fruit compared to the UTC resulting in higher yields. The largest effect on yield this season was canker management. The lack of a significant yield difference between bactericidal products prevents direct inference about HLB efficacy; however, Kocide 3000 had one of the lowest yields of the bactericides, suggesting that some of the others may have some efficacy for HLB and improvements may require a couple years to become significant.

No significant differences were found in internal fruit quality based on brix-acid ratio or total soluble solids. Insufficient scab and melanose were observed to collect useful data this year.

Fruit size and juice quality data are being analyzed and will be reported in future report.

Z. He 18-040C "Evaluation of the spatiotemporal dynamics of bactericides within the citrus tree via different application methods" – 95% Completion of Objectives (Feb. 2021 quarterly)

1) Field study has been completed based on greenhouse study results. The study included two citrus varieties, Grapefruit and Valencia orange, in two farms located in the Fort Pierce area. Oxytetracycline and streptomycin applied into/onto citrus plants by foliage spraying, tree trunk-injection and root administration methods. Samples of citrus leaves, stems, roots and fruits from all the tested plants collected on the day 0, 7, 15 and 30 after treatments and brought back to the Lab for processing.
2) All the samples collected from the field study have been processed for extraction of oxytetracycline and streptomycin analysis purpose. The procedure used for the extraction of oxytetracycline and streptomycin included washing, air drying, cutting, liquid nitrogen grinding, weighing out, and solvent extracting, SPE column cleaning up nitrogen-air dying and bring up to targeting volume with special solvent for HLPC-MASS analysis.
3) The extracted samples for oxytetracycline and streptomycin analysis have been sent to cooperated institute, the Citrus Research and Education Center (CREC), UF for analysis purpose. However, the analysis has not been finished yet.

The work planned for the next quarter (March 1 to May 31, 2021):

The major research tasks for the next three months include:

1) Completing the analyses of the extracted samples
2) Processing of the data from the field trials.
3) Write the final report for this project.
4) Preparation of manuscripts from the data obtained from the greenhouse and field experiments.

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" – 50% Completion of Objectives (March 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 continued that was initiated in Dec 2020 (horticultural and plant health assessment; CLas and Frd titer determination). Although a stimulation of leaf growth was observed in some of the Frd treated plants, effects were thus far not statistically significant. The viability of the Frd bacteria was confirmed by plating leaf extracts on culture media and determining CFUs.

A small experiment was conducted with severely HLB-affected citrus plants in which Frd was applied by leaf infiltration. Although there were no clear reductions in CLas titers, plants that received Frd through leaf infiltration produced more new leaves and lateral buds than the mock-infiltrated plants. Currently an investigation is being conducted to determine if this may have been caused by the endophyte's effect on plant hormonal balance. Also, whether there were more viable Frd cells in the leaves after leaf infiltration than after leaf spray was determined.

An injection method suitable for injecting small diameter grafted greenhouse plants was optimized for controlled injection of liquid materials. The effectiveness of Frd applied by injection will be compared against oxytetracycline, which in recent field trials showed significant HLB curative effects.

Larger-size CLas infected (grafted) plants are in the process of being prepared for further experimentation.

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

This experiment will only be conducted if Frd applications (foliar or by injection) show efficacy in restoring health of infected plants.

Spray and injection treatments, collection of horticultural information, and CLas and Frd titer determination will continue.

b. Diagnostics

M. Irey 19-001C "Continued Support for the Southern Gardens Diagnostic Laboratory"
108% Completion of Objectives, based on volume of testing (March 2021 quarterly)

This report is for the continued support of the Southern Gardens Citrus Diagnostic Laboratory that provides testing for citrus greening for researchers, growers, and homeowners. The current report is for the 3rd quarter of year 2 of a 2-year project. For the 3rd quarter of year 2, a total of 10,230 samples were processed and assayed. This brings the total for the project-to-date to 48,550 samples assayed, or 3,550 over the budgeted amount for the whole project (8% over). The projection for the full 2-year period is for the lab to have processed ~23% more than the budgeted amount. There is a growing trend for sample submitters to request copy number determination instead of just a Ct value (52 % of the samples were provided with copy number for this quarter, 59% the previous quarter). This is likely due to the fact that the majority of the samples being submitted are coming from researcher trials where quantitative data are desired. All of the samples for the current period were plant samples. No ACP samples were submitted.

It is anticipated that a higher than budgeted amount of samples will continue to be submitted. This is based on an increase in the number of trials being established that require extensive sampling and to several researchers bringing in backlogged samples.

Given that we are running 8% above the budgeted amounts for the whole project (123% over if prorated for 7 quarters), it is likely that we will be over the budgeted number of samples for the project. As has been done in the past, we will wait until the end of year 2 of the project and adjust the final invoice either up or down depending on the total number of samples run during the project. If the number of samples exceeds the budgeted amount, the final invoice will be Increased upwards to cover the cost of the consumables. If the number of samples is below the budgeted amount, the final invoice will be reduced to reflect the reduced amount of consumables used.

2. ASIAN CITRUS PSYLLID VECTOR INTERVENTION

a. **Asian Citrus Psyllid management**

L. Stelinski 18-056C "Functional IPM for Asian citrus psyllid under circumstances of chronic HLB" – 100% Completion of Objectives – Final included on last report. PI presented findings to the Board.

L. Stelinski 19-002 "Why spray if you don't need to? Putting the IPM back into citrus IPM by ground truthing spray thresholds" – 66% Completion of Objectives (Feb. 2021 quarterly)

This project is investigating the potential of a usable economic threshold to increase sustainability of Asian citrus psyllid (ACP, Diaphorina citri) management in citrus under conditions of high huanglongbing (HLB) incidence. The year-long study began in the spring of 2020 in a young planting of "Hamlin" sweet orange grove under standard agricultural practices for citrus, located in Lake Alfred, Florida. ACP management strategies were tested in a randomized complete block design with four replicates. Insecticidal sprays for ACP were based on either of three nominal thresholds of 0.2, 0.5, and 1 adult per stem tap and ACP treatment sprays were comprised of two rotations of different mode of action (MoAs) designated as rotations A and B. 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. ACP were monitored by calculating the mean number of adults per tap across all four plots for each treatment. If the means reached or exceeded the target economic threshold, all replicate plots assigned to that treatment were sprayed. ACP sampling occurred every 7 to 15 d. If the mean ACP density for a threshold treatment was within ± 0.05 per tap below the target threshold, a decision was often made to spray that treatment rather than waiting until the following week. Sprays were made after plots were sampled, and insecticide susceptibility to thiamethoxam was determined. Thiamethoxam was chosen as bellwether to test for resistance development given that resistance in ACP is predominantly metabolic and this MoA typically predicts subsequent multiple resistance within populations of this pest. An insecticide program was designed to maintain ACP numbers as close to zero as possible in the treatments considering potential impacts on resistance risks. Broad-spectrum insecticides were restricted to the 'dormant' season, and when activity of natural enemies was expected to be reduced, whereas more selective insecticides were used during the primary growing season.

The average number ACP eggs, nymphs, and adults counted was higher in plots where the 1 adult/tap threshold was implemented than in plots where the 0.2 or 0.5 D. citri/tap economic thresholds were implemented for both rotation A and rotation B. There were no differences in treatment efficacy between the two rotations. Overall, there were no statistically significant changes in susceptibility of D. citri following the completion of the either rotation schedule triggered by either of the three treatment thresholds tested. Furthermore, GSTE1, GST1, EST6, CYP4D1, and CYP4C67 gene expression levels were not significantly different in ACP collected from different threshold treatment populations as compared with the susceptible control. These results allow us to conclude with confidence that resistance was effectively kept in check throughout the trial with proper insecticide rotation.

The incidence of HLB was determined by the level of CLas pathogen in each of the economic threshold treatment plots. There was 100 % infection of trees before the first application in the early spring. Therefore, the scoring and decline index of HLB was observed after harvesting and nutritional applications. All 480 trees were scored for each economic threshold treatment after harvest on a 0-4 scale, where category 0 = no HLB symptoms, normal growth flushes; category 1 = some HLB symptoms, mostly normal growth of flush; category 2 = some HLB symptoms, some normal growth; category 3 = obvious symptoms with no flush growth; category 4 = obvious HLB symptoms, including small leaves and dead wood and no new growth. The decline index was calculated for each treatment. Furthermore, for rotation A, greater input of insecticides (lower action threshold) was correlated with lower incidence of HLB.

The PI determined insecticide application costs and fruit drop rate for each economic threshold treatment. Insecticide costs were compiled from the University of Florida extension reports and the 2020-2021 Florida Citrus Production Guide. All prices were based on the products used in our tests. For fruit drop counts, each treatment threshold, 20 trees were counted per replicate plot and 80 trees total were counted per economic threshold treatment to determine mature fruit drop. Dropped fruit numbers were counted weekly for four weeks before harvesting. To estimate the level of CLas pathogen infection among treatment plots, three trees were randomly chosen for sampling per plot for a total of 24 trees for each rotation x treatment threshold treatment combination. Every tree sampled from each treatment plot was CLas positive. Similarly, all trees exhibited HLB symptoms. The results indicated that trees had similar symptoms of HLB in all treatment plots and ranged between categories 1-4.

The input costs of spraying at the 0.2 adults per tap economic threshold were estimated at $451.50 and $451.93/hectare for rotations A and B, respectively. The costs associated with the 0.5 adults per tap economic threshold were estimated at $288.88 and $284.38 per hectare for rotations A and B, respectively. Finally, at the 1.0 adult per tap economic threshold treatment, costs were estimated at $101.12 and $35.62 per hectare for rotations A and B, respectively. There were no significant differences in fruit drop between each economic threshold treatment compared. However, we have not yet finished analyzing the yield data that were collected.

Summary results indicate that an economic threshold could be implemented as a decision tool for timing insecticide applications in Florida and is compatible with a range of possible rotations of available insecticides for ACP management. Such rotational programs can maintain psyllids below population levels that negatively impact yield but can reduce the number of insecticide sprays needed per season. In the current investigation, although psyllid populations were reduced more effectively with the lower threshold that necessitated the most insecticide sprays per year (7), there was no difference observed in fruit drop or tree health between plots treated 2 times/year using the 1.0 psyllid / tap threshold and those sprayed 7 times/year using the 0.2 psyllids/ tap threshold. As we continue this research, we will continue to validate use of economic thresholds in mature trees and determine how use of economic thresholds impacts secondary pest and beneficial arthropod populations. Also, plans are underway on computing profits between the management programs evaluated by including the yield component into our calculations.

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" – 75% completion of objectives (Jan. 2021 quarterly)

This project is focused at developing, propagation and evaluation of new citrus rootstocks with enhanced tolerance to HLB in Florida conditions.

Coronavirus restrictions instituted by USDA has delayed some data collection and evaluation of plant material.

Create new candidate hybrids. Emphasis is being placed upon the hybridization among parents with superior tolerance to HLB, CTV, Phytophthora, along with outstanding influence on fruit yield and quality. Some of the best performing of the newest hybrids in current Stage 1 field trials are hybrids of US-942 and US-802, so additional hybrids with this parentage are being selected for further testing. Seedlings from these crosses are being grown-out in the greenhouse in preparation for propagation, testing, and establishment of seed trees.

Propagate and plant new field trials. Budwood increase trees of selected scions were grown, in preparation for budding trees for new rootstock trials. Nursery trees for Stage 1 with Valencia orange, and Stage 2 rootstock trials with Valencia, Hamlin, and Star Ruby grapefruit are being prepared in the greenhouse for field planting in 2021.

Collect data from field trials. 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 six replicated trials with Hamlin scion. Early fruit drop data was also collected from Valencia scion trials. Assessments of tree health and measurements of tree size were completed on 10 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.

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). 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. Performance data continues to be collected, but it is anticipated that 2-3 of the most outstanding of these will be officially released in 2021-22.

F. Gmitter 18-011 "Part A - The UF/CREC Core Citrus Improvement Program"
70% Completion of Objectives (Jan. 2021 quarterly)

This project is focused on the core citrus improvement at UF CREC and has 4 major reporting areas.

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. Authorization has been obtained to distribute seeds to licensed nurseries for the UFRs, and to collaborative nurseries and other organizations within Florida, U.S., and globally, to establish advanced trials to compare our best rootstock selections with industry standards. The existing rootstock trial files have been updated and two new files added to the website (https://crec.ifas.ufl.edu/citrus-research/rootstock-trials/), currently there is information from 26 locations. Seeds from 2020 diploid and tetraploid rootstock crosses have been planted into calcareous, high pH soil inoculated with two species of Phytophthora, the first step of the `gauntlet' screening. Rooted cuttings from more than 50 gauntlet rootstock candidates have been plant into citripots in preparation 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. Embryo rescue from 37 interploid crosses made using selected HLB tolerant plants in 2020 was completed, including 10 crosses for each of red grapefruit, sweet orange and mandarin. Shoots have been generated from many of the excised embryos, and flow cytometry was used to verify triploidy among some of the hybrid populations. Micrografting of the earliest to germinate normal shoots has begun and will continue through the next quarter. Seeds harvested from 2 diploid crosses made for sweet orange improvement were planted and have germinated.

