Citrus Research and Development Foundation, Inc.

CRDF-Funded Projects Summary Progress Report FY 2021-2022
Quarter Ending June 30, 2022

RESEARCH TOPICS COVERED IN THIS REPORT

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1. *CLAS* PATHOGEN INTERVENTION

a. Bactericides/antimicrobials

S. Santra 18-020 "Novel multi-metal systemic bactericide for HLB control" – 100% Completion of Objectives (February 2022 final report)

Various multi-metal formulations of Mg-hydroxide and/or Zn-hydroxide were successfully loaded with Cu-chelates using a sol-gel strategy. The average hydrodynamic radius of the MM50C50M(Mg-Cu), MM25C75Z(Zn-Cu), and MM25C75M(Mg-Cu) formulations determined by DLS were 880, 580, and 450 nm, respectively. XRD results suggested the successful substitution of Cu in the MM25C75M Mg-OH brucite lattice structure.

All multi-metal formulations demonstrated a superior or equivalent performance against model pathogens such as Xanthomonas alfafae relative to copper standards in vitro. Systemic movement results suggested significant uptake of Mg for all multi-metal formulations. XRF and AAS results suggest the systemic movement of Cu was also detected in the leaves, roots, and stems at significant levels that exceed the MIC of Cu. Approximately 24-hours post treatment, the Cu-Zn treatment was detected primarily in the stems and leaves. Within 72-hours after application significant increases of Zn and Cu was detected in the roots. All Zn-Cu and Zn control formulations significantly reduced Canker lesion sums as compared to the untreated controls in a series of greenhouse trials.

MM25C75M(Mg-Cu), MM25C75Z(Cu-Zn), and MM10C45M45Z(Mg-Cu-Zn) treated grapefruit trees in the first field trial were observed to have 0% of fruit with canker lesions present throughout this trial, which was significantly better than the untreated control (0.8%, 1.2%, 4.6%, and 8.2% at successive disease ratings). The yield of MM25C75M(Mg-Cu), MM25C75Z(Cu-Zn), and MM10C45M45Z(Mg-Cu-Zn) treated grapefruits was 50.6, 60.5, and 49 lb/tree respectively. The yield of MM25C75Z(Mg-Zn) treated grapefruits showed a statistic difference compare with untreated grapefruits (43.8 lb/tree). The mean fruit diameter for MM25C75M(Mg-Cu), MM25C75Z(Mg-Zn), and MM10C45M45Z(Mg-Cu-Zn) treated grapefruits of 86.9, 83.6, and 85 mm, respectively, was not significantly different from the untreated controls (85.8 mm). No significant differences were found in internal fruit quality based on brix acid ratio or total soluble solids.

All experimental treatments significantly improved yield with respect to the untreated control. The untreated control group was observed to yield an average of 157 lbs/tree. The Kocide copper standard control groups reported an average of 171 lbs/tree. Although not statistically different from the Kocide control, laboratory grade Cu-ZnO, laboratory grade TMN 113-MgSOL-Cu, and Bz1.1-Cu, and Bz1.1 reported average yields of 202, 203, 189, and 207 lbs/tree, respectively. Laboratory grade ZnS-Cu did outperform all materials significantly with an observed yield of 229 lbs/tree in this years trial. All Mg:Cu ratios evaluated in the MM25C75M(Mg-Cu) subtreatment group promoted yields statistically similar to each other and the Kocide control.

Publication: Multifunctional Surface, Subsurface, and Systemic Therapeutic (MS3T) Formulation for the Control of Citrus Canker. Stephen L. Smith, Maria G. N. Campos, Ali Ozcan, Hajeewaka C. Mendis, Mikaeel Young, Monty E. Myers, Michael Atilola, Mitsushita Doomra, Zon Thwin, Evan G. Johnson, and Swadeshmukul Santra. Journal of Agricultural and Food Chemistry 2021 69 (37), 10807-10818 https://doi.org/10.1021/acs.jafc.1c03323

U. Albrecht 19-027 "Large-scale testing of the endophytic bacterium Frateuria defendens, a potential biocontrol agent of HLB" – 100% Completion of Objectives (Sept. 2021 final)

Frateuria defendens (Frd) is a recently discovered endophytic bacterium belonging to the family of Rhodanobacteraceae. The application of Frd by foliar spraying and soil drenching was documented to reduce the symptoms associated with phytoplasmas and liberibacters in various crops, including grapevine and carrots. It is thought that Frd has multiple modes of action including secretion of antimicrobials and priming of host defenses.

