Citrus Research and
Development Foundation, Inc.

CRDF-Funded Projects Summary Progress Report FY 2019-2020
Quarter Ending June 30, 2019

RESEARCH TOPICS COVERED IN THIS REPORT

1. ***CLAS* PATHOGEN INTERVENTION**
    a. **Bactericides/antimicrobials**

K. Pelz-Stelinski 18-018 "Disrupting transmission of Candidatus Liberibacter asiaticus with antimicrobial therapy" – Objective 1: Quantify the effect of citrus antimicrobials on vector fitness. We expect to observe reduced longevity, reduced fecundity, and longer development times among ACP exposed to antimicrobial treatments as compared with unexposed ACP. We previously reported (March 2019) on the effect of dietary antibiotic treatments on ACP survival (Obj. 1.1). During the past quarter, we evaluated the fecundity and fertility of ACP exposed to antibiotic treatments. The reproductive output of *D. citri* exposed to oxytetracycline or streptomycin treated plants was evaluated in a greenhouse assay. Five-month-old *Citrus* plants reared in an insect free greenhouse without exposure to insecticide received foliar applications of streptomycin (FireWall 50WP (Agrosource), or oxytetracycline (Agrosource,) or 1.0 mg ml-1 imidacloprid (Bayer CropScience LP), and water for a control. Females were allowed to oviposit on citrus plants with new growth (flush) over a 25-d period. Total eggs laid (fecundity) were counted under a stereoscope each 5-d period, then transferred to newly treated plants with flush to encourage oviposition. To determine if compounds containing oxytetracycline or streptomycin had an effect on the number of hatched eggs (fertility), plants were maintained as previously described for 6-d after adult removal. The total number of nymphs on plants were counted every 3-d under a stereoscope and recorded. This experiment is currently wrapping up and data analysis is underway.

Objective 2. Determine the effect of antimicrobials on Las transmission. We expect that ACP feeding on antibiotic treated infected citrus plants will be less likely to transmit Las. We initiated field experiments to evaluate the hypothesis that ACP will be less capable of transmitting Las after feeding on antimicrobials, because trees treated with antimicrobials are more likely to have lower Las titers for acquisition (Objective 2.2). An experiment was initiated in mature, infected citrus trees located in a research at the CREC to determine whether field applications of foliar antimicrobials are also capable of suppressing acquisition of Las. Trees will be received an initial treatment with streptomycin, oxytetracycline, or receive no antimicrobial treatment. Ten insects from uninfected laboratory cultures were caged on young leaf growth (flush) of antimicrobial-treated or untreated infected trees using mesh bags for oviposition. Treatment were be replicated 10 times on individual trees. Survival of females is be monitored for two weeks. Females will be collected at the end of this quarter (late June) and preserved in 80% ethanol at -20°C for subsequent analysis and CLas detection. Egg clutches will remain on trees enclosed in mesh sleeves. After the nymphs reach the adult stage (approximately 15 days), adult psyllids and three leaves exposed to the psyllids will be collected for analysis.

S. Santra 18-020 "Novel multi-metal systemic bactericide for HLB control" – Systemic characterization of three Mg-Zn gel based multi-metal formulations containing Cu A.I. [Cu:Mg:Zn (10%:45%:45%, MM10C45M45Z), Cu:Mg:Zn (15%:42.5%: 42.5%, MM15C42.5M42.5Z) and Cu:Mg:Zn (20%:40%: 40%, MM20C40M40Z)] is in progress. The hydrodynamic size of as-prepared materials was measured through dynamic light scattering (DLS). MM10C45M45Z, MM15C42.5M42.5Z, and MM20C40M40Z showed increased hydrodynamic size with higher Cu content (290.2 nm, 334.7 nm, and 354.2 nm respectively), suggesting Cu could induce the aggregation of primary particles. The primary particle size of multi-metal formulations (MM25C75M, MM25C75Z, MM10C45M45Z, MM15C42.5M42.5Z, and MM20C40M40Z) will be evaluated through electron microscopy. The electron microscopy results for the multi-metal formations will be included in the next report.

The antimicrobial efficacy of materials was tested in *vitro* against *Pseudomonas syringae*. To determine the minimum inhibitory concentration (MIC) of the materials and selected controls, a macro-dilution assay was performed (*Nature Protocols*, 2008, 3, 163-175). The MIC value was determined by measuring the optical density at 600 nm after 48 h incubation at 27 °C under shaking (150 rpm). The MIC value of MM25C75M, MM15C42.5M42.5Z, and MM20C40M40Z was 80 ppm of metallic Cu, which was similar to copper controls (copper nitrate and Kocide 3000). The MIC value of MM10C45M45Z was 40 ppm of metallic Cu. The enhance antimicrobial efficacy may due to the smaller particle size. The MIC value of MM25C75Z was 5 ppm of metallic Cu. This result suggests that MM25C75Z formulation might contain ultra-small size particles. To determine the minimum bactericidal concentration (MBC) of these materials and selected controls, a colony-forming unit (CFU) assay was performed (*Nature Protocols*, 2008, 3, 163-175). The MBC value of MM25C75M, MM25C75Z and MM10C45M45Z was 80, 10 and 40 ppm metallic Cu respectively. All multi-metal formulations showed enhanced bactericidal efficacy when compared with commercial copper bactericide control (Kocide 3000, MBC: 320 ppm metallic Cu).

To evaluate the systemic mobility of the Cu A.I., a plant uptake study was performed using *Citrus reshni* (Cleopatra mandarin) seeding in a growth chamber (Panasonic Environmental Test Chamber, Model MLR-325H, Kadoma, Japan). Temperature and relative humidity were maintained at 35 ºC and 85%. The citrus seeding was foliar-sprayed using a hand-pump sprayer with 300 ml of MM25C75M, MM25C75Z, MM10C45M45Z (500 and 1000 µg/ml metallic Zn concentration) and deionized water as a control. After 48 h incubation in the plant growth chamber, citrus seedlings were removed from the soil and gently washed with 1% cleaning detergent (Alconnox ®, Alconnox Inc.) and 0.1% HCl. Leaves, roots and stem sections were separated after washing and left in an oven for 48 h (45 ºC). Then, the dried leaves, roots, and stems were separately acid digested (EPA method 3050 B "Acid Digestion of sediments, Sludge, and Soil). Cu, Zn and Mg contentions in leaves, stems, and roots will be quantified with Atomic Absorption Spectroscopy (AAS). The AAS results will be included in the next report.

Z. He 18-040C "Evaluation of the spatiotemporal dynamics of bactericides within the citrus tree via different application methods" – The purpose of this project is to reveal the mechanisms of bactericide uptake and transport in citrus trees by (1) comparing the delivery efficacy of bactericides with three application methods (foliar spraying, truck injection, and root administration) based on the uptake and dynamic movement/distribution of the bactericide within the tree; (2) to clarify the systemic movement and transportation mechanisms of bactericides within the phloem of tree; and (3) to investigate the effects of citrus variety and age on the delivery efficacy of bactericides in the greenhouse and field. Prior to conducting these experiments, a sensitive and accurate quantifying method of bactericides (oxytetracycline and streptomycin) in citrus tissues is needed for simultaneous determination of both oxytetracycline and streptomycin in citrus plant tissue samples, but it is not available in literature. Therefore, we designed a method based on the extraction of other antibiotics in plant samples. We replaced methanol with acetonitrile and used lower concentrations of formic acid for the extraction of antibiotics in citrus plant tissue samples. Laboratory tests demonstrated the potential and feasibility of the newly designed method for simultaneous analysis of oxytetracycline and streptomycin in citrus plants, but matrix interference with antibiotic analysis was still significant. To further improve this method, a solid-phase extraction (SPE) method was developed to clean up the sample matrix, and analytical conditions of instrumentation were modified by changing the gradient profile of LC and lowering the operation temperature of MS/MS. These modifications substantially improved instrumental sensitivity for detecting oxytetracycline and streptomycin plant samples. This newly developed method has now been successfully applied to the simultaneous detection of oxytetracycline and streptomycin in citrus leaf samples.

b. Diagnostics

M. Irey 17-002C "Continued Support for the Southern Gardens Diagnostic Laboratory" – Over the two-year grant period, 49,362 samples were run (down from 63,486). Of the samples run, 95% were plant samples (mostly leaves but some roots) and 5% were psyllid samples. Approximately 58% of the samples were run during year 1 of the funding (28,478) and 42% were run during year 2 of the funding (20,488). The great majority of the samples were from research trials. The fact that most of the samples were from research trials is similar to the previous funding cycle but represents a change from the early years where the samples were primarily diagnostic in nature. Since the samples were from trials, sample submissions tended to come in based on the timing required in the trial (i.e. twice a year, quarterly, monthly, etc.). Also depending on the trials, the samples tended to come in as large groups as opposed to individual small lots of samples. At times, samples arrived in groups of a 500 or more, making scheduling a little difficult as the lab never knew when and how many samples were going to arrive. Sample turnaround varied from under a week to 4-6 weeks depending on the sample load.

During the previous funding period and continuing this funding period, and at the request of the customers, the SGCDL began quantifying the amount of DNA in the sample and running a standard curve in order to provide copy number in addition to the raw CT values. As time progressed, more and more of the samples were reported in this matter. For the current funding period, copy number was reported on approximately 50% of the outside samples (does not include internal Southern Gardens samples). As a result of having to perform the extra steps necessary to provide the DNA quantification and copy number analyses, sample turnaround sometimes was a little longer than for samples that were not quantified.

The total number of samples was 49,362, down approximately 14,000 compared to the previous funding cycle. This is in part due to the effects of Hurricane Irma (which changed the planned sampling for many trials) and to a decline in the sampling of some of the CRDF trials (antibiotic trials?), which traditionally has been the largest customer of the laboratory.

1. **ASIAN CITRUS PSYLLID VECTOR INTERVENTION**
    a. **Asian Citrus Psyllid management**

C. Vincent 16-020C "Dyed kaolin to repel Asian citrus psyllid in field conditions" – The long term field trial continues with weekly psyllid counts and quarterly CLas infection testing. Treatments continue to have similar effects on ACP counts. Plants in both of the kaolin treatments continue to show higher growth rates than the other two treatments. The red treatment has the highest growth rate, trunk cross-sectional area, and canopy volume. Kaolin treated trees that are infected grow more than untreated-infected trees, but less than treated uninfected trees. The field trial will continue until the project ends, when we expect to have the first economic yield.
We are now performing follow-up repetitions of the MS student's thesis work. We anticipate publication submission of this work in the Fall.

L. Stelinski 17-001C "Insecticide resistance management in Florida citrus production" – The current study was conducted as a risk assessment of potential evolution of insecticide resistance phenotypes and genotypes in Asian citrus psyllid (ACP) populations to fenpropathrin as a result of laboratory selection. We also investigated cross resistance of ACP to fenpropathrin with to other insecticide modes of action. The obtained results should contribute to development improved resistance management strategies.