3. Screen our ever-growing germplasm collection for more tolerant types and evaluate fruit quality of candidate selections. Evaluations of existing breeding populations in the field were somewhat restricted by UF COVID travel regulations, but we nonetheless went through our materials and made selections for further evaluations and testing. Four new HLB tolerant red grapefruit hybrids, one sweet-orange like hybrid, and three seedless easy-to-peel mandarins were selected and submitted to the DPI Parent Tree Program for cleanup and production of certified budwood for future trials. 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 by numerous sources to be nearly resistant to CLas attacks, by collecting detailed HLB phenotypic data, including Ct values and other tree health measures. The frequency of CLas-negative trees remains unexpectedly high.

4. Conduct studies to unravel host responses to CLas and select targets for genetic manipulations leading to consumer-friendly new scion and rootstock cultivars. Selections of approximately 450 mandarin hybrids for GWAS studies have been made, 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.

5. Other related activities. We have continued testing the alternating temperature chamber regime to produce CLas- and CTV-free lines of promising new scion and rootstock candidates for propagation and to hasten their use in advanced field trials. Five Vernia seedling selections, one grapefruit hybrid, and ten rootstocks were treated, and all were PCR negative, and will now be tested for CTV.

T. McNellis 18-016 "Testing grapefruit trees expressing an anti-NodT antibody for resistance to HLB" 75% Completion of Objectives (Feb. 2021 quarterly)

This report covers the period of December 1, 2020 - February 28, 2021. The objective of this project is to test transgenic 'Duncan' 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 to assess trees, take samples, and send samples for qPCR analysis at Southern Gardens. Chad visited Fort Pierce for more than one month from late December, 2020, to late January, 2021. He took samples from a field trial natural inoculation, an Asian citrus psyllid (ACP) infection in the greenhouse, and a graft challenge with FT-scFv scions grafted to HLB-infected rough lemon rootstocks. These samples were prepared and sent to Southern Gardens for qPCR detection and quantification of 'Candidatus Liberibacter asiaticus' (CLas) within the tissues. Data are anticipated to be received from these samples in March, 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. However, these data are still being analyzed and we anticipate full analysis in the next reporting period. A second run of this experiment was initiated by additional grafting of transgenic and control scions to infected and uninfected rough lemon during Chad's most recent visit in order to determine whether this effect is replicable. Finally, our publication of the accelerated blooming phenotype of FT-scFv transgenics was published in the February issue of Plant Biotechnology Journal and was honored with being selected as the cover feature of that issue. A copy of the finalized publication and journal cover is included in this report.

Work for next quarter:
qPCR data from the field, graft, and psyllid-transmission HLB challenge tests will be received from Southern Gardens in March, 2021. These data will be analyzed during the next reporting period. Additional sampling of plants from the psyllid-inoculated and field grown trees will be performed with help from collaborators at University of Florida and the USDA USHRL. These will be sent to Southern Gardens for qPCR CLas quantification for an additional infection time point. In March, 2021, we plan to submit a request for a second, six-month, no-cost extension in order to complete the time courses for CLas quantification and characterization of any potential HLB tolerance of the FT-scFv transgenic lines. This no cost extension request will be especially critical to completing the field test of the transgenics, since so far we do not detect CLas infections in those trees. In addition, it will be important to re-test the graft-transmission response of the FT-scFv scions to CLas infection, since it appears that they may have some tolerance to CLas infection. A no-cost extension

would also enable a summer visit by a student or the PI to Fort Pierce to perform additional sampling and tree assessments.

3. Please state budget status (underspend or overspend, and why):
Budget spending is on track, considering delays due to COVID-19. An amended budget by was developed and submitted in February, 2021.

Z. Mou 18-017 "Establish early-stage field trials for new HLB-tolerant canker-resistant transgenic scions" – 40% Completion of Objectives (March 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) Transgenic citrus plants for field trials were maintained in the greenhouse. These plants will be transplanted into the field on May 20, 2021.

2) Cloning the citrus gene encoding 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS). We have constructed a vector based on citrus DNA sequences for generating cisgenic or intragenic citrus plants. However, the transformation efficiency of the vector is extremely low. We plan to develop a transformation selection method based on citrus DNA sequences to facilitate this process. It has been shown in other plant species that an EPSPS variant is able to provide tolerance to glyphosate. We thus cloned the citrus EPSPS gene full-length coding sequence from sweet orange total cDNA. This gene will be mutated to create a similar citrus EPSPS variant.

3) Optimizing conditions for analyzing nicotinamide adenine dinucleotide-binding activities of a group (10) of citrus lectin receptor kinase proteins using Monolith NT.115. Four different buffers with a pH value in the range of 5-8 have been tested. Although binding activity was detected for some of the proteins using the binding test model, a reliable Kd value has not been achieved. Our goal is to find the functional citrus nicotinamide adenine dinucleotide-binding receptor for generation of intragenic or cisgenic citrus plants. We are testing the transgenic citrus plants expressing the Arabidopsis nicotinamide adenine dinucleotide-binding receptor for HLB resistance/tolerance.

E. Stover 18-022 "Delivery of Verified HLB-Resistant Transgenic Citrus Cultivars"
65% Completion of Objectives (Feb. 2021 quarterly)

Objective 1, Mthionin Constructs: Assessment of the Mthionin transgenic lines is ongoing. As the most proven of our transgenics, we continue to use them as a reference in detached leaf assays, as well as studying them in established greenhouse and field trials. Greenhouse studies (With 9 Carrizo lines and 4 Hamlin lines, 98 total plants with controls) include graft inoculation of Carrizo rooted cuttings with CLas+ rough lemon, no-choice caged ACP inoculation of Carrizo rooted cuttings, and no-choice caged ACP inoculation of Hamlin grafted on Carrizo with all combinations of WT and transgenic.

Data collection has continued for the two Mthionin field plantings. The first plantings of transgenic or control Carrizo grafted with non-transgenic rough lemon scions (45 total plants) have shown transgenics maintaining higher average CLas CT values (2.5 CT higher @ 18 months), but with a high degree of variability. The larger second planting of transgenic Carrizo with WT Hamlin scions, transgenic Hamlin on non-transgenic Carrizo and WT/WT controls (205 total plants) have shown very encouraging results; with the transgenic Hamlin on WT Carrizo having statistically greater trunk diameter, tree height and canopy volume compared to controls. Scheduled assessments for both plantings have been prioritized under pandemic conditions, allowing the 36-month field assessment of the first planting and 24 month assessment for the second planting to be completed. Leaf samples associated with these assessments have also been collected and are being processed for CLas quantification.

Additional grafts of WT Hamlin and Ray Ruby scions to Mthionin rootstock were made and are included in the ongoing chimera planting discussed in Objective 2. The Mthionin construct has also been extensively transformed into Valencia, Ray Ruby and US-942 to provide transgenic material of these critical varieties. The first 56 putative lines from these transformations are in soil and undergoing expression analysis.

Objective 2, Citrus Chimera Constructs: Detached leaf assays with CLas+ ACP feeding have been conducted for 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 3rd generation Carrizo lines is complete and the analysis of phloem specific and scion-type lines 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). The best performing 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 in the CLas+ plants. However, many plants remained CLas negative due to low inoculation efficiency. In June, 150 plants representing the best performing 7 lines of `188' and 6 lines of `74' were no-choice caged ACP inoculated using a new protocol to improve inoculation rates. At 3 months, control plants tested positive at twice the rate of the earlier inoculation; 6-12month tissue samples are now collected and processed, awaiting qPCR analysis.

A large greenhouse study is 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 leaf collection will be collected in March. An additional ~1200 rooted cuttings have been made from those same lines for matching ACP-inoculated greenhouse and field trials. An earlier field planting of 1st and 2nd generation lines (~400 plants of Mthionin, `74', and `188') is also underway. The first 165 plants (WT Hamlin and Ray Ruby on transgenic Carrizo) went into the soil in August 2020, a second set of 70 WT Valencia on transgenic Carrizo are ready to be moved to the field this season and the remaining 200 transgenic Hamlin on WT rootstocks are being grafted.

Eighteen new transformations, totaling over 6200 explants, have been completed to generate sufficient events of Valencia, Ray Ruby, US-942, and Hamlin (when not already complete) lines expressing `74', `188', TBL, TPK and other advanced chimera constructs. Over 325 new putative transgenic 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 and undergoing expression analysis.

Objective 3, ScFv Constructs: greenhouse studies on 5 scFv lines inoculated with Clas+ ACP has 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 will begin in March 2021. An additional 129 rooted cuttings are propagated for follow up plantings with a Hamlin scion. A second greenhouse trial (150 plants from 12 lines) have been bud inoculated with HLB+ RL. A third set of 370 plants for greenhouse trials has been propagated with the first 54 plants to reach a suitable size ACP-inoculated using the improved protocol. Tissue from both trials for testing CLas titer has been collected and processed; now awaiting qPCR analysis.

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. Several peptides screened through this assay have since been confirmed to reduce CLas titer by foliar application to grapefruit trees in tests performed by CRADA partners. Citron, Hamlin and Valencia trees have been selected and blocked for trunk application trials using these peptides. 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 has undergone several small revisions to improve sensitivity and maintain consistent inoculation by adjusting feeding period and ACP numbers. Analysis of ACP bodies to has been expanded to include quantification of major endosymbionts (Wolbachia, Profftella, and Carsonella) 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 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) and Carrizo/Mthionin (2 lines) have been returned certified clean.

In addition to the use of the AMP Mthionin and its chimeric variants, new strategies have been implemented. 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 for their ability to kill ACP. From these, 12 lines (4 event 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 (55 Onyx and 131 Topaz plants) that will enter greenhouse trials as soon as the plants are of appropriate size. The available Onyx transgenic material is being expanded with 40 Hamlin and Carrizo plants transformed with the phloem specific version and 33 Ray Ruby and Valencia plants with the constitutive version undergoing expression analysis.
Down regulated DMR6 Carrizo transgenic citrus, either by expression of specific hairpin RNA or by specific Cas9-sgRNA were generated, cloned, and are ready to be assessed. Since DMR6 is a broad immune suppressor, downregulated transgenic plants will be first evaluated for Canker resistance as a quicker assay. For that, clones from 5 selected lines are being inoculated with Xanthomonas citri and data will be collected soon. As an effort to accelerate the development of non-transgenic HLB resistant plants through gene editing, early flower gene (carrying FT-SCFV gene) was transformed into plants with the DMR6 targeting CRISPR construct. A set of 30 plants resulted from this gene stacking effort will be evaluated for the presence of both genes.

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" – 65% Completion of Objectives (Mar. 2021 quarterly)

1. Stated project objectives and what work has been done this quarter to address them:
This project is reporting on two major objectives.

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 minirhizotron imaging system continued in all trials.

Leaves were collected in trials 2 and 3 for CLas detection; samples are being analyzed. Trials 2 and 3 third-year horticultural data collection (tree size, health ratings, etc.) was completed. We also counted and collected fruits for fruit quality analysis and yield determination in trials 2 and 3. There were very few fruits in trial 2, but fruit quality analysis was completed for trial 3. We are working on completing the analysis of the year 3 data of trials 2 and 3 to be included in a publication of all three years of data.

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. We are still working on the PCR and ELISA assays.

2. Please state what work is anticipated for next quarter: We will continue with our minirhizotron root imaging and other data collection. We will continue with the statistical analysis and interpretation of all data collected. A Citrus Industry Magazine article will be prepared summarizing findings of this project.

3. Please state budget status (underspend or overspend, and why): Approximately 65% 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" – 65% Completion of Objectives (Mar. 2021 quarterly)

1. Stated project objectives and work that was done this quarter to address them include:

This project is reporting on two major objectives.

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. The team has conducted additional HLB foliar disease symptom and canopy color ratings at both Valencia trials for seasonal comparison. Leaves were collected for additional nutrient analysis and CLas detection. Valencia fruit samples were collected at the Basinger location for fruit quality analysis in advance of the harvesting. Fruit quality analysis was completed for these samples at the CREC pilot plant. We are working on the leaf CLas analysis. The team is continuing with the statistical analysis of all new data and are working on a manuscript for publication of three years of Valencia data.

Objective 2. Develop outreach to transfer information to growers and other industry clientele. Nothing to report in this quarter.

2. Please state what work is anticipated for next quarter: Fruits will be collected from the Valencia trees at the Lake Wales (Camp Mack) location for fruit quality analysis, and yield will be assessed.

Data analysis is continuing. A rootstock seminar including trial updates will be given in June at SWFREC (or virtual, depending on the Covid-19 situation).

3. Please state budget status (underspend or overspend, and why): Approximately 65% 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" – 56% Completion of Objectives (Feb. 2021 quarterly)

The Millennium Block trials are addressing the need of establishing field plantings to generate regional, updated information for the Indian River (IR) Citrus District. 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.