This project was part of a collaborative effort between US (UF/IFAS SWFREC) and Israel (ARO) to develop an environmentally safe, affordable, and effective solution for the management of citrus HLB. It aimed to test whether Frd can effectively reduce CLas titers and HLB disease symptoms in citrus trees and can be developed as a commercial biocontrol agent. The project was originally intended as a 3-year study but was revised to conduct a 1-year feasibility study.

The overall goal of the project was to test the efficacy of Frd in reducing HLB disease effects in Florida citrus. The specific objectives were 1) to test the curative effects of Frd against HLB under greenhouse conditions, and 2) to optimize delivery of Frd and determine suitability for large-scale control of HLB in field conditions. Objectives had been modified slightly from the original proposal to make the study feasible within the reduced time frame of the funding period.

The results showed that, under the conditions of the study, Frd had difficulties establishing in citrus leaves. This effect may have been associated with Frd-unfavorable biochemicals in the leaves and seemed to have been exacerbated by CLas infection.

There was no clear evidence that Frd interacted with CLas directly (i.e., Frd applications did not reduce CLas titers) and/or had a curative effect against HLB disease. However, Frd applications had positive effects on plant growth and seemed to be able to mitigate some of the damage caused by HLB or to retard disease progression of severely affected plants under greenhouse conditions.

The direct delivery of Frd via leaf infiltration or trunk injection was more effective than foliar sprays. A second experiment using the larger set of infected plants is in preparation to examine efficacy of Frd by trunk injection; the injection method will be optimized before commencing with the experiment.

The spray experiment will continue with collecting horticultural information and determining CLas and Frd titers.

b. Diagnostics

M. Irey 21-002C "Continued Support for the Southern Gardens Diagnostic Laboratory"
102% Completion of Objectives, based on volume of testing (June 2022 report)

This project provides HLB testing for researchers, growers and homeowners. A total of 9,118 samples were run during the 3 month third quarter period. All samples were plant samples, mostly from large scale research trials. To date 24,704 samples have been run. It is expected that we will be close to the budgeted amount of 28,750 samples for the end of year 1.
During the third quarter, the lab experienced some technical difficulties which were resolved by working with Dr. Ozgur Batuman's lab by running comparison samples. The problem was traced to using EDTA in the elution buffer. Although the elution methodology has been the same for years, for some unknown reason it became an issue. Suppliers of the affected samples were notified and the problem was corrected. It is suspected that the supplier of the master mix changed their formulation which resulted in the inhibition by EDTA (which has been reported in the literature as a potential inhibitor of PCR reactions). The lab operations are back on track at this time.
Fourth quarter of year 1 of Project 21-002C - A total of 4,512 samples were run during this 3 month-period. All samples were plant samples, mostly from large scale research trials. To date 29,216 samples have been run, which is slightly over the budgeted amount of 28,750 samples for year 1.
Anticipated: It is expected that sample volume will be slightly higher in quarters 1 and 2 of year 2 due to CRAFT projects which are just starting to come online. It is expected that the number of samples will slow down for the last quarter of year 1 as this is the time of year when symptoms are not evident and the titer of the bacterium in the plant declines.
Budget status: For year 1, the project was essentially on budget. A total of 29,216 samples were run versus a budget of 28,750. As has been done in previous years, the number of samples run at the end of year 2 will be reconciled against the budgeted amount and the last payment will be adjusted up or down as needed.

2. ASIAN CITRUS PSYLLID VECTOR INTERVENTION

a. Asian Citrus Psyllid management

L. Stelinski 19-002 "Why spray if you don't need to? Putting the IPM back into citrus IPM by ground truthing spray thresholds" – 100% Completion of Objectives (November 2021 final report)

The PI and team have investigated the potential of a usable economic threshold to increase sustainability of Asian citrus psyllid (ACP) management in citrus under conditions of high huanglongbing (HLB) incidence. Specifically, they measured efficiency of three nominal thresholds by relating ACP densities to cost a of application and yield. Moreover, two spray programs of insecticide mode of action (MoAs) were compared in a region of Florida where insecticide resistance in ACP was previously identified to evaluate the need for an effective dormant season spray and to combine integrated pest management (IPM) with integrated resistance management (IRM) practices.