First, assays and selection were performed with adults from a field-collected strain from Wauchula, FL on July 15, 2018 (WF). The bottle bioassay technique was used to determine susceptibility levels of adults of WF strain ACP to fenpropathrin. The chemical residues were achieved by pipetting 200 µL of acetone into a bottle and by rotating the bottle until the acetone evaporated. Subsequently, 5- 10 ACP adults were aspirated and transferred to the treated bottle. The LC50 was determined. Surviving individuals were reared on plants for eight generations. For each generation, adults of the WF strain were exposed to the LC50 concentration. We determined the risk assessment of ACP phenotypical resistance to fenpropathrin. After eight selected generations, the realized heritability of resistance (h2) to fenpropathrin was determined. The estimated h2 to fenpropathrin was 0.10 by the end of selection. The h2 of fenpropathrin resistance was 0.17 and 0.44 during the first and the second rounds of selection, respectively. The h2 values obtained at second round of selection are very high and could indicated a high level of risk in the field population for development of resistance to fenpropathrin. The results also suggest that a brief selection experiment may be sufficient to detect the potential for the development of resistance.

Second, we investigated level of resistance to pyrethoids in laboratory and investigated the possible mechanism involved cross resistance to two relatively commonly used insecticides, dimethoate and imidacloprid. Results indicated that there was no evidence of high cross resistance to imidacloprid (RR = 1.54) and dimethoate (RR = 4.36) for the WF fenpropathrin-resistant strain. At this point, rotation of fenpropathrin, dimethoate and imidacloprid should not increase insecticide resistance. These results are particular important for verifying the effectiveness of the rotation schedules we are putting into practice in the field.

Third, we investigated pyrethroid resistance levels and the associated molecular mechanisms in fenpropathrin resistant strain of ACP. The relative gene expression of six cytochrome P450s (CYP6A1, CYP6A2-1, CYP6A13, CYP6A14, CYP6J1 and CYP6K1) and four glutathione S-transferases (GST1, GST2B, GST3 and GST4) were quantified in the selected population, and compared with the laboratory susceptible population. qRT-PCR analysis showed that expression of CYP6A2-1 had significantly increased in the selected population relative to the laboratory susceptible population. Our results indicated that increased target insensitivity and cytochrome P450 metabolic detoxification could be mechanisms responsible for the ACP resistance to the pyrethroid fenpropathrin.

The results further confirmed that the multiple resistance mechanism following artificial selection on the field strain did not confer significant cross resistance to insecticides with other modes of action. Thus, we are able to recommend, based on a large body of evidence, that fenpropathrin, imidacloprid and dimethoate can be effectively rotated in sequence as an effective resistance management protocol for ACP.

L. Stelinski 18-056C "Functional IPM for Asian citrus psyllid under circumstances of chronic HLB" – The objective of this study was to develop an insecticide rotation with different modes of action as a resistance management strategy for Asian citrus psyllid (ACP) in Florida. We selected two large scale experimental sites in two citrus groves in central Florida. One site was in Lake Alfred (Polk County) on CREC property planted with the cultivar 'Hamlin'. The second site was in Wachula (Hardee County) and was planted with 'Valencia' selections. The Lake Alfred site has trees that are bearing fruit. At the Wauchula site, the trees are non-bearing. At each location, three rotation schemes were established ion 4.9 acres each in Lake Alfred and on 4.2 acres each in Wachula. For sampling, we established 6 replicates per treatment in Lake Alfred and 4 replicates in Wachula.

The insecticides were applied in two rotational schemes with five different modes of action. The third treatment was to apply different neonicotinoids with no change in the mode of action. Rotation A consisted of dimethoate followed by cyantraniliprole, fenpropathrin, diflubenzuron and imidacloprid. Rotation B consisted of fenpropathrin followed by dimethoate, imidacloprid and diflubenzuron. No rotation consisted of thiamethoxam, clothianidin, thiamethoxam, imidacloprid and clothianidin. At this point we have completed two applications at both locations.

Prior to the insecticide applications, the plots were monitored for ACP adults using tap sampling. Weekly monitoring was initiated on March 20, 2019 in Wachula and April 1, 2019 in Lake Alfred. When the average number of adults per tap reached 0.4 insects the appropriate insecticide was applied using an airblast sprayer by the cooperator. Also, ACP adults were collected and a baseline insecticide susceptibility was determined and compared with our susceptible laboratory population using a leaf dip assay. Five to six concentrations of each insecticide was tested and replicated 5 times. We found low to moderate resistance for thiamethoxam (RR > 20), imidacloprid (RR > 10), clothianidin (RR > 10) and dimethoate (RR > 10). We found very low resistance to the other insecticides in the field populations (RR < 5).

Finally, before application, morphological measurements on adult ACP were made At least 50 individuals were measured. Body length, abdominal length, wing length, femur length and head width were measured for laboratory susceptible cultures and for both field populations. The results indicated that the abdominal length, wing length and femur length was greater in the laboratory population compared with both field populations.

The current investigation is ongoing. It will continue to monitor ACP management and resistance among the treatments and locations described above. Our goal is to find a more refined method of effective insecticide resistance management of ACP that also shows highest efficacy. Our newly developed protocols on suppression of ACP populations by stabilizing or reducing resistance will be communicated with growers when the results are properly verified experimentally.

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

T. Eyrich 16-016C "Use of RNAi delivered by the Citrus Tristeza Virus Viral Vector to control the Asian Citrus Psyllid" –1. In April all trial trees were sampled and tested using ELISA to detect the presence of CTV and gel electrophoresis and rtPCR to detect the presence and stability of CTVvv-RNAi.
2. All trial trees were also sampled and tested using qPCR for the presence of HLB in April.
3. Aphid scouting continues on a biweekly basis. The presence of brown aphid has not been detected.

2. CITRUS HOST INTERVENTION

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

F. Gmitter 15-010 "Development and Commercialization of Improved New Disease Resistant Scions and Rootstocks - the Key For a Sustainable and Profitable Florida Citrus Industry" – Objective 1. Development of rootstocks that can impart HLB tolerance/resistance to grafted scions. Seedlings grown from 12 unreleased rootstocks, based on their abilities to control tree size, support good fruit loads, and to have minimal HLB symptom expression, were budded with sweet orange scions for field planting at the St. Helena site in 2019. As part of the `gauntlet' screening, 75 new candidate rootstock hybrids produced from HLB-tolerant parents in 2017 with HLB+ Valencia sweet orange were stick-grafted with wood from HLB infected trees; Files from more than 80 sites were reviewed, conditions of the trials were noted, and plans for 2018-19 field data collection prioritized based on this information. Efforts to review and summarize data continue for inclusion in the website.
https://citrusresearch.ifas.ufl.edu/rootstock-field-data/ Summaries of 10 trials are currently posted. Summaries are intended to inform growers that the most promising new rootstocks are being promptly moved forward in evaluation, and to enlist their support in identifying promising rootstocks.
Objective 2. Breeding of HLB tolerant/resistant processing sweet oranges and orange-like hybrids. New hybrids produced were identified for propagation and field planting next season. Fruit and juice samples of existing UF releases and new oranges under consideration for release were displayed to the industry in November and December 2018. New selections of orange-like hybrids were well received. Blends of sweet orange like-selections ranked higher than most sweet orange juice samples. Promising selections were entered into the DPI PTP for cleanup and certification.

Objective 3. Screening of the UF-CREC germplasm collection to identify and validate HLB tolerant or resistant selections. Observations from fall-winter 2018 combined with previous seasons' data, were reanalyzed to more accurately identify and characterize tolerant individuals. This information was used to target specific genome regions that may harbor genes for tolerance, for use in other projects.
Objective 4. Advanced field trials, release and commercialization of promising HLB tolerant/resistant scion and rootstock cultivars. In fall 2018, trees in 18 different field trials were evaluated for HLB responses and overall tree health, and fruit from several trials were harvested for juice quality analysis. Yield data were also collected from some of these trials. In 2018, an effort was undertaken to rescue promising individual trees of diverse scion and rootstock germplasm from a 50-acre research block at the UF-GCREC in Balm. Trees of ~1800 selected individuals displaying potential for HLB tolerance were propagated for planting at a new location in 2019.

K. Bowman 18-004 "Development of SuperSour and other outstanding rootstocks with tolerance to HLB" – Objective 1. During this time period, hybrid seed from 2018 rootstock crosses were planted, including SuperSour type parentage of unique HLB tolerant types. One new replicated rootstock trial with Star Ruby grapefruit was planted on the East coast in April which included several advanced hybrid rootstocks that have demonstrated outstanding field performance in other trials in comparison along with several commercial standard rootstocks.
Multiple trees of about 150 selected new rootstock hybrids were budded and grown in the nursery, in preparation for field planting new rootstock field trials later in 2019. These nursery trees included many of the most promising SuperSour hybrids identified in ongoing trials established in previous years, as well as several commercial standard rootstocks. These also include new and different hybrids chosen based on available information about parentage and characteristics best associated with outstanding traits. Three new field trials with sweet orange scions will be planted from these trees in 2019, including one trial in the East coast, one in the Central ridge, and one in the Southwest.
Objective 2. Seventeen rootstock trials planted prior to summer 2018 (as described in the Proposal Appendix ii) were monitored and used for data collection on field performance, as appropriate during this quarter for the scion involved. Yield and fruit quality data were collected from multiple Valencia rootstock trials, including cooperative trials with Larry Black in Polk County and Duda in Hendry County. Fruit yield, fruit quality, tree size, and other metrics from the USDA cooperative Valencia trial in Polk County were summarized in a 4 page handout distributed at the CRDF Rootstock Field Day in April 2019. Three SuperSour rootstocks identified in the trials as particularly outstanding in potential yield per acre and yield efficiency are US-2122, US-2101, and US-2134, and these rootstocks will be established in additional trials and examined more carefully for potential upcoming commercial release. Seed trees of all the most promising new SuperSour hybrid rootstocks have been planted at the USDA research farm in Ft. Pierce and the Whitmore Farm to facilitate availability of seed for new rootstocks at the time of release.
The three new USDA rootstocks released in November 2018, identified as US SuperSour 1 (SS1), US SuperSour 2 (SS2), and US SuperSour 3 (SS3), are being propagated in nurseries for numerous planned commercial plantings. An informational sheet with performance data on the three rootstocks was prepared and distributed widely to industry.

F. Gmitter 18-011 "Part A - The UF/CREC Core Citrus Improvement Program" – 1. Develop new rootstocks that impart HLB-tolerance to scion cultivars. Trees that were selected from the gauntlet screen from 2018 crosses were stick grafted with CLas-infected Valencia budwood for further selection of tolerant types and are now under observation. Several crosses were made in spring 2019 for rootstock improvement at the diploid and tetraploid levels using parents that have previously demonstrated good genetic combining ability. They will generate new families that combine tolerance of high pH calcareous soil and Phytophthora tolerance with appreciable tolerance of HLB in grafted scions. Previous good parental combinations have included AMB+HBP x Sour Orange + Rangpur, among others at the tetraploid level. Recently we have used LB8-9 Sugar Belle as a seed parent with various pollen parents and have selected several survivors in the gauntlet. Some of these crosses were repeated to increase the likelihood of finding superior performers. DNA fingerprinting techniques were used to verify the origins of the "Super-Root Mutants" that have been selected from in vitro propagations in a commercial nursery.

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. A large number of crosses were made in spring 2019 to produce families for selection of individuals that meet this objective and may be valuable for commercialization following advanced testing, as well as to continue the process of parent development to improve future outcomes from breeding. Over 25 crosses were made for grapefruit development, using at least one parent shown to be highly tolerant of HLB and citrus canker; 10 crosses were made to create families of sweet orange like hybrids, including 2 using Parson Brown as pollen parent, at the recommendation of some citrus growers, and 40 crosses for mandarin hybrid development and parent building. Some of the crosses for mandarin improvement may yield also sweet orange-like hybrids.