The four trials are: T1) grapefruit cultivars on 3 rootstocks, T2) Ray Ruby grapefruit on 36 rootstocks, T3) Glenn navel orange 56-11 on 30 rootstocks, and T4) UF-950 mandarin on 30 rootstocks.

As of this quarter, 3,400 trees were planted in September 2019 and 1,100 trees in August 2020 and the remaining trees on rootstocks UFR 7 through 14 will be available from the nursery in Spring 2021. Tree height and width and trunk diameter were measured for the 3 central trees in each plot on
February, July and November 2020, and February 2021, and canopy volume calculated. Preliminary results from October 2020 show some scion-rootstock combinations are exhibiting significant differences in canopy volume. In T1, Pummelette UF-5-1-99-2 grapefruit on US-942 was 4x larger than Star Ruby grapefruit DPI-60 on X-639. In T2, Ray Ruby grapefruit CGIP-103 on Citrus amblycarpa + Volk x Orange 19-11-8 was 3x larger than on UFR-17. In T3, Glenn navel orange F-56-11 on 2247 x 6070-02-2 was 5x larger than on Willits. In Trial 4, UF-950 mandarin on US-897 was 5x larger than on WGFT+50-7. Cultivars grafted on different rootstocks have no differences in tree phenological stage.

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, results for treatments were negative (Cycle threshold > 32), but there are several positive trees with visible symptoms by September 2020.

Asian citrus psyllids, Diaprepes root weevils, whiteflies, and other insects are at non-damaging levels in the trials, except for leafminers, 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.

The Ferrarezi Lab organized a very successful drive-thru field day to showcase the results to growers on 10/09/2020 with 49 attendees (limited by covid-19 regulations). An estimated 24,000 acres of citrus were represented at the event (70% of the current grapefruit industry acreage, highlighting the importance of the event. Attendees came from local and neighboring counties including St. Lucie, Charlotte, and Okeechobee. Another field day took place on 12/10/2020 with 4 large growers and industry leaders.
Overall, trees are building up vigorous canopies, and morphological. The study is beginning to show remarkable differences in horticultural attributes among scions/rootstocks Longer-term evaluation will be required to identify the most promising scions and rootstocks to determine their profitability and capability of meeting grower and market needs.

J. Grosser 18-039C "Part B - The UF/CREC Citrus Improvement Program's Field Trial Evaluations" – 80% Completion of Objectives (Apr. 2021 quarterly)

True sweet oranges: New OLL sweet orange candidates repeated for early-mid season maturity (consecutive years), including OLL-FB-4-13 and OLL-FB-9-33. OLL-FB-4-13, OLL-FB-9-33, and OLL-FB-1-22 were selected for inclusion in the CRDF scion trial and were entered into the Parent Tree Program. A new selection OLL-FB-4-08 was identified with January 1 maturity (first time fruiting).

Potential HLB resistance from 'gauntlet' rootstock candidates: qPCR was run on leaves sampled from more than 625 gauntlet rootstock trees at the USDA Picos Farm, and 54% tested questionable or negative for active infection. Among these, qPCR was run on roots on 60 of the best performing trees. Of these, 13 trees had root ct values of 40, meaning no bacteria was detected in the roots. Two of these also had leaves that tested negative and 4 others questionable. We also identified 19 gauntlet rootstocks in this group that were negative for active root infections, and among these 4 had no active infections in the leaves, and 9 had questionable leaf infections. Considering the severe fruit quality problems, especially from young trees the past 2 seasons in Florida, we also focused on identifying the best of these producing high brix fruit when in the early production. 3 potentially resistant rootstocks were identified producing high brix fruit: N+HBPxOrange19-12-3 (a backcross of UFR-4 onto its mom), B11-R5-T25-11-3 (a Flying Dragon hybrid with potential for high density plantings), and A+HBPxCH+50-7-12-11 (also tree-size controlling, with UFR-6 pollen parent). Several gauntlet rootstocks are also showing an apparent tolerance mechanism, as they show consistently good tree health, but had higher CLas titers in the roots than the trees above; these include a few hybrids made with SugarBelle.

Identification of probable zygotic rootstocks at St. Helena showing good HLB tolerance: 5 trees on apparent zygotic rootstocks were identified producing 3 to 3.5 boxes of Vernia/Valquarius fruit per tree with 12 - 12.5 brix. One is a tetraploid from Orange 12 (Nova+HBP-derived), two from HBPummelo x Shekwasha, and two from HBPummelo (open pollination). One rootstock genotype has been recovered, and scaffold roots of the other selections have been cut in efforts to retrieve the rootstock germplasm.

Fruit quality data was collected from the Trailer Park trial site - two combinations of 3.75 year-old trees made 6.0 and 5.9 lbs solids per box, Valencia B9-65/UFR-4 and OLL-4/UFR-4, respectively.

Yield data was collected from the St. Helena trial. There was no issue with fruit drop, and lbs. solids across more than 50 rootstocks averaged around 6.0 lbs. solids per box. Overall yield increased slightly. These positive results were attributed to the continued year-round use of CRF containing an enhanced micronutrient package. Yield data was also collected from the Peace River Valencia/rootstock trial, and the Duda trials.

Data analysis and entry onto the Rootstock Data Website with annual updates included: Duda Valencia APS rootstock trial, Duda Vernia rootstock trial, Smoak Valencia rootstock trial, Bryan Paul Doe Hill multi-scion rootstock trial, and the IMG navel/grapefruit rootstock trial. Data analyses for subsequent trial updates was performed for the following locations and are in progress: Banack multi-scion rootstock trial, Hidden Golf Trailer Park trial, Wheeler Bros. scion/rootstock trial, Orie Lee OLL clone/rootstock demonstration trial, Teaching Block scion/rootstock trial, Bryan Paul Doe Hill Grove multi-scion/rootstock trial, Citra (PSREU) scion/rootstock trial, Hammond IR Minneola rootstock trial, IR Marsh grapefruit/rootstock trial, the St. Helena rootstock survey trial, and trial and the Peace River Valencia/rootstock trial.

E. Stover 18-058C "Fort Pierce Field Test Site for Validating HLB and/or ACP Resistance" – 75% Completion of Objectives (Mar. 2021 quarterly)

Update for this quarter:
A substantial infrastructure renewal project is underway at the Picos location on USDA based funding. Renovation of the water management system has been completed and will provide improved storm protection. Drainage channels were re-dug and cleared of vegetation. Culverts were inspected and replaced if damaged. The road surfaces have also improved to provide better vehicle access. Tree maintenance and field trials have received priority support under current conditions. Full assessments have been conducted on USDA transgenic plantings by onsite personnel. UF collaborators have been permitted into the test site; samples and data have been collected. A manuscript detailing results from the canker resistance assessment of replicated trifoliate and trifoliate hybrids planted in collaboration with NCGR-Citrus/Dates and UC Riverside was accepted for publication in Hortscience as "Incidence of Asiatic Citrus Canker on Trifoliate Orange and its Hybrid Accessions in a Florida Field Planting." This site is also participating in a trial program to use drone based aerial photography for mapping and HLB assessment. The primary BRS permit, which covers the transgenic materials planted by Z. Mou, J. Jones, T. McNellis as well as USDA scientists has been renewed (AUTH - 0000043619 effective 1/27/2021).

Recent quarters:
An additional permit has been approved (AUTH - 0000043620 effective 12/17/2020) for material with "Confidential Business Information" for a project led by R. Shatters. Stover analyzed data on canker incidence for as "Incidence of Asiatic Citrus Canker on Trifoliate Orange and its Hybrid Accessions in a Florida Field Planting." Most notably: Almost all accessions with lower ACC lesion incidence were hybrids vs. pure trifoliate, though a few pure Poncirus had lower ACC than most. Based on chloroplast genome data from 57K Affymetrix SNP chip, provided by M. Roose, 11 of 33 "reported" seed parentage for hybrids was inaccurate, convention of "female first" was not followed. Of 34 hybrids validated, similar numbers had Poncirus, grapefruit, and sweet orange

chloroplasts. Chloroplast type did not affect ACC incidence, but in each year accessions with grapefruit chloroplasts had small but statistically higher ACC severity than those with Poncirus chloroplasts. Hybrids of Citrus with Poncirus have markedly reduced ACC sensitivity compared to Poncirus, indicating that this trait is readily overcome in breeding. A manuscript has been prepared and submitted from this data.

Previously established at the site:
A number of trials are underway at the Picos Test Site funded through the CRDF. A detailed current status is outlined below this paragraph. Continuation of an experiment on pollen flow from transgenic trees. FF-5-51-2 trees are slightly more than 1000 ft from the US-802, and are self-incompatible and mono-embryonic. If pollen from transgenic trees is not detected from open-pollination, it should reduce isolation distances required by BRS. Early-flowering transgenic Carrizo (flowered ex-vitro within five months of seed sowing, and used at 12 months) was used to pollinate some of the same FF-5-51-2 What should be the final samples from the C. Ramadugu-led Poncirus trial (#3 below) completed preparation and were shipped in ethanol to UC Riverside.

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

Current Plantings:
1) The UF Grosser, Dutt and Gmitter transgenic effort has a substantial planting of diverse transgenics. These are on an independent permit, while all other transgenics on the site are under the USDA permits.

2) Under the Stover permit, a replicated planting of 32 transgenic trees and controls produced by Dr. Jeff Jones at UF were planted. These trees include two very different constructs, each quite specific in attacking the citrus canker pathogen.

3) A broad cross-section of Poncirus derived material is being tested by USDA-ARS-Riverside and UC Riverside, and led by Chandrika Ramadugu. These are seedlings of 82 seed source trees from the Riverside gene bank and include pure trifoliate accessions, hybrids of Poncirus with diverse parents, and more advanced accessions with Poncirus in the pedigree. Plants are replicated and each accession includes both graft-inoculated trees and trees uninfected at planting.

4) More than 100 citranges, from a well-characterized mapping population, and other trifoliate hybrids (+ sweet orange standards) were planted in a replicated trial in collaboration with Fred Gmitter of UF and Mikeal Roose of UC Riverside. Plants were monitored for CLas titer development and HLB symptoms. Data from this trial should provide information on markers and perhaps genes associated with HLB resistance, for use in transgenic and conventional breeding. Manuscripts have been published reporting HLB tolerance associated QTLs and differences in ACP colonization. Trees continue to be useful for documenting tolerance in a new NIFA project.

5) A replicated Fairchild x Fortune mapping population was planted in an effort led by Mike Roose to identify loci/genes associated with HLB tolerance. This planting also includes a number of related hybrids (including our easy peeling remarkably HLB-tolerant 5-51-2) and released cultivars. Genotyping, HLB phenotyping and growth data have been collected and will continue to be conducted under a new NIFA grant.

6) Valencia on UF Grosser tertazyg rootstocks have been planted for several years, having been CLas-inoculated before planting, and several continue to show excellent growth compared to standard controls (Grosser, personal comm.).

7) In a project led by Fred Gmitter, there is a planting of 1132 hybrids of C. reticulata x C. latipes. C. latipes is among the few members of genus Citrus reported to have HLB resistance, and it is expected that there will be segregation for such resistance. The resulting plants may be used in further breeding and may permit mapping for resistance genes.

8) Seedlings with a range of pedigree contributions from Microcitrus are planted in a replicated trial, in a collaboration between Malcolm Smith (Queensland Dept. of Agriculture and Fisheries) and Ed Stover. Microcitrus is reported to have HLB resistance, and it is expected that there will be segregation for such resistance. The resulting plants may be used in further breeding and may permit mapping for resistance genes.

9) Conventional scions on Mthionin-producing transgenic Carrizo are planted from the Stover team and are displaying superior growth to trees on control Carrizo.

10) Planting of USDA Mthionin transgenics with 108 transgenic Hamlin grafted on wild type Carrizo (7 events represented), 81 wild type Hamlin grafted on transgenic Carrizo (16 events represented) and 16 non-transgenic controls.

11) Grafted trees of conventional sweet orange and grapefruit scions on transgenic rootstock expressing antimicrobial citrus-thionin and bacterial recognition domain fusion proteins (165 trees with controls) as a collaboration between USDA and Innate Immunity.

12) Planting was made of transgenics from Zhonglin Mou of UF under Stover permit, with 19 trees of Duncan, each expressing one of four resistance genes from Arabidopsis, and 30 Hamlin expressing one of the genes, along with ten non-transgenic controls of each scion type.

13) Transgenic trees expressing FT-ScFv (12 transgenic and 12 control) to target CLas from Tim McNellis of Penn State Univ.

14) Numerous promising transgenics identified by the Stover lab in the last two years have been propagated and will be planted in the test site.