The highest fruit yield was observed with the 0.2 ACP/tap threshold that required 7 annual sprays, while reducing the number of sprays to 5 and below with higher thresholds caused a significant decline in yield. The estimated profit obtained with using the 0.2 ACP/tap threshold was higher than with the two higher thresholds tested (0.5 and 1.0 ACP/tap) in this study, indicating that we were unable to reduce the number of sprays below 7 per year under this set of circumstances without compromising yield. Fewest adults were observed in plots with the lowest threshold evaluated (0.2 ACP/tap), which required seven annual sprays. ACP populations were lowest overall in plots where treatments were triggered by the lowest threshold evaluated (0.2 ACP/tap). The input cost of spraying at the 0.2 adults per tap threshold were estimated at $451.93/hectare ($182.89 acre). The costs associated with the 0.5 adults per tap economic threshold were estimated at $284.38 per hectare ($115.08 acre). Finally, at the 1.0 adult per tap economic threshold treatment, costs were $35.62 per hectare ($14.41 acre). There were no significant differences in fruit drop between the three threshold treatments compared. Susceptibility of ACP to thiamethoxam in treated field plots after each insecticide application was monitored with an insecticide bioassay. In treatments that were managed with the 0.2 ACP/adults/tap threshold, resistance ratios ranged between 1.63-5.25. For the 0.5 ACP adults/tap threshold, the resistance ratios varied from 1.75-5.25. For the 1 ACP adult/tap threshold, the resistance ratios ranged between 2.89-3.25. Overall, there were no statistically significant changes in susceptibility of ACP following the three treatment thresholds tested. Significant differences were observed between two insecticide rotation programs depending on whether or not an effective dormant season insecticide spray was applied near budbreak of the first seasonal flush. High counts of ACP adults were associated with the presence of feather flush structures on Valencia and Hamlin citrus trees. Also, differences in vegetative growth intensity were observed between Valencia and Hamlin varieties which could have an impact on ACP vector control programs if ACP adults are able to migrate to new groves where feather flush structures are present. Study results indicate that ACP management is most critical during the period between January to March, when citrus is characterized by flowering, fruit maturation (final stage), and the need for safe harvesting. Current results also indicate that the 0.2 psyllid per tap threshold shown to effectively predict need for ACP treatment application in mature citrus may also be useful in young trees but going above this threshold may not be possible without reducing yield. This outcome may have been affected by the frequent flushing that occurred in young trees combined with the small size of the treatment plots, which likely promoted re-colonization of treated areas due to psyllid movement. Results also suggest that combining an action threshold with an appropriate rotation program can effectively prevent development of insecticide resistance among psyllid populations. Furthermore, study results indicate that a highly effective dormant season insecticide spray targeting both adult and immature psyllids near budbreak of the first seasonal flush will be required in order to implement a low (0.2 psyllids/tap) treatment threshold during the remainder of the season. Implementation of thresholds to predict need for ACP sprays could allow for transitioning away from calendar-based spray programs and development of more sustainable citrus management programs. Further research on the consequences of using economic thresholds on populations of secondary pests and beneficial arthropod species in Florida is warranted.

The PI has submitted a comprehensive final report that fully discussed the entire study conducted over the two years of the grant. The report has a full set of study methods, graphs, tables, data and statistical analysis to fully support the above conclusions.

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

3. CITRUS HOST INTERVENTION

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

K. Bowman 18-004 "Development of SuperSour and other outstanding rootstocks with tolerance to HLB" – 100% completion of objectives (April 2022 final)

Project 18-004 reports in 6 major areas related to how the USDA breeding program is working to create new citrus rootstock that show enhanced performance in yield and/or quality of citrus being produced in an endemic HLB environment.

1. Create new candidate hybrids. Crosses were made this spring flowering season, based on parental combinations yielding the best progeny in previous trials. Hybrids from previous crosses were selected and propagated this quarter. New seed source trees for advanced selections were field planting in Spring 2022.