3. Screen the germplasm collection for more tolerant types and evaluate fruit quality of candidate selections. Fruit quality attributes of several very tolerant types were evaluated, but not tree health vis a vis HLB in the last 2 months.

4. Conduct studies to unravel host responses to CLas and select targets for genetic manipulations leading to consumer-friendly new scion and rootstock cultivars. Anatomical studies of HLB-tolerant LB8-9 and Bearss lemon demonstrated that phloem regeneration is a mechanism of their apparent tolerance.

T. McNellis 18-016 "Testing grapefruit trees expressing an anti-NodT antibody for resistance to HLB" – Two students were successfully recruited to work on the project during, March 15 - June 15, 2019. Mr. Chad Vosburg has accepted an offer of admission as a M.S. degree student in the Penn State Department of Plant Pathology graduate program. He has experience working with HLB and is currently involved in HLB research as a technician. Chad will begin his studies in August 2019 and will be working on the citrus infection and HLB disease measurement parts of the project. Mr. Jeremy Held, who was rotating in the PI's lab starting in February, 2019, has elected to stay in the PI's lab as a Ph.D. student in the Intercollege Graduate Program in Plant Biology at Penn State. Jeremy will be working on the tree molecular characterization objectives of the project. Jeremy has already successfully detected the FT-scFv protein by protein gel immunoblotting and verified the pollen viability of trees producing the FT-scFv protein. Personnel to complete this project are now in place.

The PI visited the lab of collaborator Dr. Tim Gottwald at the United States Horticultural Research Laboratory (USHRL) in Fort Pierce, Florida, on June 10 - 11, 2019. Chad Vosburg, who is from the nearby area, was also present for part of these meetings. The current status of existing trees was determined, and a plan was developed by Drs. McNellis and Gottwald and Mr. Vosburg and Mr. Earl Taylor (Gottwald Lab research assistant) for tree propagations that were needed and setting up a series of HLB resistance tests. At least 4 of the FT-scFv lines had sufficient numbers of trees available to set up a test immediately. Chad would best work at USHRL as a "guest". We plan to initiate plant propagations and one HLB resistance experiment run during the next reporting period, prior to Chad's formal beginning of studies at USHRL.

Z. Mou 18-017 "Establish early-stage field trials for new HLB-tolerant canker-resistant transgenic scions" – 1) Forty-nine transgenic plants (30 Hamlin sweet orange plants expressing transgene 1, six Duncan grapefruit plants expressing transgene 1, six Duncan grapefruit plants expressing transgene 2, six Duncan grapefruit plants expressing transgene 3, and one Duncan grapefruit expressing transgene 4) were planted on May 9, 2019 in Picos farm with the help from Dr. Ed Stover. Twenty healthy plants (10 'Hamlin' plants, 10 grapefruit plants) purchased from Brite Leaf Nursery were randomly planted together with the transgenic plants. All plants currently growing well and will be evaluated.
2) Propagation of CTV and CLas free transgenic plants continues. More budwood identified as negative for CTV and CLas has been grafted onto rootstocks. We will keep propagating plants whenever negative budwood samples are available. The propagated progeny plants will be analyzed for transgene expression by Western blotting.
3) A major citrus defense gene was cloned. This gene is expected to provide resistance or tolerance to HLB when overexpressed in citrus. Generation of cisgenic citrus plants using this gene is planned. The full-length cDNA was sequenced and cloned into a bacterial expression vector. Protein is being expressed for antibody development. The antibody will be very useful for identifying cisgenic citrus plants that accumulate high levels of the defense gene product, which would help shorten the time-consuming screening process.

E. Stover 18-022 "Delivery of Verified HLB-Resistant Transgenic Citrus Cultivars" – Objective 1) Mthionin Constructs: Assessment of the Mthionin transgenic lines is ongoing. Detached leaf assays, with CLas+ ACP feeding, have been conducted and lines with the most promising results have begun greenhouse studies. These 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 from the first round of field plantings with Mthionin transgenic Carrizo (45 plants) rootstock with non-transgenic rough lemon continues. Initial results show transgenics maintaining higher average CLas CT, significantly decreased leaf mottle and significantly increased health values after 6 months. A large group of Mthionin plants were planted in April, including transgenic Carrizo with WT Hamlin scions (81 plants) and transgenic Hamlin on non-transgenic Carrizo rootstock (108 plants) with WT/WT controls (16 plants). Additional grafts of WT Ray Ruby (118 plants) and WT Valencia (118 plants) onto transgenic rootstock are being propagated for future plantings. Mthionin construct transformations have also been completed on 250 Valencia explants to provide sufficient events for this critical variety.

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Objective 2) Citrus Chimera Constructs: Detached leaf assays, with CLas+ ACP feeding, were conducted on lines representing chimera constructs TPK, PKT, CT-CII, TBL, LBP/'74', `73', and `188'. Multiple lines from several constructs were moved forward into greenhouse studies based on these results as noted below. Definitive results for TPK, PKT, CII, and TBL were hindered by low inoculation rates. Assays for these constructs are being repeated to identify which lines of each are best suited for greenhouse studies. Detached leaf feeding assay protocols have also been adjusted to improve sensitivity (See section 4)

No-choice caged ACP inoculation has been conducted on 8 lines of citrus Thionin-lipid binding protein chimeras (`73', and '74'). Early data from CLas+ plants showed a statistically significant reduction (13x) in CLas titer for transgenics vs WT in the CLas+ plants. However, many plants shown little to no amplification of CLas DNA at 3 and 6 months post inoculation. CLas multiplication by this time would be expected from a successful inoculation, indicating low inoculation efficiency. All plants in this experiment will be re-inoculated by bud grafting with HLB+ rough lemon to allow for continued greenhouse studies. Parallel field trials for phenotyping efforts and modification of the ACP inoculation in greenhouse will focus on increasing CLas inoculum pressure. 475 rooted cuttings were previously made from Hamlin and Carrizo lines expressing constructs `74' and `188' and are now of a size appropriate for CLas exposure. 360 of these plants will be grafted with WT scions (for Carrizo lines) or rootstocks (For Hamlin lines) for field trials. The remaining 115 plants will be ACP inoculated for greenhouse studies, with the caged no-choice feeding time period extended from 7 days to 14.

Seven new transformations, totaling over 2000 explants, have been completed to generate Valencia, Hamlin and Ray Ruby lines expressing constructs `74', `188', and TPK.

Objective 3) ScFv Constructs: Greenhouse studies on the 5 scFv lines in the 1st round of ACP-inoculation has been completed with the best performing lines showing significantly reduced CLas titer over the 12 month period (up to 250x reduction) and a much higher incidence of no CLas rDNA amplification in all tissue types. The best lines have been used as rootstock for WT Ray Ruby scions and will be moved to the field after necessary permit approvals. An additional 129 rooted cuttings are being propagated for follow up plantings.

Like the '74' results discussed under Objective 2, the 2nd round of ACP-inoculations of scFv plants (150 plants, 12 lines) had a poor infection rate. The plants will be re-inoculated by budding with HLB+ rough lemon. The 370 scFv rooted cuttings already propagated for a 3rd round of ACP-inoculations will be screened using the higher pressure 14 day feeding protocol described above.

Objective 4) Screening Development and Validation: Details of the high throughput ACP homogenate assay, and its use for selecting lytic peptides for activity against CLas, has been submitted for publication and remains in use for early screening of therapeutics in the lab. The detached leaf ACP-feeding assay has undergone several small revisions to improve sensitivity and maintain consistent inoculation; increasing from 10 to 20 ACP per leaf, decreasing the feeding period (7 days to 3) and adding a 4 day incubation period between feeding and tissue collection. An array of phloem specific citrus genes has been selected for investigation as potential reference genes to improve detached tissue and plant sampling techniques. The use of a phloem specific endogene would allow for samples to be normalized to phloem cells instead of total citrus cells, more accurately evaluating bacterial titer and potential therapeutic effects with the phloem limited CLas.

Objective 5) Transgene Characterization: Transgenic Carrizo lines expressing His6 tagged variants of chimeric proteins TBL (15 lines), BLT (15 lines), TPK (17 lines), and PKT (20 lines) have been generated and confirmed for transgene expression by RT-qPCR. These plants will be used for generating data on the movement and distribution of transgene products in parallel to antibody based approaches.

U. Albrecht 18-028C "Comparison of field performance of citrus trees on rootstocks propagated by seedlings, cuttings, and tissue culture" – 1) During the second quarter of 2019, field trial evaluations of seedling, cutting and tissue culture propagated trees continued. Trees three trials are composed of Valencia scion on the three propagation types for eight different rootstocks (US-802, US-812, US-897, US-942, US-1516, X-639, Swingle, Cleopatra). The SWFREC trial (Collier County) was established in October 2017. The two commercial trials were established in April 2018 on a typical Ridge site in Polk County and on a flatwoods type-site in southwest FL (Hendry County).

2) Rhizotron root growth measurements at the SWFREC location continued in monthly intervals. Results showed some differences in root growth among propagation methods within a rootstock cultivar during the first year of growth in the field. But, when averaged across all rootstock cultivars, no differences associated with propagation method were observed. Statistical analyses are still ongoing. After one year of growth, significant differences in tree height were found among trees on different rootstocks independent of the propagation method. The largest tree size was measured for US-1516 and the lowest for US-897. No statistically significant differences in tree size were attributed to the method by which rootstocks were propagated. Other horticultural parameters such as trunk diameter and the ratio of rootstock to scion trunk diameter were significantly influenced by rootstock and by propagation method. Nutrient analysis of leaves showed no significant differences associated with the propagation method, but differences were found among trees on different rootstocks for all nutrients except nitrogen and potassium. The SWFREC trial was demonstrated during a field day in April 2019.

3) During the first year of growth in the commercial locations, no dieback of trees was observed for trees at the Polk County location. A small percentage of trees died within several weeks after transplant at the Hendry County location, but dieback was not related to the propagation method or rootstock cultivar. After one year of growth, no significant differences in tree height, canopy spread, and trunk diameter among trees were observed based on the method by which rootstocks were propagated in both trials. In contrast, significant differences were observed for trees on different rootstocks independent of the propagation method. Data analysis is still ongoing. Rhizotrons for root observations were installed during the previous quarter, and the first measurements were conducted.

Tree propagations for the additional field trial to be conducted in Indian River County were completed and trees are maintained at the US Horticultural Research Lab in Ft. Pierce until ready for planting. Trees are Valencia scion grafted on four different rootstocks (US-812, US-897, US-942, and US-1516) propagated by seed, cuttings, and tissue culture.

U. Albrecht 18-029C "Evaluation of citrus rootstock response to HLB in large-scale existing field trials using conventional and automated procedures" – During the second quarter of 2019, horticultural assessments of rootstock trials at two Lykes groves continued. In April 2019, fruit samples were collected from the Valencia trees at both the Lake Wales and the Basinger location. Because of the high cost, only fruit on rootstocks in trial that were fully replicated were analyzed for the following quality parameters: fruit weight, % juice, % solids, % acid, brix/acid ratio, and juice color. Fruit quality analyses were conducted at the CREC Processing Pilot Plant. Fruits were harvested in both locations shortly thereafter and yield data collected from trees on all rootstocks, even if not fully replicated, as this data may provide important information to the breeders.