N. Wang 18-064C "Evaluation of the control effect of bactericides against citrus Huanglongbing via trunk injection" – 75% Completion of Objectives (Apr. 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. 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. Field experiments were performed at four different groves on different aged trees with a different disease severity. 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. The 1st application of spray treatments was 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 the following 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 estimation 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 in a 3-months interval. 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 of10 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 the following 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 by Las carrying buds in February 2020. These plants are being tested for CLas infection and 4 plants were confirmed 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.

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. The samples were sent out for sequencing to monitor the mutations of OTC and STR resistance related genes

E. Stover 18-065C "High -Throughput Inoculation of Transgenic Citrus for HLB Resistance" – 78% Completion of Objectives (Mar. 2021 quarterly)

The goal for this three-year project is to evaluate citrus germplasm for tolerance to HLB, including germplasm transformed to express proteins that might mitigate HLB, which requires citrus be inoculated with CLas.

Progress January to March 2021:
The Bowman lab has established three new experiments with grafted Valencia trees on groups of rootstocks which were inoculated with ACP. Each experiment compares replications on nine different rootstocks. For that, 189 trees were inoculated with 3,780 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 monitor 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.). Six detached leaf assays experiments, involving individual 290 leaves, were inoculated using 2,900 Clas infected ACPs. Transgenic material tested in DLAs were of three constructs (ONYX and two Chimerical Thionin), and a total of 46 independent events were tested alongside WT controls.

The balance of Covid-19 restricted lab hours was spent processing the leaf and ACP samples in preparation for CLas qPCR. substantial ACP mortality from feeding on CLas-killing transgenic leaves continues to be observed.

In addition, 600 CLas+ ACP were provided to Dr. YongPing Duan of USDA.

E. Johnson #19-009 "Whole tree vs. rootstock or scion tolerance to HLB" – 35% Completion of Objectives (Jan. 2021 quarterly)

Propagation of experimental trees has proved more difficult than anticipated. Trees have been transplanted for the first repetition into split root rhizotrons and are preparing for HLB inoculation and sampling. The postdocs in Drs. Johnson's and Wang's labs have coordinated and done test runs of sampling procedures for RNA and metabolite samples, which will begin 2 weeks after inoculations.

Very high success rates for producing Valencia on Swingle trees have occurred, all other combinations that included either Sugarbelle or UFR-4 had lower than expected success rates and the trees continue to underperform compared to the Valencia/Swingle. Although the PI thought he had enough plant material for both experiments vigorously growing, some of the combinations have struggle recently. The PI is contacting nurseries to see if they can provide sufficient number of grafted trees for the 2nd rep by next spring.

*From what is being reported, it appears that the project is struggling to meet the objectives in the original proposal. Difficulty in producing sufficient number of trees for the study appears to be an issue.

N. Killiny 19-015 "Evaluation of the tolerance of newly developed citrus cultivars, on different rootstocks, to Huanglongbing" – 65% Completion of Objectives (Jan. 2021 quarterly)

In this project is focused on profiling new scions and rootstocks for their tolerance to HLB by studying the metabolite content by GC-MS, and challenging new varieties with psyllids and HLB.

Current objective(s) pursued:
1. To understand the mechanism behind the tolerance of different varieties toward HLB. The comparison between the varietal responses will allow the PI and team to determine the mechanism of tolerance to CLas.
2. To understand the role of rootstocks in citrus tolerance to HLB. The comparison between rootstock metabolites will allow for the determination of the best scion/rootstock combination for tolerating CLas.

This quarter we focused in three topics: 1) Marathon Mandarin analyses; 2) CUPS varieties; 3) "Lucky" biology and plant response.

1) For the evaluation of the new mandarin hybrid "Marathon", the team was able to sample many leaves for analysis of volatiles and polar metabolites. These samples have been run on the GCs and need integration.
2) The samples from the new varieties we collected from the CUPS and issues were noted with getting sufficient growth on these selections during the winter. The leaves collected for volatiles and metabolites are being prepared now for extraction. We estimate there will be approximately 150 samples for this phase.
3) For "Lucky" and its parents Sugar Belle and Nova x Osceola, we began the biology experiment. The experiment was completed by collecting leaf samples from all plants to assess the plant response to ACP, sprayed any remaining insects, and returned them back to their outside cage to recover. The leaf metabolites from the ACP-exposed plants will be compared to non-infested controls.

In addition to these efforts, the seeds from the USDA (US-802, 812, 897, 942, 1283, 1284, 1516) for metabolite profiling and HLB screening were received, planted, and the germination rate was good. It will be some months however before we can do anything with them in terms of sample collection.

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" – 25% Completion of Objectives (Mar. 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.

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" –

OBJECTIVES for three year USDA CRDF CAP

1. Optimize lead class of synthetic plant defense inducers
2. Develop promising microbial strains into viable product candidates
3. Determine relevance of hairy root assay for predicting field efficacy on HLB
4. Use greenhouse citrus assays to determine best methods for field testing
5. Field test most promising candidates to demonstrate efficacy against HLB

6. Investigate metabolomics as an early detection method for HLB in field
7. Define registration pathway for moving leads towards commercialization
8. Develop effective extension and outreach program

Since initiation of USDA funding in September 2020, regular meetings of all collaborators are taking place each month to coordinate activities and share findings between researchers.

Objective 1. Plant Defense Inducers
Field transfer on vegetable diseases, including *Pseudomonas sp*. and *Xanthomonas sp*., was confirmed in 2020 with additional H class candidates. New field candidates were already selected for all the citrus and vegetable trials, planned in 2021. The selection was based on greenhouse performance and Ecotoxicology profile. Several trials are also ongoing at the University of Florida (Batuman Lab). Those include a confirmation trial against HLB with the 2 first H class leaders and the evaluation of new candidates. The work plan for 2021 was established with our academic partners. Plan for new field trials with Florida Agco were also established. Finally, besides the overall very favourable Human safety profile, a risk of accumulation was recently identified for a few candidates, a regulatory concern. The team immediately set up a new Human Safety screening cascade coupled to a new chemistry program. The goal is to synthesize H-class analogs that will prevent any potential risk and to move forward with compounds having the best regulatory profile.

Objective 2. Antibacterial Microbial Strains
In primary *in vitro* screening, a set of pilot experiments were concluded to evaluate compatibility of Streptomyces cultivation with existing automated platform with emphasis in culture aggregation, and redefine the process in order to accommodate the yet-unexplored genus in the next Strep-targeted screening campaign. The gram negative bacteria Pilot was completed to identify common microbial standards to use as Negative Controls during the screening campaign. This involved HighThroughput cultivation of ~25 strains and *in vitro* bioassay for 5 targets for 3 sample types (Filtered Supernatant, aqueous and organic extracts).
Decision Science analyzed data and recommended 2 strains to include as microbial Standards to cover all combinations of SampleType/Target. Work has been performed to troubleshoot and streamline creation of working vials for the *Liberibacter crescens* in vitro assay.

In Scale up *in vitro* screening, we completed data processing of all MIC (Minimum Inhibitory Concentration) data to date (campaign 18Q1Q2, 18Q3, 19Q1) and conducted dose response analyses to prioritize strains for *in planta* testing and finalized these selections with the project team based on potency and taxonomic diversity. We completed all of 19Q2 MIC screening and started processing the data, thus finishing original screening requirements for the CRDF-Bayer agreement which had been delayed due to Covid-19.

In Vitro Screening Status										
In Silico Screening		Screening (Target 100%)			Confirmation (Target 80%)			Scale up (Target 100%)		
150K	Strains in Collection	99.6%	2490	Strains Screened	80%	176	Strains Screened	100%	250	Strains Screened

4500	Strains Selected		326	Distinct Species		50	Distinct Species		95	Distinct Species	
400	Distinct Species		3 to 4	Pathogens		3 to 4	Pathogens		3-5	Pathogens	
			2	Sample Types		2	Sample Types		2	Sample Types	

External leads for testing in HLB Screening cascade

There are currently ongoing discussions taking place with three companies to set up Material Transfer Agreements to allow for testing in lab, plants and potentially in field depending on results. Materials range from peptides and antimicrobial dyes to microbial endophytes.

Objective 3. In planta Testing

Fermentation material was produced for 2 strains for testing in Hairy Root assay (Mandadi Lab) with the goal of determining if the hypothesis of activity in *L. crescens* assay *in vitro* also results in activity in the CLas hairy root assay. CLas-citrus hairy root cultures were initiated and prepared as per established methods in Mandadi lab. The QC was also completed to validate CLas titers in the hairy roots. The extreme winter storm/weather conditions in Texas resulted in short term lab closures in February, but the testing will resume in the coming months.

Tomato clavibacter experiments performed in Bayer at the Woodland, CA site have been explored as models for testing against bacteria in Phloem/Xylem space. These assays were delayed in 2020 due to site closures but now have been completed. Improvements in consistency of infection and the injection method were made. The positive control of oxytetracycline was consistent in reducing disease severity at 2 and 4 weeks after injection. Negative control bacterial extracts did not have an effect different from untreated plants confirming that the activity observed is not a result of the extraction methods. A bacterial extract confirmed to be producing oxytetracycline showed some effect on disease severity at the highest dose tested.

Objective 4. Greenhouse Testing

Greenhouse screening is being performed during this quarter in the University of Florida (Batuman Lab) to test various microbial and plant defense inducer (PDI) compounds for phytotoxicity on citrus trees before designing experiments in plants. These compounds, depending on their target sites, are being applied onto plants through various application methods such as soil drenching, foliar spraying and trunk injection. Until now there is an indication that the highest concentration tested is showing phytotoxicity but all other concentrations look acceptable. Furthermore, phytotoxicity was correlated with the tree age (or size) and number of applications, which indicates cummulative detrimental effects on young trees if used in early developmental stages or too often. Assays are also ongoing testing PDI latest analogs at various concentrations and microbial extract leads. Treatment intervals are being investigated to inform best field practices for trials. Here, young flushes are being protected by applying PDI treatments before ACP colonization to activate plant defense (priming) and prevent bacterial infection. Some of the preliminary experiment results are encouraging and provided a substantial delay in Clas infection (~3 or more month) after 2-3 applications. Treated plants are being assessed at monthly intervals for CLas infection, and bacterial titer in plant is determined by qPCR assays.

Objective 5. Field Trial Testing

Fermentation in flasks and in tanks was completed as well as Sample Preparation for 3 strains for 2021 Q1 and eventually Q2 applications for the field trials initiated in 2020.

In the field, additional applications were made of both plant defense and microbial extract leads during the first quarter of 2021. Some phytotoxicity has been noted in foliarly applied synthetic compounds in the field which was not observed in the greenhouse. Therefore, several calls were held between Florida Agco and Bayer field trial researchers from Lyon and Monheim. As a result, the team has planned for some Florida field phytotoxicity screening to help better understand effective rates below the threshold of plant damage. Trees continue to be sampled for qPCR disease levels. Planning has also started for the first two 2021 trials to be initiated, which will be focused on testing improved PDI analogs and will include combining with a microbial treatment aimed at improving root health and managing plant stress.

Objective 6. Metabolomics testing:

UC Davis has currently been sent leaves from four trials so far, including 2 field trials and 2 greenhouse trials. All samples from Field Trial #1 and Greenhouse Trial #1 have been prepared, with corresponding metabolomics analysis completed. UCD is presently injecting samples from Field Trial #2 into the LC-qTOF instrument. Of the Greenhouse Trial #2 samples that have been sent to UCD, all have all been injected, but the chemometric analysis is in progress. See following table.

Metabolomics assay	# Samples received	# Samples processed*	Chemometrics status
Field Trial #1 (6 sampling rounds)	2,592	100%	Completed
Field Trial #2 (2 sampling rounds so far)	972	75%	In progress
Greenhouse Trial #1	210	100%	Completed
Greenhouse Trial #2	600	100%	In progress

**Processed = sample has been prepared, injected into the instrument, and a corresponding data file ready for chemometric analysis*
***UCD team was only sent leaves from half of the experimental trees in Round 2*

The UCD team has made health predictions for all trees from Field Trial #1. On average, predictions for the untreated trees (Trt-I) have always fallen into the infected category, while the covered, negative control trees (Trt-II) have largely been healthy, with few exceptions. On average, treated trees have exhibited a metabolic decline over time that corresponds to HLB infection, per UCD models. The Davis team is also tracking each tree individually and looking at its diagnosis over time. In Round 3, there has been less variability among prediction values, which is currently being investigated by the UCD team. A meeting has been set up with Southern Gardens to look at comparisons of qPCR and Metabolomics data from the same leaves of the same trees from the field trials.

M. Dutt 18-007 “Investigating the role of transgenic rootstock-mediated protection of non-transgenic scion” – 60% Completion of Objectives (Feb. 2021 quarterly)

The project objectives are: 1. Evaluate existing transgenic Carrizo and Swingle AtNPR1 overexpressing rootstocks in the laboratory and greenhouse. 2. Conduct a replicated field trial with the best transgenic rootstocks budded with non-transgenic `Valencia' and test for GMO gene products in the fruit or juice. 3. Produce additional transgenic rootstock lines and stack other gene(s) responsible for SAR using mature transformation. 4. Evaluate transgene segregation analyses of the rootstock progeny and large-scale propagation of select lines.

Evaluation of transgenic trees continued in the greenhouse and field. Trees in the greenhouse have been tested against HLB. Some lines have been detected with lower Ct but the majority remained similar to last sampling. Western blotting of several newer lines has been conducted to identify high expressing lines. Most transgenic lines with a stacked construct (NPR1 with other transgene) have been produced. seed source trees overexpressing AtNPR1 will be planted in field for production of seed as some have had to be re-budded due to failure of the bud union or tree mortality from Phytophthora infection. We have produced several trees to serve as interstocks and they will be evaluated in the next cycle.

Next quarter evaluation of additional transgenic lines and interstock trees against HLB will continue.

F. Gmitter 18-010 “Upgrading Citrus Genome Sequence Resources: Providing the Most Complete Tools Necessary for Genome Editing Strategies to Create HLB Resistant Cultivars” – 35% Completion of Objectives (Jan. 2021 quarterly)

Raw sequence data were generated for Valencia orange (S, sensitive), Ruby Red grapefruit (S), Clementine mandarin (S), LB8-9 Sugar Belle® mandarin hybrid (T, tolerant), and Lisbon lemon (T) and preliminary assemblies and analyses were carried out. Because of reduced sequencing costs, additional important genomes were entered into the pipeline, including Carrizo citrange, sour orange, and Shekwasha (an important breeding parent for HLB tolerance); these also have now been sequenced and assembled.

Hi-C sequencing were performed with the two genomes and these data incorporated with PacBio sequence of one of the target genomes resulting in improved chromosome scale assembly. The two parental chromosomes of the target genome were phased/separated using Illumina short reads from citrons, pummelos and mandarins. By genome alignment and comparison to the Poncirus assembly (see below), minor assembly errors in repetitive regions have been fixed, resulting in a high-quality assembly; transcript sequencing for annotation (i.e., identify all the genes within the genome) currently is in progress. The availability of high-quality assemblies for the 3 basic species (C. medica, reticulata, and maxima) will allow a more thorough and complete characterization of large-scale structural variation (SVs: deletions, insertions, etc.) in genomes of commercial interest. These SVs are the driving force for phenotypic diversity especially among somatic mutants (e.g., different oranges, grapefruits), and this information will become more important as we test different sweet orange mutants exhibiting enhanced tolerance of HLB. A manuscript is in preparation on this work.

As an example of the utility of these quality-improved new citrus genome assemblies, the polyembryonic allele associated with a MITE transposon insertion in the promoter of the CitRKD1 gene in the mandarin lineage, has been examined for sequence completeness. Previous reference assemblies either lacked this allele (the MITE insertion obviously is absent in the monoembryonic Clementine reference) or were missing important sequence in the promoter region of the gene (in the sweet orange reference assembly from China). Full knowledge of the polyembryony gene in citrus is important because it is the basis for rootstock propagation by nucellar seedlings, and it is an impediment to breeding by preventing the ability to make hybrids using polyembryonic seed parents. More importantly, this example demonstrates the quality of the assemblies; lacking promoter sequence for important HLB-resistance candidate genes could lead to CRISPR editing failures, which is proposed to prevent through successful achievement of the objectives of this project.

The first ever high-quality reference genome of Poncirus trifoliata was completed and published using the same pipeline used for this project. The publication in The Plant Journal (https://doi.org/10.1111/tpj.14993), and the sequence has been released to the global citrus research community through Phytozome and the Citrus Genome Database. By mining this new genome, candidate genes were identified within the previously identified chromosomal regions for HLB tolerance, including a transcription factor gene and one disease resistance-like gene that are up-regulated by CLas and positively selected in trifoliate orange. These genes are promising candidate genes for further research and were highlighted in the publication, so other researchers may also begin to explore their potential.

E. Rogers 18-019 "Phloem specific responses to CLas for the identification of novel HLB Resistance Genes" – 50% Completion of Objectives (Feb. 2021 quarterly)

Our project is examining phloem gene expression changes in response to CLas infection in HLB-susceptible sweet orange and HLB-resistant Poncirus and Carrizo (a sweet orange - Poncirus cross). We are using a recently developed methodology for woody crops that allows gene expression profiling of phloem tissues. The method leverages a translating ribosome affinity purification strategy (called TRAP) to isolate and characterize translating mRNAs from phloem specific tissues. Our approach is unlike other gene expression profiling methods in that it only samples gene transcripts that are actively being transcribed into proteins and is thus a better representation of active cellular processes than total cellular mRNA. Sweet orange, and HLB-resistant Poncirus and Carrizo (sweet orange x Poncirus) will be transformed to express the tagged ribosomal proteins under the control of characterized phloem-specific promoters; tagged ribosomal proteins under control of the nearly ubiquitous CaMV 35S promoter will be used as a control. Transgenic plants will be exposed to CLas+ or CLas- ACP and leaves sampled 1, 2, 4, 8, and 12 weeks later. Ribosome-associated mRNA will be sequenced and analyzed to identify differentially regulated genes at each time point and between each citrus cultivar. Comparisons of susceptible and resistant phloem cell responses to CLas will identify those genes that are differentially regulated during these host responses. Identified genes will represent unique phloem specific targets for CRISPR knockout or overexpression, permitting the generation of HLB-resistant variants of major citrus cultivars.

During the 1st quarter of the third year of our grant, the Stover lab has focused on producing additional transgenics of the couple lines that are still needed. They tried a new shoot elongation medium with good results and have a large number of transformants for expression testing. The Rogers lab has continued small-scale no-choice psyllid inoculation experiments. ARS employees are still been ordered to maximize telework due to the COVID-19 pandemic. This continues to slow down progress on grant milestones. We are very much hoping to be allowed to move to the next phases of reopening soon, which will allow for much more rapid progress towards grant milestones.

N. Wang 18-025 "Optimization of the CRISPR technology for citrus genome editing" – 87% Completion of Objectives (Apr. 2021 quarterly)

The purpose of this project is to optimize CRISPR technology for citrus genome editing.

Objective 1. Expanding the toolbox of citrus genome editing. In this study, StCas9, NmCas9, AsCpf1 (from Acidaminococcus), FnCpf1 (from Francisella novicida) and LbCpf1 (from Lachnospiraceae) will be adapted for genome modification of citrus.

CRISPR-LbCas12a (LbCpf1), which is derived from Lachnospiraceae bacterium ND2006, has been successfully used to edit a citrus genome. One manuscript entitled CRISPR-LbCas12a-mediated modification of citrus has been published on Plant Biotechnol J. Currently, LbCas12a-crRNA-mediated genome editing is being further optimized to make homologous biallelic mutations.

AsCpf1 and FnCpf1 are also being tested for their application in citrus genome editing and generating homologous biallelic mutations. Both homozygous and biallelic mutations have been successfully generated in the EBE region of LOB1 gene in pummelo. This work has been submitted for publication. homozygous and biallelic lines of additional citrus varieties are in the process of being generated.

Recently, multiplex genome editing toolkits have been developed for citrus including a PEG mediated protoplast transformation, a GFP reporter system that allows rapid assessment of the CRISPR constructs, citrus U6 promoters with improved efficacy, tRNA-mediated or Csy4-mediated multiplex genome editing. Using the toolkits, genome modification of embryogenic protoplast cells and epicotyl tissues has been successfully conducted. A biallelic mutation rate of 44.4% and a homozygous mutation rate of 11.1% have been achieved indicating that CRISPR-mediated citrus genome editing technology is mature and a viable approach for citrus genetic improvement. These results lay the foundation for non-transgenic genome editing of citrus. One manuscript entitled: Development of multiplex genome editing toolkits for citrus with high efficacy in biallelic and homozygous mutations has been published on Plant Molecular Biology.

Objective 2. Optimization of CRISPR-Cas mediated genome editing of citrus. In this study, different promoters will be tested including INCURVATA2 promoter, the cell division-specific YAO promoter, and the germ-line-specific SPOROCYTELESS promoter, and ubiquitin promoter for driving the expression of Cas9 and Cpf1 orthologs.

To optimize the expression of sgRNA and crRNA, multiple citrus U6 promoters and two of the citrus U6 promoters have been identified that show higher efficacy in driving gene expression in citrus than the 35S promoter and Arabidopsis U6 promoter. The mutation rate has been increased to 50%.

The CRISPR/Cas9 system has been further optimized as well. Now, the biallelic mutation rate is 89% for Carrizo citrange and 79% for Hamlin orange.

One homozygous line has been generated in the promoter region of canker susceptibility genes for Hamlin. One biallelic mutant of grapefruit has been successfully generated that is canker resistant. Multiple biallelic and homozygous mutant lines of sweet orange that are canker resistant have also been successfully generated.

Objective 3. Optimization of the CRISPR technology to generate foreign DNA free genome editing in citrus.

Transient expression of Cas9/sgRNA plasmid and Cas9 protein/sgRNA ribonucleoprotein complex have been conducted in citrus protoplasts. Citrus genome editing using Cpf1/crRNA plasmids and ribonucleoprotein complex is also being conducted in citrus protoplasts. The plasmid-transformed protoplast has 1.7% editing efficiency, and the RNP-transformed samples have approximately 3.4% efficiency. The genome modified protoplast cells are undergoing regeneration. The aim is to increase the efficiency to over 20% and eventually generate non-transgenic genome modified citrus. One patent has been filed on the CRISPR-Cas mediated genome editing of citrus.

Citrus protoplast isolation and manipulation have been optimized. More than 98% of the isolated protoplasts are alive. A transfection efficiency of approximately 66% or above is routinely obtained. Genome modified lines for canker resistance are being regenerated.

Six putative HLB susceptibility genes from sweet orange are being edited.

M. Leslie 20-015C – "Vismax™: A novel peptide-based therapeutic for mitigation of citrus diseases, including HLB" - February 1, 2021 start date.

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" – 79% Completion of Objectives (Apr. 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 orange and 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 or co-immunoprecipitation assays.

Las outer membrane proteins were 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 in the greenhouse for their effects in inducing plant defense against Las. At least four different Las PAMPs showed significant effect in inducing plant immunity. These 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 are being conducted and the data are currently being analyzed.

The control effects of different PAMPs are being tested against HLB. Three PAMPs showed strong activity in inducing plant defenses.

Greenhouse trials have been completed. Currently, different PAMP products are being sprayed on newly planted young sweet orange trees in the field.

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 via grafting or psyllid transmission will be conducted once the transgenic plants are about one year old.

For those identified receptors or targets, the promoter regions are being sequenced in Valencia, Sugar Belle, and Poncirus 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 driving using a pathogen-inducing promoter. Several plants expressing the constructs have been generated. Testing of those plants showed that they responded to canker. They will be tested to determine whether they are resistant to HLB. Right now, more plants we are being propagated for testing.

6 constructs have been made to express PAMP receptors individually or in combinations in sweet orange.

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

Screening of 30 putative Las effectors has been completed and 4 of them repressed plant defense. We are screening another 20 putative Las effectors and 3 more effectors that suppress plant defense.
Y2H for the four defense-suppressing effectors has been completed and their targets identified in Valencia orange. 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 plants showed severe HLB symptoms. The mechanism of a putative HLB S gene is being characterized. We are conducting genome editing of the identified HLB S gene of Valencia sweet orange and Duncan grapefruit to generate HLB resistant or tolerant citrus. In addition, the HLB S gene in Valencia sweet orange 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, Y2H assays are being conducted to identify putative targets for effectors hypothesized to induce plant defense in Poncirus and Sugar Belle. RNA-seq analyses of Sugar Belle have been conducted. Data analyses have identified some interesting prospects regarding chemicals to control HLB. They are currently being tested in the greenhouse.

One manuscript entitled Citrus CsACD2 is a target of Candidatus Liberibacter asiaticus in Huanglongbing disease has been accepted by Plant Physiology.

The binding sites of CsACD2 with SDE15 are under investigation. the effect of effectors in suppressing plant immune responses caused by PAMPs is being evaluated. Another important effector has been identified. In total, six promising HLB susceptibility genes have been identified.

V. Orbovic 18-066C "Support role of the Citrus Core Transformation Facility remains crucial for research" – 75% Completion of Objectives (Mar. 2021 quarterly)

In 2021, Juvenile Tissue Citrus Transformation Facility (JTCTF) started operating as an EBA unit. As a result of this transition, relationship between the clients and JTCTF is redefined in a sense that clients will be paying for the work JTCTF has done and not for the produced plants. Towards this goal, JTCTF has created a price list for the services it offers. In accordance with changed status of

JTCTF, administration at CREC has established an account that will be used for financial transaction associated with facility's operation.

In the first three months of 2021, JTCTF accepted five new orders. Four of those orders were for transgenic sweet orange plants and one for Duncan grapefruit plants. One of the orders is associated with the existing USDA grant, while four other orders will be paid for. Certain number of orders that were expected because JTCTF was designated subcontractor have not materialized because those clients have not started working on projects for which they received funding as they had difficulties in hiring personnel.