Y. Ampatzidis 18-033C "Automated root mapping to enhance field trial evaluation of citrus rootstocks in the HLB era" – In the first six months of this project, the influence of several limiting factors on the performance of a ground penetrating radar (GPR) to accurately detect HLB-affected citrus roots and determine their main structural characteristics.

1) GPR generated a hyperbola in the radar profile; from the width of the hyperbola, the diameter of the root was determined when roots were larger than 6 mm in diameter. GPR also distinguished live from dead roots, which is indispensable for studying the effects of soil-borne and other diseases on the citrus tree root system. If two or more roots were located in close proximity, GPR distinguished the roots only if their horizontal distance was greater than 10 cm and their vertical distance was greater than 5 cm. The results demonstrate that GPR is useful for studying citrus tree root systems under Southwest Florida grove conditions.
2) An automated (remote-controlled) platform is under development for the GPR apparatus. Current GPR technology requires the operator to manually complete 360-degree peripheral scans around the tree trunk (per tree) to cover the entire root system. A wheel encoder measures and records the distance covered by the GPR. This manual procedure generates many errors ("noise"), because it is difficult for the operator to follow a "perfect" circular path (360-degree peripheral scan) around the tree. The automated system will increase data collection accuracy and decrease data collection time. The first prototype is ready for field trials.

R. Ferrarezi 18-037C "Performance of newly released grapefruit cultivars and rootstocks in the Indian River Citrus District" – Project start date 6/1/19; first progress report due 9/15/19.

J. Grosser 18-039C "Part B - The UF/CREC Citrus Improvement Program's Field Trial Evaluations" – Significant progress was made in establishing the website https://citrusresearch.ifas.ufl.edu/rootstock-trials/ for presenting data from field trials. Initially data from 10 trials were posted.

Summary of data-collection activities this quarter: Trial Name and Data Type Collected
Banack (Vero Beach) - Juice quality 2018-19
Cody Estes (Vero Beach) - Tree health Ratings 2018-19
CPI Ranch 1(Immokalee) - Tree health Ratings 2018-19
Doe Hill Grove (Bryan Paul) - Tree health ratings, yield, juice quality 2018-19
Duda (Immokalee) Vernia/rootstock - Yield, juice quality, tree health ratings 2018-19
Duda (Immokalee) APS-OHS Valencia/rootstock - yield, juice quality, tree health ratings 2018-2019
Ed English (LaBelle) - Tree height, tree health ratings 2018-19
Hammond (Vero Beach) - Tree health ratings, yield and juice quality 2018-19
IMG (Vero Beach) - Yield and fruit quality 2018-2019
Lee Groves (St. Cloud) 13E Frank's Block OLL seedling/rootstock - Fruit quality, PCR 2018-2019
Lee Groves (St. Cloud) Alligator Mathew Block - Tree health and juice quality 2018-19
Lee Groves (St. Cloud) 13W Karen's Block OLL/rootstock - Yield, fruit quality and PCR 2018-2019
Heller Brothers (Fort Pierce) Vernia/rootstock - Yield, juice quality, tree health ratings 2018-19
Hidden Golf Course (CREC Variety Trial) - Tree size and health ratings 2018-19
Peace River (Larry Black) - Tree size and health, fruit count 2018-19

Post Office (Haines City) OLL/rootstock - Tree size and health ratings 2018-19
Raley Tree (Dundee) - Tree health & size ratings, yield and fruit quality 2018-19
Saint Helena (Lee Groves, Dundee) Vernia & Valencia - Yield, juice quality, tree Size & PCR 2018-19
Serenoa (Immokalee) - HLB Rating 2018-19
Smoak (Lake Placid) - Yield Data, fruit quality, tree size and health 2018-19
Steve Brewer (Land-O-Lakes) Fresh Mandarin trial - Tree Health & Size Ratings 2018-19
Teaching block (CREC) Berna /Salustiana - Yield, tree health ratings, fruit drop 2018-19
Hammond (Fort Pierce) Minneola Fresh Fruit - Yield 2018-19
Jackson Citrus (LaBelle) OLL/Valencia B9-65/rootstock reset - Tree size and health ratings 2018-19
Wayne Simmons (LaBelle) OLL/Valencia B9-65/rootstock reset - Tree size health ratings 2018-19
Wheeler Bros. (Waverly) - Yield 2018-19

K. Pelz-Stelinski 18-051C "Improving bactericide therapy for young tree protection and inoculum reduction" – Objective 1: Investigate efficacy of bactericide treatments for preventing new infections. This objective is necessary to determine the potential role of bactericides in an integrated pest management program for young tree protection. Experiments were initiated in new tree plantings in a Wauchula grove.

Initial leaf samples were collected prior to treatments to evaluate CLas titers in the uninfected trees. The following treatments were applied this second quarter of 2019:

1. Antibiotics (monthly rotation) 2. Antibiotics (quarterly rotation) 3. Negative Control (insecticide + Tree defender exclusion netting) 4. Positive Control (insecticide only)

Tree height and circumference were quantified. ACP populations were monitored biweekly (eggs, nymphs, adults). No flush, eggs, or nymphs were detected after treatment applications. Tap sampling of adults was conducted, and insects are currently being processed to determine the infection rate.

Objective 2. Determine the effect of bactericide application frequency on Las infection of citrus.

This experiment will determine the most effective application frequency for bactericides to maximize tree health. This will inform revised recommendations for the use of antimicrobials in commercial citrus groves. Experiments were initiated in new tree plantings in a Lake Alfred grove. Initial leaf samples were collected prior to treatments to evaluate CLas titers in the uninfected trees, then one week after treatment applications. The following treatments were applied this second quarter of 2019:

1. Antibiotics (monthly rotation) 2. Antibiotics (quarterly rotation) 3. Positive Control (insecticide only).

Tree height and circumference were quantified. ACP populations were monitored biweekly (eggs, nymphs, adults). No flush, eggs, or nymphs were detected after treatment applications. Tap sampling of adults was conducted, and insects are currently being processed to determine the infection rate.

Objective 3: Quantify the effect of repeated inoculation of the efficacy of bactericides. This experiment will determine whether bactericides are sufficient to overcome the pressure of repeated Las inoculation by ACP. This will inform revised recommendations for integrated pest management programs to improve use of insecticides in conjunction with bactericides in commercial citrus groves. We have procured trees and will initiate experiments next quarter.

E. Stover 18-058C "Fort Pierce Field Test Site for Validating HLB and/or ACP Resistance" – A number of CRDF funded trials are underway at the Picos Test Site. In the last quarter, the most significant advances have been:

1) 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.
2) 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.
3) Renewal and approval for BRS permit effective 9/1/19 through 8/31/20.
4) 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.
5) 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 and some fruit appear to have set.
6) What should be the final samples from the C. Ramadugu-led Poncirus trial were prepared and shipped to UC Riverside.

N. Wang 18-064C "Evaluation of the control effect of bactericides against citrus Huanglongbing via trunk injection" – 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. Three field trials have begun to investigate how the application of bactericides via trunk injection affects citrus growth, production, HLB symptom development, and Las population in different aged trees at different levels of HLB disease severity. We evaluated the inhibitory activity of OxTetCyc against Las in greenhouse and field experiments. Citrus trees were trunk-injected with OTC, leaves were inspected for Las populations and OTC residues using qPCR and HPLC assays respectively, at various times after OTC treatment so we have acquired data about the MBC of OTC in planta. A manuscript is under preparation. 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. A field trial has begun to determine the concentrations of bactericides in leaf, stem, root, flower, and fruit using HPLC at: 2, 7, 14, 28 days, 2, 4, 6, 8, 10, 12 months after injection of different doses. The leaf samples are being collected at different time points for testing. Objective 3. We will determine whether trunk injection of bactericides at three different doses could decrease Las acquisition by ACP in greenhouse and in the field. We are conducting the experiment right now. Objective 4. To monitor resistance development in Las against bactericides and evaluate potential side effects of trunk injection of bactericides. Las-specific primers were designed to target the putative binding sites of OTC in 16SrRNA gene of Las. Plant genomic DNA was extracted from citrus trees received OTC injection for three years. PCR were performed with the primers and DNA samples, and the products were purified and subjected to DNA sequencing. No mutation was identified yet. We will continue to monitor the resistance development against OTC and Streptomycin. We have collected more samples from multiple citrus groves.

E. Stover 18-065C "High -Throughput Inoculation of Transgenic Citrus for HLB Resistance" –
1) Over 7000 infected ACP were used in the last quarter, in part to screen 450 trees, but also for other related uses. The Stover lab used 1700 ACP in no-choice inoculation of transgenic citrus. 2700 ACP were used for detached leaf assays in which leaves of putative CLas killing transgenics and related controls are exposed to CLas-infected ACP for 4 days, allowed 3 days for ACP-free metabolism and then assessed for CLas titer in leaves and the ACP. One thousand ACP were used in an assay in which CLas+ ACP are used to develop a uniform homogenate for rapid testing of putative CLas-disrupting peptides

2) The Bowman lab has transitioned to use of grafted trees with a commercial scion in 2.5 liter pots. The first group of test plants will be removed from ACP inoculation the second week of July to begin post-inoculation evaluation. Subsequent groups of test plants for rootstock evaluation are being prepared.

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

Y. Li 16-001 "Enhancing Genetic Transformation Efficiency of Mature Citrus" – (March:)
1) Methods development to produce gene-edited and transgene-free plants derived from mature citrus tissues continues. The approach is to create a chemical resistance to reduce chimeras, but resistance has not been observed. Alternatively, a different strategy to create chemical resistance requires new gene constructs that will be tested for their efficacy.
2) Further experiments were conducted to address inconsistent results for genes regarding their effects on mature citrus tissue transformation. The effects of several chemicals on transient and stable expression and regeneration efficiencies were completed. The results confirm some improvement of shoot regeneration for callus derived from mature tissues of citrus.
3) Young shoot tissues from mature citrus shoots can be regenerated through vegetative propagation but the efficiency is low. Efficiency of Agrobacterium-mediated transformation of young tissues generated from mature tissues is also low. To circumvent these problems, additional genes are under evaluation for their effectiveness using both juvenile and mature tissues.
4) For in-planta transformation method of mature citrus tissues, low efficiency of Agrobacterium infection in transient and stable transformation assays was observed. Low de novo regeneration efficiency was also observed. Hence, additional genes are being evaluated to enhance regeneration efficiencies for in-planta transformation from mature plants. Final reports due 7/30/19 – PI expecting additional data.

N. Wang 16-005 "GFP labeling of Candidatus Liberibacter asiaticus in vivo and its applications" – Objectives:1) GFP labeling of Candidatus Liberibacter asiaticus. 2) Elucidation of plant-Las interaction through real-time monitoring of Las movement and multiplication in planta using GFP labeled Las. 3) Investigate the effect of different control approaches on the dynamic population of Las in planta using GFP labeled Las. Previously, the reporter plasmid, pBAM1::R-PgyrA-GFP, composed of Tn5 and narrow host-range origin was constructed and therefore the GFP gene can be inserted into the genome of

bacteria. However, it was only successfully transferred into a genome of Pseudomonas fluorescence with low transformation efficiency and failed with other bacteria including Escherichia coli DH5α, Sinorhizobium meliloti Rm1021, and Liberibacter crescens BT-1. Recently, pDH3::PgyrA-GFP was constructed which has a wide bacterial host range replicon, repW, but cannot be inserted into a genome. Transformation of E. coli by PEG mediated method with pDH3::PgyrA-GFP showed higher transformation efficiency (~2 x 104 CFU/µg of DNA) than with previous reporter plasmid (failed). Following application with L. crescens BT-1 by electroporation was also successful (1.9 x 103 CFU/µg of DNA). Transformants and the GFP expression in L. crescens BT-1 were confirmed by PCR and fluorescent microscopic analysis, respectively. As L. crescens is a phylogenetically close species to Ca. L. asiaticus, there is a possibility that pDH3::PgyrA-GFP would be useful for GFP labeling of Ca. L. asiaticus. We have further confirmed the Lcr-GFP using western blot. The GFP plasmid is being used to transform Las. To facilitate Las transformation, we have tested multiple novel methods of culturing. Las population was observed to decrease at the beginning, and then increase slowly. Repeated experiments show similar patterns which suggest we might be able to acquire enough Las cells for transformation after further optimization.1) Especially, we are testing co-culturing Las with citrus tissue culture and psyllid tissue culture. Currently, we are in the process of establishing a pure psyllid cell culture. We have used two approaches to label L. crescens. Preliminary data show one approach works for Las in vitro. We are testing whether we can label Las in vivo and observe its movement. 2) We have conducted Las movement and multiplication in planta based on qPCR method. We have tested approaches to prevent Las movement in planta. In addition, based on the movement of Las in planta, we have developed a method for targeted early detection of Las before symptom expression. 3) We have been testing the effect of different control approaches including application with bactericides. One manuscript entitled: "Control of Citrus Huanglongbing via Trunk Injection of Plant Defense Activators and Antibiotics" has been published by Phytopathology. In addition, based on the movement of Las in planta, we have developed a method for targeted early detection of Las before symptom expression. A manuscript entitled, “Targeted Early Detection of Citrus Huanglongbing Causal Agent 'Candidatus Liberibacter asiaticus' Before Symptom Expression” has been published by Phytopathology. We determined the in planta minimum inhibitory concentration of oxytetracycline against Candidatus Liberibacter asiaticus effective for control of citrus Huanglongbing which has been accepted with revision by Phytopathology.
In addition, we further characterized the movement of Las in planta and the treatment effect of antibiotics using trunk injection and foliar spray. Two manuscripts are being prepared.

Y. Duan 16-007 “Field evaluation of the selected variants of Ruby Red grapefruit volunteer seedlings for greater HLB resistance/tolerance” – 1) Based on two year's graft-inoculation assays in greenhouse with two HLB bacterial isolates and the performance of individual selections of Scott Grove's seedling variants in the field, four lines of the seedlings (with greater HLB resistance/tolerance) were selected for further propagation on three different rootstock (commercial sour orange, newly selected USDA-sour orange and 942). Fruit quality (Brix, sucrose, glucose and fructose, soluble solids, pH, % TA and total ascorbic acid) of the four selected seedlings showed no significant difference from their maternal trees.
2) The first group of the propagates on three different rootstocks from the selections of Scott Grove's seedling variants were grown at our research farm, Picos Farm, where the plants are under extreme high HLB disease pressure with very aggressive HLB pathogens. These new plantings (July, 2017; Nov, 2017; and May, 2018) showed different disease index, the longer the planting was in the field, the

higher the disease index, which was also highly correlated with the titers of Ca. Liberibacter asiaticus (Las) in infected plants. It is worth noting that the new HLB isolate from Picos Farm caused severe HLB disease on most of grapefruit selections of seedlings and bud sports in our latest greenhouse evaluation. Those selections were either resistant or tolerant to the previous HLB isolates maintained in greenhouse. Preliminary data showed some of the selections are better than the others with either lower disease index or better canopy growth. In general, the average infection rate was 20.85% for one group of grapefruit plants (470) 20 months after planting, and 5.46% for the other group of grapefruit plants (366) 15 months after planting.
3) The second group of the propagates on three different root stocks mentioned above (Ca. 750 plants) have been budded and grown in the greenhouse, and over 400 plants in 12 combinations have been planted in Scott Grove. The remaining plants will be planted in September. All of the propagates have been tested for the presence of Las. Propagates carrying a low titer of Las will be planted separately and serve as the experiment for "cross-protection".

E. Triplett 16-009 “Developing second generation antimicrobial treatments for citrus greening disease” – In collaboration with Dr. M. Rogers, we are conducting laboratory, greenhouse, and field experiments to determine whether glyphosate can control citrus greening disease. Glyphosate inhibits aromatic amino acid synthesis in many plants and many bacteria, including L. crescens, that produces these compounds. In the laboratory, we determined the levels of glyphosate that inhibit L. crescens when the cells are cultured in the presence of aromatic amino acids suggesting that there may be another site of action for glyphosate in addition to the ESPS protein. Based on those experiments, we choose 8 mM and 25 mM concentrations of glyphosate to test for citrus toxicity in the greenhouse; 25 mM glyphosate was quite toxic to Valencia citrus. The plants survived 8 mM glyphosate but it delayed growth of flush by two months. Two sprays of 8 mM glyphosate one month apart caused considerable leaf drop two weeks after the second spray. Two sprays of 8 mM glyphosate two months apart hurt the growth of flush but the flush is returning three weeks after the second spray. These interval sprays will continue monthly until the first and last sprays are six months apart. We expect that sprays three months apart will not significant effect citrus growth and yield. These greenhouse trials will inform us as to the best interval for spraying the field in 6 weeks.

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” – Link to presentation to CRDF Board of Directors, June 12, 2019: https://citrusrdf.org/wp-content/uploads/2019/07/2019-06_Bayer-Project-Update_BoD-Mtg_Manker-Denise.pdf

M. Dutt 18-007 “Investigating the role of transgenic rootstock-mediated protection of non-transgenic scion” – Transgenic lines have been budded with non-transgenic HLB free Valencia budwood. It has been observed that the bud take frequency is higher on cuttings made from juvenile transgenic lines when compared to mature lines. Seed source trees, budded onto US802 rootstock have been produced for field evaluation and are being sized up. Transgenic NPR1 lines, that have been observed to be tolerant to HLB under field conditions, are also being propagated to use as interstocks in this study. ELISA protocols are being developed to rapidly test for transgene products in the fruit and juice. An application for a USDA-APHIS permit has been filed for field evaluation of these transgenic lines.

F. Gmitter 18-010 "Upgrading Citrus Genome Sequence Resources: Providing the Most Complete Tools Necessary for Genome Editing Strategies to Create HLB Resistant Cultivars" – The first steps in this project have taken place. The very latest DNA sequencing technologies will be used to produce nearly full-length genome sequences. This requires the highest quality DNA preparation as starting material for library construction for sequencing. Young leaves and young flush were collected from the five cultivars selected for sequencing. This required multiple samplings because it was not always possible to get the ideal tissue types; just slightly more mature vegetative tissue than the optimum did not yield the purity and the quantity of high-molecular weight (HMW) DNA required. Previous protocols were optimized to produce quantity and quality required. HMW DNA samples were sent to UC Berkeley Genome Sequencing Laboratory for library construction.

E. Rogers 18-019 "Phloem specific responses to CLas for the identification of novel HLB Resistance Genes" – 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 x 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 (TRAP) strategy 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 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.

This is the first year, 2nd quarter progress report; our grant started December 1, 2018. In the last three months, we have brought on board our post-doctoral researcher, Dr. Tamara D. Collum. Objective 1 (development of transgenic constructs) has been completed. For objective 6 (Additional Approach: Phloem limited citrus tristeza virus vectors will be used to express the His-FLAG-tagged ribosomal protein in healthy and CLas infected citrus) Dr. Dawson's lab has the necessary constructs and has begun moving them into citrus. The majority of our efforts in the 2nd quarter were focused on objective 2 (production of transgenic citrus lines). The Stover lab has performed Agrobacterium-mediated transformation of seedling epicotyls from all three citrus genotypes indicated in the grant (Carrizo, Poncirus and Hamlin sweet orange) with the His-FLAG tagged RPL18 (ribosomal protein L18) under the 35S promoter and all three phloem promoters pSUC2, pSUL and p396ss. Carrizo putative transgenic plants with three promoters are already rooted and established in the greenhouse; transformation was initiated with the fourth construct. Putative transgenic plants of Poncirus are also in hand, with only one construct in soil and the other three in rooting and/or shoot induction and selection media. Hamlin transformation was initiated with three constructs; explants are still in shoot induction/selection media. shoots are already evident on the shoot induction/selection medium for p35S::HF-RPL18 (35 shoots). More in vitro germinated seedlings are growing for additional Hamlin transformations. Likely the Carrizo and Poncirus transformations already created will be sufficient for this project. Since Hamlin has a much lower transformation efficiency, some transformations will likely need to be repeated in the next quarter. Gene insertion and expression will be verified for many plants in the coming months. Plans are underway for transfer of the first plants to Ft. Detrick

N. Wang 18-025 "Optimization of the CRISPR technology for citrus genome editing" – The purpose of this project is to optimize the CRISPR technology for citrus genome editing.

Objective 1. Expanding the toolbox of citrus genome editing. As a proof of concept, CsPDS and/or CsLOB1 were chosen for targeting through transient expression of Cas9-sgRNA or Cpf1-crRNA using Xcc-facilitated agroinfiltration. CRISPR-LbCpf1, derived from Lachnospiraceae bacterium ND2006, was employed to edit the citrus genome. First, LbCpf1 was successfully used to modify Duncan CsPDS via Xcc-facilitated agroinfiltration. Next, GFP-p1380N-35S-LbCpf1-crRNA-lobp and GFP-p1380N-Yao-LbCpf1-crRNA-lobp were constructed to edit the PthA4 effector binding elements in the CsLOB1 promoter (EBEPthA4-CsLOBP) in transgenic Duncan grapefruit. In total, seven GFP-p1380N-35S-LbCpf1-crRNA-lobp-transformed Duncan plants were generated, designated as #D35s1 to #D35s7, and ten GFP-p1380N-Yao-LbCpf1-crRNA-lobp-transformed Duncan plants were created, designated as #DYao1 to #DYao10. LbCpf1-directed EBEPthA4-CsLOBP modification was observed in three 35S-LbCpf1-transformed Duncan (#D35s1, #D35s4 and #D35s7). However, no LbCpf1-mediated indels were observed in the Yao-LbCpf1-transformed plants. Importantly, transgenic line #D35s4, containing the highest mutation rate, alleviates XccΔpthA4:dCsLOB1.4 infection.

Objective 2. Optimization of the CRISPR-Cas mediated genome editing of citrus. One promotor showed higher efficacy in driving gene expression in citrus than 35S promoter and Arabidopsis U6 promoter. The promoter is being tested for efficacy in driving sgRNA and in genome editing of citrus.

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 has been achieved 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 efficacy to over 20% and eventually generate non-transgenic genome modified citrus. One manuscript is under preparation and a patent filed for this technology.