At this time, JTCTF has four employees but one has not worked since December of 2020 because of an unresolved immigration status. Another employee is working during the night which has allowed us to return to the level of attendance of pre-COVID period. As a result, the facility operated at about 75% capacity.

Between January and April, JTCTF produced 10 transformants. Those include five Duncan grapefruit plants transformed with HGJ87 and HGJ88 plasmids. The rest are sweet orange plants transformed with pXH-H1 and pXH-H2 plasmids.

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

The objective is to produce disease resistant, commercially & agronomically acceptable, mature citrus transgenics and intragenics using Agrobacterium and biolistics.

The research is focused on improvement of Agrobacterium and biolistic transformation of mature citrus, so that protocols become more efficient which can decrease costs for scientists and contribute to financial self-sufficiency of the Mature Tissue Transformation lab. Progress was made in Year 2 by increasing transformation efficiency of some cultivars. A citrus selectable marker as an alternative to the nptII gene in citrus transformation was also accomplished in Year 2.

One genetic construct submitted by a faculty member did not work, so no transgenic tissues or plants were produced. Another three genetic constructs submitted by another faculty member produced shoots, but only wanted photos of the shoots and did not want these transgenic shoots micro-grafted. Because these orders produced no plants no significant monetary gain was made. However, ~41 transgenic scion shoots were produced for testing a new marker selection protocol. Alternative rootstocks to Carrizo developed by Dr. Grosser for micrografting were tested to reduce losses in micrografting transgenic shoots.

Different tissue culture media for grapefruit were tested and found to work better for regenerating shoots than the standard sweet orange basal medium. The next step is to test whether Agrobacterium-mediated transgenics of mature grapefruit cultivars can be produced using this medium.

Numerous biolistic transformations have been conducted to test different variables for enhancement of transformation efficiency. Bombardments were conducted with different vectors, cultivars, and treatments. Results from these trials are pending.

A spontaneous mutant of Early Valencia 1 (EV1) was discovered in the growth room. The leaves are enormous compared to wild-type EV1, so perhaps it is a tetraploid. This plant had undergone the process of shoot-tip grafting for plant introduction but there no 2,4-D in the shoot-tip grafting medium was used that might induce mutations. Apparently, Valencia selections frequently undergo spontaneous mutations. This mutant and an OLL20 mutant from FDACS will be budded to determine whether they have HLB disease resistance. Mutations are the most frequent source of new citrus cultivars.

Next quarter, work will continue for two scientists who have submitted several vectors. Another scientist might be interested in testing Agrobacterium transformation of grapefruit now that the medium has been optimized for shoot production. Two manuscripts on the plant selectable marker and enhanced transformation of mature citrus are under development.

CRDF funding is sufficient, however there will probably overspend of the Director's account since not very many plants have been produced for monetary gain, unless more plants are produced in upcoming quarters.

d. Horticultural Practices

E. Triplett 18-024 "Foliar phosphate fertilization: a simple, inexpensive, and unregulated approach to control HLB" – 65% Completion of Objectives (Feb. 2021 quarterly)

We proposed to test foliar fertilization with potassium phosphate to alleviate HLB symptoms and increase yield of citrus.

Foliar phosphate fertilization field trial (Hamilton and Immokalee)

We are currently about one year into scheduled spraying of our mature citrus plots. Four concentrations of potassium phosphate 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 is continuing to decline in the 2mM phosphate treatment group after a year of spraying. 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 this year.

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 micronutrients 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. While we have confirmed citrate reduction of 50% in phosphate treated plants of the first time point, we are currently running HPLC for our July and October 2020 samples.

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. We have recently analyzed the titer change of CLas in the plants.
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%.

Work that's anticipated for next quarter:
Field trials continued. Yield and canopy measures expected next quarter. q PCR analysis on first 15 months of samples will be done and analyzed.

Again, Harder than expected to get field trials underway but now full speed ahead. Spending at the expected rate now.

F. Alferez 18-032C "Preventing young trees from psyllids and infection with CLas through use of protective netting" – 70% Completion of Objectives (Feb. 2021 quarterly)

Objective 1. Assessing tree growth and absence of psyllids and HLB disease symptoms (including CLas bacteria titer) under individual protective covering (i.e., IPC). As in the last quarter, we have continued monitoring trunk diameter and canopy area as well as flushing and blooming dynamics in the new plots (700 trees planted last year). After IPC removal in the original plot in August 2020, we have been monitoring CLas infection of the uncovered trees in real time. We are now processing these samples for real time PCR CLas detection so we can document the rate of infection once IPCs are removed from trees.

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 continued monitoring the new 700 trees mentioned in the Objective 1 planted last year that are arranged in an alternated pattern we are monitoring for CLas in trees adjacent to the IPC-covered trees. Also, we have continued working with several commercial collaborators who are also evaluating different netting layouts under the CRAFT program.

Objective 3. Monitoring the transition from vegetative to reproductive stage in the covered trees as compared to the uncovered. We are continuing data collection on Bingo, Early Pride, and Tango trees. This is the second year in documenting blooming on these varieties. We have also documented different blooming rate in the trees from our first experiment that were uncovered last August. These trees are blooming more profusely than the always-uncovered trees. We are counting flowers and we will assess fruit set in the coming weeks.

Objective 4. Comparing IPC with CUPS-like systems. We have performed for the second year deficit irrigation. As in last year, we have induced more bloom. We are also finishing regular quality analysis from fruit that matured this year.

Outreach for this quarter:
-Batuman, O. Individual and direct contact with CRAFT applicants to establish and evaluate IPC trials for psyllid and HLB control.
-Gaire, S., Albrecht, U., Batuman, O., Qureshi, J., Zekri, M., Alferez, F. 2020. Horticultural performance of citrus trees grown under Individual Protective Covers (IPCs). Crop Protection, under review.
-Individual Protective Covers' by Alferez, F, Gaire, S., Albrecht, U., Batuman, O., Qureshi, J., Zekri, M., 2021-2022 Citrus Production Guide, EDIS. Under Review.
-Gaire, S. Evaluation of individual protective covers for preventing vector transmission of Candidatus Liberibacter asiaticus and effects on growth and physiology of young citrus trees. Master's Thesis Defense, March 11, 2021, UF, Horticultural Sciences Dept.
-Gaire, S. Evaluation of individual protective covers for preventing vector transmission of Candidatus Liberibacter asiaticus and effects on growth and physiology of young citrus trees. Oral Presentation at the Southern Fruit Workers 3 Minute Thesis Competition, ASHS, 2nd Prize winner.
Anticipated work for next quarter:
Objective 1.We will continue monitoring parameters described in the first section. Also we will continue monitoring HLB progression after IPC removal in the first experiment and fruit yield and quality to compare fruit from IPC and non-IPC trees.
Objective 2. We will continue collecting data on psyllid populations and HLB incidence in the different netting layouts.
Objectives 3 and 4.We will start collecting data on bloom and fruit set for this second season of deficit irrigation treatments.

Outreach:
-Alferez, F. Invited speaker at the Citrus Institute 2021. Virtual. April 6.Individual Protective Covers (IPCs) influence on tree performance, fruit production, pests, and diseases.

Budget status:
As mentioned in the last report, we are on track with activities and spending after the COVID pause. Budgeted amounts for salaries and student stipend and tuition are being spent as predicted.

L. Duncan 18-036C "Cover crops and nematicides: comprehensive nematode IPM across the grove landscape" – 70% Completion of Objectives (Feb. 2021 quarterly)

Nematicide effects on nematode populations were measured in December, as previously described. The average number of sting nematodes in aldicarb plots was 10% higher (NS) than that in untreated plots, whereas oxamyl, Syngenta product and Nimitz plots had 34%-42% fewer sting nematodes (P<0.05) than in untreated plots. Nematode numbers in the plots treated with the other nematicides ranged from 89%-93% of that in untreated plots, none of which were significant differences. The average cumulative numbers of sting nematodes measured 60 days following each of the four seasonal applications thus far during the project (areas under the curve) were 55%, 54%, 37%, 21%, and 8% lower than those in untreated plots for the Syngenta compound (P=0.05), oxamyl (P=0.05), Nimitz, (P=0.05) Salibro, and Velum Prime, respectively. Cumulative sting nematodes in aldicarb treated plots were 16% more numerous than in untreated plots.

Average fibrous root mass density for all nematicide treatments ranged between 7%-47% greater than for non-treated plots. Unlike during the previous June, no treatment resulted in significant root increase during the fall period. The growth of the tree trunks during 2 years between February 2019 and January 2021 was 26% greater for trees treated with oxamyl than that of untreated trees (Dunnett, P<0.05). Growth rates of trees treated with other nematicides were between 2-13% greater than the untreated trees, but the differences were not significant. There remained a strong inverse linear relationship (r= -0.75, n=7, P=0.05) between trunk growth during 2 years and the average sting nematode population density (log-transformed) during that time. The harvested fruit in were counted. The number of oranges per tree in both 2020 and 2021 were very highly correlated (r=0.68 and r=0.64, P=0.001) with trunk cross-sectional area measured at the beginning of the trial (2/2019). Larger trees initially produced more fruit, irrespective of treatment. The average trunk area differed greatly between treatments at the beginning of the trial, therefore we determined the % increase in number of fruit per treatment between2020 and 2021. The yield/tree declined by 22% in untreated trees between 2020 and 2021, but increased in all of the nematicide treated plots. Aldicarb and oxamyl plots increased (P=0.05) by 166% and 126%, respectively while plots treated with combinations of Velum Prime and Nimitz increased by 44% and those treated with combinations of Salibro and the Syngenta product increased by 23%.

In a second trial comparing untreated trees to trees treated with aldicarb in April 2019 and 2020, there were no differences in the trunk cross-sectional areas of either treatment at 20 months after the first treatment (untreated=2403 mm2 vs aldicarb=2409 mm2). Nor was there a significant increase in growth since summer 2020. There was no treatment effect on the very small number of fruit per tree (10.2 untreated vs 6.2 aldicarb). Although young tree growth in this grove is very uneven, the cause(s) are other than sting nematodes which were below detectable levels in most plots.

In the perennial peanut trial, sting nematodes in the native vegetation middles increased to 85 nematodes/250 cc soil from an average of 51 nematodes during the previous 6 months. However, the populations remained below 10 nematodes in the plots planted with peanut regardless of whether there was some incursion of weeds in some of the peanut plots. Indeed, during the monthly measurement the past 6 months, sting nematodes in peanut middles averaged just 7.7% ± 2.02% (mean and standard error) of those in native vegetation middles. Unlike the previous spring, oxamyl did not significantly reduce the sting nematodes in the plots during the autumn (just 25% reduction). Citrus fibrous root density in plots without peanut middles or oxamyl treatment was 0.21 mg/g soil, compared to 28 mg/g in plots treated with oxamyl, 29 mg/g in plots with peanut middles, and 30 mg/g in plots with both peanut and oxamyl. There was an interaction (P=0.01) between oxymyl and peanut effects on trunk size. Trees were 14% larger (P<0.1) when peanut rather than native plants were in the row middles. Trees treated with oxymyl in peanut plots were 26% larger than those not treated (P=0.03), but were not different than untreated trees in plots with weed middles. Trunk size was highly correlated with number of fruit per tree, but there were no significant effects or interactions between fruit number and either treatment.

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.

D. Kadyampakeni 18-042C "Development of Root Nutrient and Fertilization Guidelines for Huanglongbing (HLB)-Affected Orange and Grapefruit" – 90% Completion of Objectives (Apr. 2021 quarterly)

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. Fruit yields and juice quality were evaluated at both the Ridge and Flatwoods sites in March 2021 and no significant differences were observed between treatments. All samplings for the second 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. There were no significant differences in tree height, canopy volume, root length, and root diameter. Yield and fruit quality data were collected in February and are being analyzed.

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. Graduate student Tanyaradzwa Chinyukwi working with Dr. Kadyampakeni graduated on May 3, 2021. Portions of her work will be published in two Citrus Industry Magazine issue of May 2021 and Florida State Horticultural Society Conference Proceedings of 2020 and 2021. Addition outputs from Tanyaradzwa's work will be published in refereed journals.

Lukas Hallman will defend his MS Thesis in July 2021. Portions of his work will be published in refereed journals and IFAS/EDIS extension bulletins and trade journal articles this summer/fall, as well as presented at ASHS and FSHS planned for August and September 2021.

Budget status We are on track with our planned budget spending but would need a no cost extension to get a fourth harvest from our experimental blocks.

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 quarterly – final comprehensive report in progress by PI)

Objective 1 - Determine the effect of the ratio and concentration of Fe^{2+} + organic acid on hydroxyl radical production and stability. Objective complete.

Objective 2 - Determine the phytotoxic levels of Fe^{2+} + organic acid solutions on citrus. Complete.