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" – The goal is to understand how citrus interacts with Candidatus Liberibacter asiaticus (Las) infection.

Objective 1. Identification of the receptors for Las PAMPs in susceptible and tolerant citrus varieties. 21 outer membrane proteins have been cloned and the putative targets in citrus are being identified using the Yeast 2 hybrid (Y2H) system. 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 (named LasPil85, LasPil95, LasPil105 and LasPil115) were cloned under 35S promoter and the Arabidopsis phloem specific promoter SUC2 and introduced into Agrobacterium. Multiple receptors were identified for the aforementioned PAMPs and are in the process of being confirmed. The receptors will be tested in tobacco and citrus.
Objective 2. Generate transgenic/cisgenic citrus expressing PAMP receptors recognizing Las. Selected PAMP receptors are overexpressing in citrus.
Objective 3. Investigate the roles of effectors in HLB disease development. For the 10 selected SDEs, Y2H was conducted to identify their targets in Valencia sweet orange. The targets are in the process of being confirmed using other approaches including BiFC and co-IP assays. Y2H and SPR assays are being conducted to identify their targets in Poncirus.

V. Orbovic 18-066C "Support role of the Citrus Core Transformation Facility remains crucial for research" – The Citrus transformation Facility (CTF) received 7 orders in the second quarter of 2019. Six of the orders required production of transgenic Duncan grapefruit plants and one was for production of transgenic Mexican lime plants.
Altogether, CTF produced 67 plants: 8 Duncan BB3, 7 Duncan plants BB4, 7 Duncan HGJ74, 6 Duncan ZM3, five Duncan ZM6, two Duncan ZM12, 11 Duncan JJ8, 3Mexican lime LM2, 5 Mexican lime M2SF, 3 Pineapple orange AL2, 9 Pomelo HGJ68, one Valencia BB4.
A new employee was hired in April to work for the next 6 months on the CRDF-funded project lead by Jeff Jones. With the departure of one employee at the end of June, there are presently six staff at the CTF.
Fruit of Duncan grapefruit and Valencia orange stored in the cold room at CREC provide a steady source of seeds. Other seeds required were purchased from the Lyn Citrus Nursery in California.

J. Zale 18-067C "Continued Funding for the Mature Citrus Facility to Produce Disease Tolerant, Transgenic Citrus" – The Mature Citrus Facility (MCF) produced ~85 transgenics this quarter, which is a significant increase in productivity compared to last quarter. Use of the gfp reporter in some transformations, which makes transgenics easier to identify likely contributed to this increase and the fact that it was spring when productivity seasonally increases. Although more transgenics were produced not all grew large enough for micrografting. Of the 57 transgenics micrografted, 30 survived.

Scion cultivars from the UF Plant Improvement Team, EV2, WP Murcott, Valquarius, OLL4 were introduced into the MCF. OLL4 and Valquarius have high Agrobacterium transformation efficiency. UF 15, UF 17, X639, US 942 rootstocks were introduced and are being tested in Agrobacterium transformations. We also recently introduced several grapefruit cultivars (Flame, Marsh, Ray Ruby and Duncan). In the past, grapefruit (Ray Ruby, Grosser's Red Grapefruit, & Ruby Red Grapefruit) were recalcitrant using the standard Agrobacterium protocol.

d. Horticultural Practices

J. Grosser 15-013 "Understanding and Manipulating the Interaction of Rootstocks and Constant Nutrition to Enhance the Establishment, Longevity and Profitability of Citrus Plantings in HLB-Endemic Areas" – Greenhouse Experiment: This experiment studied the effects of high-quality controlled release fertilizer formulations containing various enhanced micronutrient packages on HLB+ Valencia trees on 5 rootstocks (Swingle, x639, UFR-3, UFR-15 and WGFT+50-7 tetraploid citrumelo). Treatments were: 1. Harrells 16-5-10 nursery mix (no boron); 2. Harrells nursery mix plus TigerSul Mn; 3. Harrells nursery mix + Florikan polycoated boron; 4. Harrells nursery mix +; 5. Harrells St. Helena mix (12-3-9); and 6. Florikote IFAS blend (12-3-8). Infected Valencia trees were produced by stick-grafting entire budwood sticks of Valencia from symptomatic, PCR+ field trees. Trees were grown in an air-conditioned greenhouse that favored CLas replication. Trees were evaluated by qPCR to monitor CLas, and by a subjective tree health index. Leaf nutritional analyses were also conducted. Nutritional treatments 1 & 2 had weak micronutrient packages, and the lowest performances. The four treatments that contained enhanced micronutrient packages clearly performed the best, producing trees among all rootstocks with lower bacterial titers and higher health ratings. Treatments 1 (control) and 2 did not contain boron, and both produced trees with statistically significant higher CLas titers across all rootstocks, and poorer health indexes among the more tolerant rootstocks. This data proves that boron is essential to suppress CLas infections in recovering/tolerant trees. The data also show that an overdose of manganese was not effective in improving tree health and reducing CLas titers without at least some baseline level of boron. When Mn was combined with boron (treatments 3 & 4), tree health was improved with CLas titers reduced, even without enhanced levels of the other micronutrients. Treatments 5 & 6 that contained complete enhanced micronutrient packages also performed well. High levels of manganese and zinc were found in leaf samples from all treatments. Among the rootstocks, the unreleased somatic hybrid WGFT+50-7 (tetraploid citrumelo) was clearly the most HLB tolerant, followed by Swingle, x639, UFR-3 and UFR-15. The data also suggest that different rootstocks may have different micronutrient requirements, and bacterial titers were not always correlated with tree health indexes.

Field Experiments (Fort Mead and Arcadia). Ten acres of Valencia sweet orange on Swingle rootstock trees were used at two locations. The 10-acre blocks were divided into 2 trials. Fertilizer was applied by hand into the microjet wetted zone every 3 months from 2016-2019. Tiger Citrus Mix (67%S, 9%Fe, 4.5%Mn, 3%Zn) was used in both trials. Trial 1 focused on the evaluation of Tiger Citrus Mix for improving health of HLB-affected trees; Trial 2 added micronutrients to the trees as a standard management practice, since the CRF that was being evaluated did not contain these micronutrients. Thus, all the 6 treatments in Trial 2 were getting the same amount of soil-applied micronutrients. Results for Trial 1: Yield: In 2018 and 2019 we observed significant improvement in yield with soil applied micronutrients as compared to foliar micronutrients. Interestingly, the control (conventional fertilizer treatments with no supplements) was the worst performer each year at both sites; however, the best treatments were different according to the site. Overall, these results suggest that soil applied micronutrient in form of Tiger-Sul product were beneficial for the trees. In 2019, the best treatments yielded about a box per tree more fruit- suggesting a substantial increase in profit/acre. These data support our data/conclusions from other experiments that micronutrients delivered to the roots can correct the HLB-induced deficiencies and improve production. Results for Trial 2: In 2018 and 2019, a significant improvement in yield was observed with the higher level of CRF. A positive linear correlation between % CRF and yield was observed. Overall, the results from trial 2 indicate that intermediate to high levels of CRF are best for improving yield in HLB+ trees, and this response improves over time. The positive result was more apparent at the Arcadia site, again indicating site-specific differences. It takes time for the nutrient deficiencies caused by HLB to be corrected with improved tree health and fruit yield. The best soil-applied micronutrient programs provide micronutrients to the roots year-round.

The economics of using CRF is an important deciding factor, and more research is needed to develop optimized formulations and the most economically viable delivery systems.

A. Schumann 15-023 "Citrus nutrition studies for improved survival of HLB-affected trees" – Our main goal was to find the reasons for inconsistent responses of HLB-affected citrus to Enhanced Nutrition (EN) programs and to develop feasible and economical remedies that can consistently replicate successful HLB mitigation with ENs in Florida groves. The two research objectives are summarized below.

Research Objective 1: Establish nutrient sufficiency guidelines for leaf tissues of HLB-affected trees

Using a survey data collection method of quarterly sampling in multiple groves of three Florida citrus production regions (Ridge, Indian River and Southwest), we assembled a large database of leaf, soil, and tree characteristics from a wide variety of HLB-symptomatic trees. Trees were classified into 5 HLB-severity classes and also grouped into "responding" or "non-responding" classes. Data were analyzed with ANOVA, DRIS, segmented regression, Cate-Nelson partitioning, and artificial neural networks in order to evaluate every possible dimension and interaction to elucidate linkages between measurements and tree performance or HLB severity. Using both segmented regression and Cate-Nelson methods with DRIS allowed us to identify new critical threshold concentrations (CTCs) for leaf nutrients of HLB-affected trees that can immediately be used to make tentative recommendations that supercede the somewhat aged CTCs published in UF/IFAS SL253 and elsewhere.

There is definitely a need to update the IFAS guidelines for interpretation of sweet orange tree leaf nutrient analysis and we now have proof. When making decisions pertaining to the nutrient needs of HLB infected trees, it is important to realize that healthy trees and diseased trees behave differently. The evidence gathered in this objective shows a real need to modify these guidelines to reflect the increased need for all 11 nutrients, specifically potassium, magnesium, boron, zinc, manganese, iron and copper. With a larger database of responding and non-responding tree values these values could be improved. The results of this research were presented at grower events: Citrus Nutrition Day, Bartow; Citrus School, Arcadia; Citrus Institute, Avon Park, and published in the April 2019 Citrus Industry magazine.

Research Objective 2: Determine soil conditions that favor root hair and VAM proliferation in HLB-affected trees

Using solid-phase tricalcium phosphate (TCP) as the sole source of phosphorus in a liquid culture experiment with Carrizo trees, we were able to limit the amount of available phosphorus in solution (< 1 ppm) and thereby stimulate the development of abundant root hairs by a factor of nearly two compared to a standard control with about 10 ppm soluble phosphorus. After exposing the experiment to HLB-carrying psyllids for two months, the TCP-treated trees had the lowest CLas titer, and we continued to see and measure healthy root hair development (now a 32x difference in root hair abundance, post-HLB) on the TCP treated tank solution plants compared to the control. The control tree's roots were nearly devoid of root hairs after trees became HLB positive, while the TCP-treated trees only suffered a minor reduction in root hair density.

We conclude that these differences in root hair abundance could significantly improve tree health if the results could be replicated with soil-applied and incorporated TCP in the field. A paper on this research was presented at the FSHS conference in June.

E. Triplett 18-024 "Foliar phosphate fertilization: a simple, inexpensive, and unregulated approach to control HLB" – 1) In Gainesville, a greenhouse experiment is in progress to assess the effect of phosphate fertilization on citrate levels in phloem. In one treatment, 2mM potassium phosphate at pH 6.5 is sprayed onto Valencia leaves. In another treatment, 2mM calcium phosphate at pH 6.5 is drenched onto Valencia roots. The idea is that the plants fed calcium phosphate to roots will need to acidify the soil matrix with citrate in order to solubilize the phosphate and make it available. If we spray the leaves with potassium phosphate, no solubilization of phosphate will be required. Samples from this experiment will be given to Nabil Killiny soon for citrate analysis in phloem. At Lake Alfred, Nabil Killiny has established a greenhouse experiment. He has grafted seedling to infect plants with CLas. He is supplying the plants with the two sources of phosphate as described above. In five months or so, we will assess CLas titer and examine HLB symptoms with the prediction that foliar potassium phosphate fertilization reduces titer and HLB symptoms while the root fertilization with calcium phosphate increases CLas titer and HLB symptoms.
2) A field trial led by Christopher Vincent was established In Polk County and the first sampling has been completed. Nian Wang is helping with sampling and the determination of CLas titer. These plants will be sprayed with foliar phosphate for the first time this week. Recently, Dr. Vincent located a flatwoods site at the Southwest Florida REC in Immokalee. The experimental plots at that site will be set up next week. Initial sampling and the first spray of trees will be done shortly thereafter.