Objective 3 - Determine the effect of Fe^{2+} + 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 (Fe^{2+}) 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” – 65% Completion of Objectives (Mar. 2021 quarterly)

In this quarter, monitoring and management activities continued in all of the Integrated Pest Management (IPM) programs for ACP which include:

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.

The dormant spray applications in February included Danitol in programs 1 and 3, Pyganic + 435 oil (2%) in program 2, and 435 oil (2%) in program 4. The March sprays included Microthiol in program 1, 435 oil (2%) in program 2 and 4, and Movento in program 3. Nine samplings were conducted to monitor for ACP and beneficial arthropods. February sprays conducted in the middle of the month kept psyllids below treatment threshold of 0.1 adults per tap sample across all programs until the second week of March. After March sprays a decreasing trend in the populations of ACP was seen in all programs and numbers dropping from 0.2-0.3 adults per tap sample to 0.1 adults per tap sample by the end of the month. Within the predator guild, spiders and lacewings continue to be the dominant groups and among lacewings Ceraeochrysa cubana most dominant. In the laboratory, C. cubana larvae were more tolerant to imidacloprid than C. claveri or D. citri. Ceraeochrysa cubana seems to have a significant potential to be used in biological control against D. citri. A preliminary laboratory study testing a predator prey ratio of 1:1 (mite: ACP egg) showed that the predatory mite Amblyseius swirskii provided 50% reduction in ACP. Follow up experiments were initiated in the greenhouse using different densities of this predator against ACP. Residual toxicity of March sprays was evaluated against A. swirskii. Three days after sprays leaves were collected from the treated trees and exposed to the predatory mite in the experimental arenas in the laboratory. After, 24 h of exposure 12% mortality was observed in the Movento treatment, and 8% in the Microthiol and 435 oil treatments. Amblyseius swirskii were also released in the field within a week of March sprays.

Approximately 5,000 mites per plot and total of 20,000 per program were released. No predatory mites were observed in a sample conducted before the release. After release, 20% of the sampled trees in program 3 sprayed with Movento were found to contain predatory mites, 30-32.5% in the program 2 and 4% sprayed with 435 oil, 35% in program 1 sprayed with Microthiol and 40% in the program 5 which is untreated control. Colonies of field collected ACP from all programs were established in the laboratory and their subsequent generations tested for tolerance to dimethoate, fenpropathrin, and imidacloprid in comparison with a laboratory colony. The concentration required to kill 50% of the field population averaged 2, 4 and 8 times more for dimethoate, fenpropathrin, and imidacloprid, respectively, than the concentration required to kill same percentage of ACP from the laboratory colony. Leaf samples were collected from all programs and submitted for HLB analysis in March. Analysis of the leaves collected in Fall 2020 showed that 80-95% samples collected across all programs were HLB positive. Although, no Tamarixia radiata was released in the programs during this quarter due to the problems with the colonies at the FDACS-DPI, Dundee, facility, the previous work on the parasitism activity of T. radiata tested at different times of the day and temperatures was written and a presentation on the same work given at the Southeastern Branch Meeting of the Entomological Society of America and Florida Citrus Grower Institute.

J. Qureshi 18-055C "Optimizing Benefits of UV Reflective Mulch in Solid Block Citrus Plantings" – 55% Completion of Objectives (Mar. 2021 quarterly)

This project evaluates young tree protection from ACP/HLB using approaches to integrate ground cover, insecticides, and irrigation management at three locations 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. Treatments of 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 are being implemented to the trees on UV reflective and bare ground. The irrigation deficit treatments are also functional at the SWFREC and CREC locations to trees on UV reflective and bare ground to synchronize flush to target spray applications on major flushes. Sampling was conducted to monitor flush abundance and psyllid populations at three experimental sites. At the Vero Beach location, significantly more flush was observed in the trees on mulch compared to the trees on bare ground averaging 4.09 and 3.47 per plant, respectively. The diameter of the trunk of the rootstock and scion of the trees on the mulch was significantly more than the trees on the bare ground. An average rootstock diameter of 33.79 mm on the mulch and 28.20 mm on the bare ground was observed. Scion diameter averaged 24.04 mm on the mulch and 21.12 mm on the bare ground. At the CREC location, on average, there was also significantly more flush observed on mulched trees than on bare ground trees. Analysis of data from September 2020 to March 2021 showed significantly fewer adults on trees with mulch than with no mulch. Trees with insecticides applied based on a calendar schedule also had fewer adult ACP than those that experienced insecticide applications based on flush. ACP adult abundance was weakly but positively dependent on flush abundance. Peaks of adult counts occurred between March to June as well as September to October in 2020 and this is where treatment differences were starkest. Significantly fewer eggs and nymphs were observed on the trees with mulch than those on the bare ground. At SWFREC, trends on flush abundance across treatments and ACP response to the treatments were similar to other two locations. During this quarter, the mulched and non-mulched treatments' soil moisture contents varied between 0.11±0.02 m3 m-3 and 0.10±0.01 m3 m-3 showing not much difference in water storage with the root zone. Overall, canopy density appears to be greater on mulched than non-mulched trees. In the next quarter, we will do leaf and soil nutrient analysis and canopy size determinations to see changes if any in the treatment effects. At SWFREC, several experiments were conducted using the remote-controlled and manual ground-penetrating radar (GPR) operation to evaluate the effort needed to perform the GPR scanning, measure the required time to complete the scanning process, evaluate precision and accuracy of the scanning circles, and determine if the remote-controlled system can perform better for non-invasive root detection than the manual process. The scan line data collected from both the remote-controlled and manual process were compared after processing the data using an auto root detection software (Tree Radar Inc., Maryland, USA.) The results of root detection for both processes were almost the same, and there were no significant differences between them. The depth of the detected roots in the remote-controlled process was same as of the manual process. These experiments show that the remote-controlled process can reduce the required application time by 3 times when compared to a manual process. It can also reduce the human effort required and increase the precision of the data collection process. A Qualtrics survey to obtain growers input on the reflective mulch technology was developed and distributed through several channels.

S. Strauss 18-059C "Citrus row middle management to improve soil and root health" – 80% Completion of Objectives (Jan. 2021 quarterly)

Objective 1: Determine how different cover crop mixtures impact soil and root health and weed cover in established commercial citrus groves.
The next set of cover crops were planted at the end of Nov 2020. These included sunhemp, Austrian winter pea, daikon radish, oats, and winter rye. Analysis of the soil nutrient and microbial samples collected in August 2020 indicates similar patterns to Year 1: increased bacterial abundance in cover crop treatments, and increased abundance of nitrogen cycling genes with cover crops. In addition, preliminary analyses 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. There has been an increase in the abundance of bacteria under the canopy of trees in the legume+non-legume treatment. Analysis of weed data from 2020 shows that cover crop planting has resulted in suppressing weeds up to 98% in one location and 69% in the second location. The observed trend in weed suppression is in line with the previous year. 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. 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. After 1 year of study, we have not yet observed differences in fruit yield, fruit quality, canopy volume, and trunk size. This is not unexpected, as trees of this age could take at least two years to show responses to treatments. We will continue to assess canopy volume and trunk size, and harvest data will be collected again in Spring 2021.

Objective 4: Identify the economic benefits of using cover crops
The cover crops survey was drafted and has IRB approval. The survey was also reviewed but faculty, the project team, extension agents, and is waiting one grower's comments. Qualtrics was engaged to administer the survey and next steps are outlined. Our initial estimates are that cover crop establishment is cost prohibitive. Therefore, the survey includes a question on willingness to participate in cost-share programs to help with establishment costs. The partial budgeting process is ongoing.

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. Information on cover crops and preliminary data was included in two articles for the Citrus Industry magazine in September. Discussions are underway about how to host a field day, or a virtual field day, in the spring of 2021.

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. DNA from soil samples collected in August 2020 has been extracted and microbial analysis has been completed (see above). Weed data analysis will be continued and weed density data collection for the 2021 spring planting will occur in early Summer 2021. Canopy and trunk size measurements and leaf nutrient status along with root image collections and soil moisture monitoring will continue, and quantitative differences reported in the next quarter. The next set of cover crops are scheduled to be planted in May 2021. The composition of the mixes is still being discussed. The economics team expects to execute the adoption survey and begin analysis. They will also construct the framework for partial budgeting and assessing the cost of cover crop use and continue to collect data. The cover crop survey will be administered, and data analysis will begin. Partial budgeting analysis will continue.

Budget status: We are on track with our planned budget spending.

T. Vashisth 18-061C "Evaluating sustainability of yield and fruit quality of sweet oranges with use of controlled release fertilizer and micronutrients" – 80% Completion of Objectives (Feb. 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 successfully collected data on tree health, leaf and soil nutrition. We also applied fertilizer for spring at both sites. Due to low soil pH, we have skipped Tiger sul application and instead applied regular micronutrients. We are continuously monitoring soil pH and will resume Tiger Sul application when pH is high enough.

In addition we recently harvested Arcadia site. The data on fruit yield, fruit size, juice quality has been collected. 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.

We are also getting ready to harvest second trial site. We anticipate harvesting it in next 2-3 weeks. This quarter the results of field trial were presented at virtual OJ break.

R. Ferrarezi 20-003 "Nitrogen fertilization of 4 to 7-year-old trees planted in high density" – 5% Completion of Objectives (Mar. 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 nitrogen application in young trees planted in high-density plantings affected by Huanglongbing (HLB).

The PhD student and Research Assistant for the project were hired with a short delay due to Covid-19 and are now working on the project.

Contact has been made with grower collaborators and include IMG Citrus (Brian Randolph), Peace River Citrus (Larry Black Jr.), Agromillora Florida/ Lost Lake Groves (Clay Pedersen), Graves Brothers (David Howard). Site visits have scheduled and/or performed to all locations have been completed.

The planting densities and variety/scion combinations were chosen, maps generated, experimental units identified with permanent signs erected, and current information regarding nitrogen fertilization has been requested to proceed with the treatment applications.

It is anticipated that ordering the fertilizer will be completed by the end of this month to proceed with the May/June fertilization as scheduled.

The first tree size data collection will be obtained in the next quarter. AgerPoint has been contacted to potentially collect tree size data using Lidar sensors but cost appears prohibitive.

The project is on schedule.

E. Johnson 20-004 " Organic acids compared to conventional acidification for improved nutrient uptake and root physiology" – 10% Completion of Objectives (Mar. 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.
We are preparing plant material for CLas inoculation to initiate 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 identified multiple candidate field sites and are currently working on assessing soil characteristics to make sure they fit within the desired starting conditions, especially pH, for the trial.

Anticipated work for next quarter:
In the next quarter we anticipate inoculating greenhouse trees with CLas for objective 1.
We anticipate finalizing field site selection, layout of plots, preliminary plot data collection, and initiation of treatments for objective 2

Budget status :
The budget is in underspend because we need final field site selection before determining the injection equipment and amount of chemical for application based on row length.

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" – 85% Completion of Objectives (Mar. 2021 quarterly)

The objectives for this proposal are 1) Conduct field trials of new products and fungicide programs for Postbloom fruit drop (PFD) management as well as validation trials for the Citrus Advisory System (CAS); 2) Investigate the reasons for the movement of 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.

The two validation trials for CAS have been laid out in the Fort Mead area and a small amount of disease has been observed on early bloom in at least one site. We hope for at least one positive site. There were 3 weekly applications made and 2 applications according to the PFD-FAD. The fungicide trial had one application made on May 8th after a rain period. There were no triggers for disease this year at the site and this was the most likely infection period. Fruit data should be recorded in June or early July. A manuscript is in preparation for all the years of CAS validation.

The majority of analysis for the leaf wetness models is now completed and manuscript preparation is continuing. We compared the output of leaf wetness sensors to combinations of predictive models for accuracy and sensitivity. As for all models, reliability was heavily influenced by the quality of the weather station data. Reasonable accuracy was found for one, three, or four models in combination but with just two models, the predictive capacity was poor. We are in discussions for how this information could be used to improve CAS predictions. We are working on evaluating PFD risks via an analysis for prediction accuracy.

So far, we have tested floral extracts of pinhead, popcorn, and open citrus flowers. All floral extracts stimulated Colletotrichum abscissum conidial germination, and stimuli were compared among these treatments. We analyzed the sugar composition of the floral extracts. Then, we prepared sugar solutions with the same sugar concentration and composition to that of the floral extracts. The germination stimulus of the sugar solutions was higher than the water control, but less than the floral extracts. We tested the stimulus posed by leaf extracts prepared with new and old leaves on the pathogen. Leaf extracts were as good as floral extracts in stimulating the pathogen. As leaves are easier to obtain and available year-round, we decided to investigate the leaf extracts further, to pinpoint what portion of it was responsible for the stimulus considering polarity. We found that the highly polar portion of the leaf extract was responsible for the stimulus. An untargeted metabolomic analysis revealed that there are thirteen main components of the high-polarity portion of the leaf extract. These components will be tested soon.
We have been in conversation with the USDA and found out that they have only just repaired their wind tunnel and had slightly more access to the facilities. In the meantime, as we were unsure how soon we could use the equipment, we decided to build a similar tunnel at CREC. We have done a complete set of experiments with the new tunnel with inoculated leaves and flowers with and without rain. We are analyzing the data and adding it to what we observed with the USDA wind tunnel. The manuscript is in preparation and we may do a few follow up experiments as questions arise during the analysis

b. Citrus Black Spot

M. Dewdney 18-006 "Understanding the underlying biology of citrus black spot for improved disease management" – 75% Completion of Objectives (Feb. 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. From initial scouting, it appears that black spot severity is greater this year and there may be more differences among the treatments that will hopefully support the conclusions from the first year. 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. We will be collecting the data at the end of March, 2021.