F. Alferez 18-032C "Preventing young trees from psyllids and infection with CLas through use of protective netting" – This project investigates feasibility of individual tree covers (IPC) systems in citrus .The project started in December 2018 with 4 main objectives: Objective 1. Assessing tree growth and absence of psyllids and HLB disease symptoms (including CLas bacteria titer) under protective covering. 2. Assessment of alternative netting approaches involved in `targeted', `alternated' or `patterned' setup of IPC in groves for more cost-effective protection. 3. Monitoring the transition from vegetative to reproductive stage in the covered trees as compared to the uncovered. 4. Comparing individual tree protection with CUPS-like systems. Objectives: 1: We continued assessing the trees (Valencia on Swingle) planted in our pilot study 18 months ago for incidence of CLas. Today, virtually all of the uncovered trees in the trial (80-100% depending on the treatment) are already PCR-positive for CLas whereas all trees covered with IPC have tested negative. Cumulative leaf drop data of April through June show no significant increase in leaf drop in IPC-covered trees. 2: We have identified a collaborator in SW Florida to evaluate different layouts of IPCs and compare with the grove that we are setting up in Polk County. 3: We have measured this season's blooming and are now monitoring fruit set and development. We saw more blooming in uncovered than in covered trees. There are two possible reasons for these observations: 1) we measured more vegetative growth in IPC trees, which is known to delay transition to the reproductive stage, or 2) uncovered trees are more stressed by HLB than IPC trees, which may result in more flowering. 4: We finished planting the trees in our CUPS facility and we started to perform different treatments to study fruit set in 'Tango', `SugarBelle', and `US Early Pride' trees. These treatments consist of different applications of gibberellic acid for overcoming absence of external pollinators. We will be assessing fruit set and fruit development in the coming months.

Outreach, Professional Presentations and Extension Activities:

-Citrus Industry, January 2019. "HLB reduction strategies".

-IRCHLB VI meeting, California, March 2019. "Individual Protective Covers (IPCs) prevent young citrus trees from psyllids and infection with CLas, and promote vegetative growth".

-Spring Citrus Field Day at SWFREC April 2019. Demonstration of the SWFREC IPC experimental grove with an attendance of over 60 growers and industry representatives.

-FSHS ANNUAL CONFERENCE, Orlando, FL, June 2019. "Effect of Individual Protective Cover (IPC) on Physiology and Growth of Citrus Trees".

-Florida Citrus Industry Annual Conference. Educational Session, June 2019. "Protective Covers on Young Trees".

L. Duncan 18-036C "Cover crops and nematicides: comprehensive nematode IPM across the grove landscape" – Two trials, (1) factorial combinations of perennial peanut in row middles with nematicide in irrigated zone and (2) efficacy against sting nematode of oxamyl, aldicarb, fluensulfone, fluopyram, fluazaindolizine, and an experimental compound, were treated with recommended rates of each nematicide. All nematicides except aldicarb and oxamyl were applied in dedicated irrigation lines by irrigating (microjet) for 30 minutes, injecting the nematicide for 60 minutes, followed by 30 additional minutes of irrigation. Prior to treatments, dye was injected sequentially into the manifold controlling each treatment to ensure the plumbing was treating all of the experimental trees. On May 30 and 31, two months after treatments were initiated, soil samples were taken from all trials and processed (sucrose centrifugation) for nematode determination. Two nematicides reduced sting nematode populations by more than 70%, with no significant effect yet by four compounds. Because there is evidence that some of the new compounds move in the soil very little after application, the injection period will be extended to 2 hours in an effort to increase efficacy. Effects on fibrous root density are being processed. Treatments of most products will be resumed in the autumn. Perennial peanut is establishing reasonably well in all plots since the onset of the rainy season. The large effort of watering and weed management required of the sod laid during the spring dry season argues for starting such plots during the summer. Similarly, the sunn hemp crop did not establish well during the dry season and is will be replanted in early July. A second nematicide trial was initiated at a second young orchard by treating with aldicarb on 26 April, with all remaining treatments to be initiated in autumn after the plots are plumbed.

E. Johnson 18-041C "Characterizing HLB-pH interaction to improve management of root function and tree health" – Objectives: 1) Identify optimal pH range for root function and minimize root turnover on HLB-affected rootstocks and 2) determine 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 maintained.
We are in the final stages of rhizotron construction which was slightly delayed because of the late Valencia harvest this year for other projects combined with an unexpected loss of a staff member that will soon be replaced. The Masters student has assisted a member of Tripti Vashisth's lab with the 2nd repetition of the experiment that created the foundation of this project to become familiar with

techniques that will be important for maintaining pH and collecting data. We expect to initiate treatments before the end of May.

D. Kadyampakeni 18-042C "Development of Root Nutrient and Fertilization Guidelines for Huanglongbing (HLB)-Affected Orange and Grapefruit" – The purpose of the project is to develop new guidelines for restoring root health and improving overall tree nutrition in Florida oranges and grapefruit. The objectives of the project are to:
1. Determine optimal nutrient concentrations in roots and leaves for multiple grapefruit and orange varieties. 2. Compare and contrast fertigation, soil, and foliar fertilization to identify best application method for uptake of nutrients into both underground and aboveground components. 3. Investigate the relationship between root and leaf nutrient contents to tree health, yield, and fruit quality as well as bacteria titer. 4. Generate updated and new guidelines for optimal nutrient contents for roots and leaves for HLB-affected trees.
Progress: The project is being implemented at three sites at Citrus Research and Education Center (CREC), Southern Gardens Citrus near Clewiston, FL and Indian River Research and Education Center (IRREC). First preliminary data collection (on yield, fruit size, juice quality, canopy size, HLB and other disease ratings, soil characteristics and root growth and nutrition) has been completed at the 3 sites in the first and second quarters. First nutritional treatments have been imposed in spring 2019 and will be continued as scheduled. Data collection will continue and analyses will be done as needed. Updates and data will be presented in future extension meetings after about a year or two of data collection and validation of results to get feedback from growers and the citrus industry.
Plans for Next Quarter: The team will continue with data collection and reporting on the progress of the project.

R. Niedz 18-050C "The effect of the ionization state of iron and citric acid on the health of HLB-infected trees" – Objective 3, Effect of Fe2+ and citric acid treatment on HLB titer of model HLB system. This experiment tests Treatment Solution B reported in Patent US 8,945,631, Liquid for treatment of citrus greening disease and treatment method. Greenhouse-grown citron plants propagated from HLB-infected citron were tested by RT-PCR. Thirty-six plants that tested positive and showed HLB symptoms were planted into Ruck's pots. The experiment included 3 treatments applied to three groups of twelve plants. The treatments included Treatment Solution B at two concentrations - 30 ppm Fe (the concentration used in the field experiment of the patent) and 150 ppm Fe, the upper limit of Treatment Solution B the patent's authors thought was possible without significant phytotoxicity. Treatment Solution B is applied to foliar and soil at 150 ppm Fe weekly. For soil, 40 mls is added to each pot. The testing of F11 as described in the Feb. 2019 report continues. A third experiment to determine the phytotoxicity threshold of F11 and Treatment Solution B was initiated. Citron cuttings were taken, planted, and uniform plants were selected.
There were eight iron treatments with 12 replicates each arranged in a randomized block design (RCD). The 8 iron treatments included 1) F11 applied to foliar-only to runoff; 2) F11 @2X to foliar + soil; 3) F11 @10X to foliar + soil; 4) Fe chelate mixture applied to foliar + soil; 5) Sequestrene 138 (a standard Fe treatment) to foliar + soil; 6) Treatment Solution B applied to foliar + soil; 7) a proprietary iron mixture with high Fe2+ to foliar + soil; and 8) an untreated control receiving standard recommended citrus nutrition.
With the exception of the Fe chelate mixture (#4) and Sequestrene 138 (#5), all Fe treatments are high in Fe2+ and meet the patent's specifications for the use of Fe2+ to manage HLB. To determine the

number of replicates, a power analysis was conducted using tree quality and fruit yield data from the Florida Research Center for Agricultural Sustainability, Vero Beach, FL, where the field experiments will be conducted. For tree condition, measured on a 0-4 scale, an 18% difference was required and for yield in boxes/tree, a 124% difference between treatments can be reliably detected using the standard 5% significance and 80% power thresholds. Based on this analysis, 96 uniform Red Grapefruit trees on USDA 897 rootstock (planted 12/19/18) have been randomly assigned to one of the eight treatments in 12 replicated plots using an RCB design. Trees were allowed time to establish root systems and exposure to native population of ACP before treatment applications. This supports Objective 7, Determine the effect of Fe2+ + organic acid solutions on newly planted (<2 years old) field trees.

J. Qureshi 18-052C "Sustainable Management of Asian citrus psyllid (ACP) and Citrus Production" – This project is focused on developing Asian citrus psyllid (ACP) management programs for conventional and organic growers. Programs include sprays of organic or conventional insecticides alone or combined and with use of biological control. Studies were initiated in a 15-acre block of mature Valencia orange in the Gulf region. After the harvest in spring 2019, the experimental block was treated with insecticide Portal at 32 oz per acre rate to bring the psyllid populations to the same level throughout the block before dividing into different management programs. Four Integrated Pest Management (IPM) programs focused on controlling the ACP were initiated. These included 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 and 5) Only biological control program. Programs 1 and 3 received Apta 17 oz per acre rate and program 2 and 4 received HMO at 3% of the total application volume which was 100 gallons per acre. A total of 360 tap samples were conducted to detect ACP adults and predators and 694 shoots examined for infestation with ACP immatures. Psyllid adults averaged 0.3 per tap sample in the program 5 where no insecticide or HMO sprays were conducted compared to a range of 0-0.06 adults per tap sample across programs 1-4, showing a significant reduction of 80-100%. Shoot infestation with ACP immatures averaged less than 10% between programs 1-4 significantly less than 40% in program 5. Spiders averaged 0.03-0.08 per tap sample and ants averaged 0.06-0.81 per tap sample across all programs. Postdoctoral associate and temporary assistant were hired.