We were unable to set up the second planned fungicide trial this year because of the COVID-19 shut down. We will be collecting pre-treatment data at the end of March and plan to move forward with the trial.

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. 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. We aim to sequence 6 isolates from each of the 5 South African provinces where CBS is found (North West, Limpopo, Mpumalanga, Kwa-Zulu Natal and Eastern Cape) and 6 each from the other countries (Argentina, Australia, Brazil, China, Swaziland and USA) by end of March.

Objective 3 (Survey for the MAT-1-1 mating type and two closely related Phyllosticta spp.). AStudies on the diversity of Phyllosticta spp. associated with citrus in Florida has progressed. Our collection of P. citricarpa isolates were obtained from citrus fruit in different areas under quarantine from 2010 to 2020. These isolates were previously screened by morphology to remove nonpathogenic P. capitalensis isolates. We are now screening the remaining 202 isolates using molecular techniques to determine if we have cryptic species that may have been misidentified as P. citricarpa. To date we have screened 125 isolates by amplifying and DNA sequencing the tef-1α (Translation elongation factor-alpha) locus. This screen has revealed that two isolates (Gc-6 and Gc-7) match to a Phyllosticta species not previously reported in association with citrus. The best sequence match based on ITS and tef-1α is to a species reported previously as a pathogen on a member of the Amaryllidaceae family (Hymenocallis littoralis) in Australia. Numerous inoculations of citrus have determined that these isolates do not cause citrus black spot. Multilocus analysis and leaf inoculations on Hymenocallis species are ongoing. These studies will allow us to conclusively determine the identity of these two isolates. Vegetative and sexual compatibility assays (sandwich mating) were performed to determine the mating type of the two isolates. The results showed that Gc6 and Gc-7 isolates are heterothallic and belong to the same mating type, as neither isolate was a capable of producing pseudothecia in solo cultures or in paired cultures with one another. 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.

We screened an additional 26 isolates of our P. citricarpa isolates for mating-type (MAT1-1 and MAT1-2). During this period we have screened an additional 26 new isolates and, as shown in Figures 1, only the MAT1-2-1 mating type is detected in new isolates collected in 2020. We conclude that the MAT1-1 mating type is still absent in the Floridian population.

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" – 60% Completion of Objectives (Feb. 2021 quarterly)

This project is a continuation of previously funded CRDF grants to TWO BLADES focused on utilizing multiple strategies to produce canker-resistant citrus plants plus the addition of a new strategy using gene editing. The 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. Grapefruit transformed to modify the bs5 gene to enhance resistance to the citrus canker bacterium is also being tested.

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. In late March 2019, the transgenic material was planted in the field at Fort Pierce in collaboration Dr. Ed Stover. Citrus canker has developed on plants in the field and the trees were rated for disease in November 2020. There was considerable defoliation on all trees including JJ5. We also observed disease on all susceptible Duncan trees, but no evidence of disease on the transgenic JJ5. The disease at this point was difficult to rate on Duncan given the defoliation. Next evaluation will be in June. As for developing a different transgenic, ProBs314EBE:avrGf2 has been placed in a vector acceptable for future transgenic purposes. The previous constructs contain an additional selectable marker for identifying putative transgenics with a higher success rate. Given the concern about the additional marker, the new construct contains only NPT as a selectable marker. The construct is with 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. Two most promising EFR transgenic plants (based on ROS activity) have been grafted onto the rootstocks US 812 and sour orange and planted them in the field at Fort Pierce. They were planted in the field in last March and rated in late July. Data analysis is underway. Additional transgenics from plants received from Dr. Vladimir Orbovic have been identified and grafted onto rootstocks. They and the original EFR transgenic plants were tested last October for susceptibility by pinprick inoculation. Leaves from all EFR positive grapefruit leaves were susceptible with typical pustule formation similar to the wild-type Duncan control. Lesions on leaves of one of the EFR positive sweet orange trees were smaller. EFR gene expression was also evaluated, and it was determined that all EFR grapefruit and orange had similar gene expression levels. Young leaves from representative trees of each transgenic event were inoculated by spray inoculation in February and the leaves will be rated for susceptibility.

Objective 3. To determine if bs5-generated transgenic Carrizo plants are resistant to citrus canker bacterium and to generate transgenic grapefruit carrying the pepper bs5. Budwood was recently received from UC Berkeley representing two transgenic events and a third was from a tree that was run through the transformation process, but negative for the gene. The latter was to serve as the negative control as it had undergone the transformation process. The buds have been grafted onto rootstocks and several have developed shoots. Recently leaves from the two CRISPR events, C1 and C2, were inoculated by pinprick inoculation and assessed for pustule size in December. Pustules on C2 leaves were similar in size to non-edited leaves while pustule formation on C1 leaves was reduced. When new leaves develop additional inoculations will be conducted this spring.

d. Lebbeck Mealybug

L. Diepenbrock 20-002C "Developing near and long-term management strategies for Lebbeck mealybug (Nipaecoccus viridis) in Florida citrus" – 35% Completion of Objectives (Mar. 2021 quarterly)

Work completed during the reporting quarter included areas in the original proposal:

(1b) Adjuvant screening preliminary trials have been conducted with adjuvants alone to determine their lethality to lebbeck mealybug. A total of 9 adjuvants, including and 4 from Helena Agri Enterprises were mixed with DI water at label rates and sprayed until dripping on Volkamer lemon leaves with mealybugs attached. Eight of the 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 at a 10-acre commercial citrus planting that was heavily infested in 2020 was initiated. First insecticide applications occurred as a pre-bloom prophylactic spray of a systemic material, with a second spray planned in the end of April.

(1d) Ant Management. A variety of methods have been tested to remove red imported fire ants (Solenopsis invicta) from citrus groves. Removing the ants can allow natural enemies to prey on and parasitize lebbeck mealybug without interference and provide more effective biological control.

Research goals are:

1. Reduce abundance of fire ants associated with lebbeck mealybug (Nipaecoccus viridis) in central Florida citrus groves.
2. Determine long-term efficacy of different treatment types at reducing fire ant abundance.
3. Assess time and cost of different treatment types.
4. Determine effects of ant treatment on predator abundance within trees and within N. viridis clusters. Currently collaborating with the King Lab from the University of Central Florida and are conducting the experiment in the grove of a local citrus grower. Four experimental treatments will be 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 will be assessed by counting the number of surviving colonies, and by determining presence/absence of foragers with pecan sandie baits. Natural enemy abundance will be assessed by dissecting mealybug clusters for predator larvae. Sampling for each of these methods will take place every 2 weeks for a total of 3 months post-treatments. Currently, all treatments have been applied and we are conducting follow-up surveys to determine their efficacy.

(1e) Evaluate management options for individual protective covers (IPCs). A study was recently completed to evaluate several commercially available entomopathogenic fungi (EPF) products as a potential tool to control lebbeck mealybug infestation on young citrus trees within IPCs. Findings of the study indicate that EPF can cause death of mealybugs and EPF conidia are viable on citrus trees within IPCs up to 42 days after treatment. Thus, EPF can offer control of mealybug for up to 1.5 months after treatment. In February, the team began infesting trees in our research planting to run a similar comparison of topical insecticides for clean-up, with pretreatment drenches planned prior to the next set of trees to be infested. Additionally, a controlled greenhouse comparison of drench materials including aldicarb is planned once trees develop sufficient leaf mass to enable testing of residual post application of all chemistries used in addition to impact on mealybugs. A methods trial was conducted and found that malathion lasted 2-3 times longer under IPCs compared to open field settings, suggesting that materials sprayed in IPCs that are known to have rapid UV breakdown may persist much longer and remain effective longer under IPCs.

(2a) Predator assessments. Predatory insects have been reared from locally infested plant materials the past two years, with the majority of predators emerging being parasitic flies, which consume a variety of piercing-sucking pest insects, mealybug destroyers, and one species of lacewing. A primer has been developed and evaluated to enable the detection of mealybugs in the guts of predators. Post-feeding retention time in a known predator was assessed and we feel confident that this tool will now enable screening of field caught arthropods that may play a role in the suppression of lebbeck mealybug. The commercially available predators Cryptolaemus montrouzieri, Orius insidiosus, Adalia bipuctata, and Hippodamia convergens 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 predators have also been screened, including Harmonia axyridis and larva of the genus Ceraeochrysa (colloquially called trash bugs). Both adult and larval of C. montrouzieri readily feed on lebbeck mealybug larvae and ovisacs, as do larval Ceraeochrysa. 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 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 current 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.

(2d) Develop tools to minimize spread. Killing lebbeck mealybug with isopropyl alcohol. A study to test different concentrations of isopropyl alcohol to determine how lethal they are to 1st instar lebbeck mealybugs has been conducted. 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. Two 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 at the CREC, an experiment using steam to treat adults and ovisacs at 100- and 120-degrees Fahrenheit for 5, 10, and 15 minutes to determine mortality has been completed. Previous preliminary experiments showed steam treatments at 130 degrees F 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 degree 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 also functionally 0%. However, mortality rose to 100% at 120 degrees F for 10 and 15 minutes. Currently, we are running trials on adults and ovisacs treated at 130 degrees F for 5 and 10 minutes, and preliminary results show 100% mortality.

Work planned for next quarter.
(1b) Adjuvant screening will continue to determine optimal adjuvants to work in synergism with insecticide sprays.

(1c) Continue treating and scouting the grove we have started a management comparison trial at.
(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, looking towards conventional materials for management and spray penetration by tractor mounted sprayers.
(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.
(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) Continue evaluating sanitation options and are planning to look at vehicle sanitation in the next quarter.

e. Phytophthora

E. Johnson 19-010 "Determining new cost-benefit guided Phytophthora propagule treatment thresholds for HLB-affected citrus" – 35% Completion of Objectives (Ja. 2021 quarterly)

HLB is known to make citrus roots more susceptible to Phytophthora root rot. 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 has been collected from Hamlin sites and the Valencia harvest is being planned currently. Phytophthora samples have been taken and this year's treatments will begin in April. plot by plot microjet irrigation systems have been built that connect to our trailer mounted handgun sprayer to mimic irrigation injection of materials for ease of application. A split plot factor is currently being added to the plot design to begin foliar brown rot sprays in midsummer through October.

Seedlings inoculated with HLB are being prepared for an additional 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. Inoculation will proceed when enough CLas+ budwood for graft inoculations or a large enough field population of psyllids after the spring flush to do a psyllid inoculation is available, whichever comes first.

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 (Jan. 2021 quarterly)

Citrus Nematode Exp 1 - The experiment was terminated in early January. Plants were removed from pots and weighed (tops and rinsed/blotted roots). Aliquots of fibrous roots were collected for metabolomic analysis and CLas determination, nematode infection rate, and moisture content determination. Females and offspring were separated from roots by comminuting in a dilute sodium hypochlorite solution; juvenile and male nematodes were extracted from soil on Baermann funnels. The females/g root (458) on the susceptible rootstock Carrizo citrange exceeded the damage threshold by more than twofold and those on the resistant rootstock Swingle citrumelo were about half the number considered damaging (103). Roots also exhibited symptoms of infection by Phytophthora nicotianae and the presence of the oomycete in soil was confirmed in all treatments. The contaminant originated from the orchard in which the nematode inoculum was obtained. The effects of rootstock, treatment and their interaction were all highly significant and therefore the results from each rootstock were considered separately. Treatment with citrus nematode or CLas reduced the root weight of Carrizo citrange by 57% and 55%, respectively and by 60% when combined. An expected additive interaction between these pathogens would reduce roots by 80%.

Therefore, the interaction of these organisms on root weight was antagonistic. Citrus nematode and CLas reduced the root weight of Swingle citrumelo by 20% and 26%, respectively and by 4% when combined. An expected additive interaction between these pathogens would reduce roots by 41%. Therefore, the interaction of these organisms on root weight was antagonistic. In the nematode susceptible rootstock there was a highly significant inverse relationship between the root weight and the nematode offspring recovered per gram of roots; however, HLB had no significant effect on nematode infection. The nematode resistant rootstock had significantly fewer females in roots (P=0.01) and offspring per root weight (P=0.001) when infected by CLas. qPCR measurement of CLas infection in roots and tops of plants is ongoing.

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