J. Qureshi 18-055C "Optimizing Benefits of UV Reflective Mulch in Solid Block Citrus Plantings" – This project is focuses on young tree protection from HLB through reduction in populations of vector ACP and irrigation management for improved tree health. Metalized polyethylene mulch and irrigation management can alleviate the problem by repelling ACP and controlling flush cycles to improve ACP spray timing and efficiency. Replicated experiments planned for the Ridge, Gulf and River regions using a split plot factorial design to compare costs and benefits of bare ground to mulch (main plots), and two ACP management programs (subplots) based on soil applied insecticides compared to spraying on flush were initiated. The mulch experiment at Ridge location (Lake Alfred) is already planted. The orange trees planted were Valencia on Carrizo rootstock with 19 ft spacing between rows and 6 ft spacing between trees. Mulch is also installed at the Gulf location (Immokalee) and plants ready to be planted in August. Mulch and plants are also ready for the River (Vero Beach) location which will also be planted in August. We are using 96-inch wide metalized reflective mulch (Shine N' Ripe XL, the original MRM) instead of 72-inch wide mulch used in the previous experiments and therefore expect more reduction in ACP populations over longer period. Data collection will include periodic pest scouting and counts, HLB incidence, soil moisture/temperature and tree growth variables. Specific objectives include 1) assessment of effects of UV reflective mulch on ACP control, HLB incidence and severity, tree growth and ultimately fruit production, and 2) assessment of ACP control and resistance to insecticides in response to flush synchronization for ACP control using mulch/drip irrigation system on three different soils types. Economic analysis summarizing 3-year and projected costs and benefits of mulch system with and without flush control will be conducted.

S. Strauss 18-059C "Citrus row middle management to improve soil and root health" – The spring cover crop mix was planted in late February and early March in both locations. This mix included Daikon radish, white clover, crimson clover, buckwheat, oats, and sunflower. Daikon radish germination was particularly good in both locations. Dataloggers and soil moisture probes continued to record soil moisture every hour. A rain gauge will be installed at each site to record rainfall data. Preparations are underway for the next assessment of root growth via the mini-rhizotron tubes installed in both groves. Weed emergence in row-middles and tree-rows were collected in mid-March from North grove location. Weed emergence data in South grove location will be collected in mid-summer.

Yield and juice quality data was collected for the North grove location March 28-April 4 and for the South grove April 11-18. Since treatments had only just begun, this yield and juice data will serve as baseline data for comparison with subsequent years of the project. The summer cover crop mix is being planted at both locations in the next two weeks. This mix will include dove millet, buckwheat, brown top millet, and sunnhemp.

A postdoctoral research associate will be joining the project in July and a graduate student for Drs. Kadyampakeni and Kanissery will begin this summer. Dr. Wade's graduate student will begin this fall. The next collection of samples for soil and leaf measurements will occur this summer.

T. Vashisth 18-061C "Evaluating sustainability of yield and fruit quality of sweet oranges with use of controlled release fertilizer and micronutrients" – This project is a continuation of an objective of existing CRDF funded project (# 00124558; which ended in March 2019) with some added treatments to be evaluated. The added treatments are:

1. CRF + Tiger Micronutrients+ Mn 50%
2. CRF + Tiger Micronutrients+ Zn 50%
3. CRF + Tiger Micronutrients+ Fe 50%
4. CRF + Tiger Micronutrients+ B 50%
5. CRF + Tiger Micronutrients+ Mn +Zn 20%
6. CRF + Tiger Micronutrients+ Mn +Fe 20%
7. CRF + Tiger Micronutrients+ Zn +Fe 20%
8. CRF + Tiger Micronutrients+ Zn +B 20%
9. CRF + Tiger Micronutrients+ Fe + B 20%
10. CRF + Tiger Micronutrients+ Mn +Zn 50%
11. CRF + Tiger Micronutrients+ Mn +Fe 50%
12. CRF + Tiger Micronutrients+ Zn +Fe 50%
13. CRF + Tiger Micronutrients+ Zn +B 50%
14. CRF + Tiger Micronutrients+ Fe + B 50%

The treatment for objective 3: 1.CRF + Foliar Micronutrients + Tiger 90; 2.CRF + Tiger Micronutrients

These treatments have been initiated at all sites. The pre-treatment data and first round of fertilizer application have been made. The leaf and soil samples have been sent out for nutrient analysis. Overall, the trees are looking good and they have a good crop load. The trees seem uniform in symptom levels. The second application of the fertilizer will be made in late June-early July. A consumer taste panel was performed in April on 4 of the best performing treatments based on the yield.

4. **OTHER CITRUS DISEASES**
   a. **Post-Bloom Fruit Drop**

M. Dewdney 16-010C "Enhancement of Postbloom fruit drop control measures" – (7/31/19 project termination; final reports are due 8/30/19)

M. Dewdney 18-034C "Improved postbloom fruit drop management and exploring PFD spread in Florida" – The objectives for this proposal are 1) Conduct field trials of new products and fungicide programs for PFD management as well as validation trials for the Citrus Advisory System (CAS); 2) Investigate the reasons for the movement of Postbloom fruit drop (PFD) to new areas and recent major outbreaks; 3) Evaluate methods for initial inoculum reduction on leaves so that early fungicide applications could be more effective and identify the constituents of the flower extracts using "omics" techniques.
Objective 1 was mostly covered by project 16-010C and activities are also reported there. In 2019, a field trial was set up and treatments were applied in a Valencia grove in Ft. Meade. There were few predicted infection events this year and only one application was made based on the PFD-FAD or CAS model predictions. Button counts were collected. There were also two CAS model validation trials and button data were also collected for both of these. Fruit data should be collected by the end of July for all the trials.

The experiments with the wind tunnel located at the ARS facilities in Ft. Pierce have commenced although access is still limited as progress is made for the USDA screening process. We hope to finish the screening process in the very near future for greater access. Preliminary trials with Colletotrichum acutatum in the wind tunnel indicate that the spores are able to move considerable distances in the wind. The modelling to indicate possible distances and gradient are very preliminary but indicate that some modifications to the experimental set up may be needed including increasing the number of repetitions since the variability was high.
Work on modelling of leaf wetness to better predict PFD outbreaks in Florida is on-going. Five FAWN weather stations were selected for this work and are equipped with leaf wetness sensors. The recorded data were compared to the output of four leaf wetness estimation models, singly and in combination. The most accurate models were considered for modifying the citrus advisory system (CAS). Analyses to look at the number of recommended sprays and model accuracy are underway.
Experiments to assess the effect of available fungicides on the secondary conidiation of C. acutatum on citrus leaves. In the initial experiment, pyraclostrobin was used and no difference was seen in the secondary conidiation regardless if sugar or water were used for stimulation of conidia. In subsequent experiments, ferbam was used instead. To stimulate sporulation, leaves were exposed to sterile water or 2.5% sucrose solutions with or without ferbam. Once the treatments were complete, the leaves

were coated with nail polish and the conidia and appressoria stripped from the leaf surface and counted. Ferbam affected the viability of the spores but not the number produced. It also significantly reduced the number of appressoria. These techniques are being used to assess the effect of the water, methanol, and ethyl acetate extracts of the flowers. Preliminary trials with the extracts are underway to make sure that the experimental procedures developed for the fungicides will be adequate or if modifications will be necessary.
Flowers were collected and extracted using water, methanol and ethyl acetate. The yields of all the extracts have been calculated for future reference. All the extracts have been dried for testing their antifungal effects. Once we receive the antifungal effects of the extracts, we will start the composition analyses. Preliminary metabolomics analyses are planned once some data from the fungal tests are available.

b. Citrus Black Spot

M. Dewdney 18-006 "Understanding the underlying biology of citrus black spot for improved disease management" – Objective 1: Evaluate the optimal spray timing for Florida and investigate if tree skirting or alternative products improves fungicidal control of citrus black spot.

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, the existing asexual population may be more diverse than currently measured and it will affect management practices. 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.

In the fungicide and skirting control trial, three applications of fungicides have been completed. They are applied approximately every 28 days. Skirting was planned for early June. We expect to continue with the applications through the summer and into the fall.

The two potential groves for the fungicide spray trial were scouted for CBS incidence and symptom severities in in March 2019. The selected grove was chosen for the trial based on the higher incidence of CBS. To keep treatments consistent, only mature trees were rated for CBS. Twenty-five fruit per tree were rated and three trees were grouped based on the average rating values into a replication. There are five replications per treatment and each treatment consists of at least one replicate at each CBS incidence level. The trial is spread out over eight rows, each consisting of 117 trees, plus guard rows on both sides of the rows containing the treatments. A total of 10 treatments were applied with a handgun at 200 psi. The first application was done over a two-day period starting on May 16th, this was a two-week delay from the original plan due to unforeseen grove activities. The second application was done over a two-day period beginning on June 3rd. The first two applications were completed as planned without any weather delays; although there was significant rainfall five hours after the last treatment of the second application. The third application is planned to begin on June 24th.

The CRDF-funded project has already officially started but the sub-contract between UF and CRI must still be concluded. Funding has therefore not been allocated yet. Nonetheless, a postdoctoral researcher has been appointed from 1 March 2019, and preparations for genotyping-by-sequencing of a collection of Phyllosticta citricarpa isolates from USA have been initiated. Eighteen isolates (4 from South Africa and 14 from USA) were sequenced (600nt read length) using the Ion Torrent System. Thus far, the genomes of eight isolates (3 from SA and 5 from the USA) have been successfully assembled and analyzed using a customized bioinformatics pipeline. The following available reference sequences were used: GC12: 5748 contigs (Florida, USA) and GCMC3: 6716 contigs (Zhejiang, China). The rest of the isolates will be sequenced before comparative analysis commences.

Jeff Rollins has been able to secure most of the necessary permits for him to be able to travel to Cuba and collect isolates to determine the mating type and species identifications. As the travel rules change based on Federal government policy, a few more details need to be worked out and final trip arrangements. We received MAT 1-1 DNA from South Africa to use as positive controls for our experiments. Trees are fruiting for some on-tree experiments.

c. Citrus Canker

J. Jones 18-013 "Using a Multipronged Approach to Engineer Citrus for Canker Resistance" – Objective 1. To determine if Bs3-generated transgenic grapefruit plants are resistant to diverse strains of the citrus canker bacterium or to alternate target susceptibility genes in greenhouse experiments and to the citrus canker bacterium in field experiments in Fort Pierce. As stated in a previous report, transgenic Duncan grapefruit containing the Bs3-executor transgene shows a high level of resistance to an array of strains representing a worldwide collection. Furthermore, real time PCR was used to validate that the gene is activated by one or more TAL effectors and that there is minimal activation without these genes. Two other transgenics with activity have been received from Vladimir Orbovic. Both responded to infiltration with a high concentration of bacterial cells by exhibiting a hypersensitive reaction within 4 days of infiltration. One of the transgenics appeared to have a growth defect, but recently has developed normal shoots. Both transgenic trees contain the avrGf2 gene (based on PCR for detection). These transgenics will be grafted onto rootstocks once they reach the appropriate size. During the past 3 months we have placed our construct in a different vector that is acceptable for future transgenic purposes. The previous constructs contain an additional selectable marker that allowed for identification of putative transgenics that contained the targeted construct. Given that there was concern about the additional marker, the new construct contains only NPT as a selectable marker. The construct was sent to Vladimir Orbovic, who is in the process of screening putative grapefruit and sweet orange transformants. Additional transgenics are expected later this year. The one transgenic plant with activity was grafted onto two rootstocks (812 and sour orange) and in late March planted in the field at Fort Pierce in collaboration Ed Stover.
Objective 2. To determine if EFR-generated transgenic grapefruit plants are resistant to the citrus canker bacterium in field experiments in Fort Pierce. The most promising EFR transgenic plants (based on ROS activity) have been grafted onto two rootstocks (US 812 and sour orange) and planted in the field in late March at Fort Pierce in collaboration with Ed Stover. The additional transgenics received from Dr. Orbovic will be grafted onto rootstocks in the near future.

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