2. Propagate and plant new field trials. One new Stage 2 field trial with Hamlin with selected released rootstocks and the best of the next generation hybrids was field planted this quarter. Nursery trees for one new Valencia Stage 1 trial with 60 new rootstocks planned for field planting in 2022 with a cooperator was delayed due to completing the required MTA, and will be planted next quarter. Budwood increase trees were grown in preparation for budding trees for new rootstock trials. Trees with Valencia scion and HLB-tolerant Microcitrus interstocks were planted into a field trial as a preliminary analysis of interstock feasibility.

3. Collect data from field trials. Measurements of fruit crop and fruit quality data were collected from 9 rootstock trials with Valencia scions. The USDA researcher assisting with the analysis of fruit quality from USDA rootstock trials retired in December, so responsibility for this aspect of the work shifted entirely onto the Bowman program. Because of the Bowman program emphasis on rootstock trials, it is anticipated that this change will allow for an increase in the pace of fruit quality analysis associated with the rootstock trials.

4. Evaluate effectiveness for seed propagation of new rootstocks and develop seed sources. As the best rootstocks are identified through field trials, seed sources are established. Studies continued to evaluate seed propagation for the most promising SuperSour hybrid rootstocks, using morphological and SSR analyses of seedling progeny for trueness-to-type. Additional effort was initiated on evaluation of seed propagation for the SuperSour rootstocks as a UF graduate student project.

5. Field trial results for grower access. The USDA rootstock trials produce large amounts of information that is useful to help growers make informed decisions about rootstock choice for new plantings. A manuscript has been prepared that has a detailed comparison of field performance for Valencia on 50 SuperSour rootstocks and other commercial rootstocks and has been provided for review to selected citrus growers. It is expected that this manuscript will be submitted for publication in the coming quarter, and key findings distributed broadly to citrus growers. During this quarter, updated trial summaries from the 2021-22 season were prepared for uploading to the USDA citrus rootstock program website https://www.citrusrootstocks.org/, and information was provided to individual growers and groups, as requested.

6. Release of superior new rootstocks for commercial use. Release of new USDA rootstocks is based on robust data from multiple trees in replicated field trials over multiple years. Several of the SuperSour rootstock hybrids in the Valencia field trial being prepared for publication have exhibited superior performance in comparison with commercial standard rootstocks and have supporting superior performance from one or more other trials. Performance data continues to be evaluated and is being used to critically compare the new hybrids with each other and existing rootstocks. It is anticipated that 2-3 of the most outstanding of the new rootstocks from this set of SuperSour hybrids will be proposed for release in late 2022.

A full final report has been received that fully outlines accomplishments for the program over multiple years.

M. Dutt 18-007 "Investigating the role of transgenic rootstock-mediated protection of non-transgenic scion" – 100% Completion of Objectives (October 2021 final)

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

Obj 1: Transgenic rootstocks (n=12) that were budded with non-transgenic scion side grafted with HLB infected budwood and maintained in the greenhouse. Six months after budding trees were tested for the presence of HLB and were also evaluated for PR1 gene expression. PR1 is a SAR marker. All scions (with transgenic rootstock or non-transgenic control) were infected within a year after inoculation. There was no statistical difference between the treatments and control for the first 18 months. After 18 months of infection, Ct values did not decline in several transgenic rootstock lines at the same rate as in controls. In several transgenic rootstock - non transgenic scion combinations, there was an enhanced expression of the PR1 gene, which indicated an active defense mechanism. At the termination of the project, all lines with enhanced PR1 gene expression were alive, albeit infected. Four of the 12 controls died while 6 exhibited enhanced HLB symptoms. Only one control could be considered an outlier with mild HLB symptoms at the termination of the project.

Obj 2: This objective was in progress at the termination of the project with rootstock lines clonally propagated in the mist bed and ready to be budded with non-transgenic scion.

Obj 3: 61 transgenic lines (Carrizo, US942) with different genes stacked with NPR1 were produced by the mature transformation lab. Most of these tested were determined to produce adequate trans-protein. At the termination of the project, all lines were being clonally propagated in the greenhouse. Additionally, several select lines were budded onto standard trifoliate rootstocks for field planting. This was being planned for seed production.

Obj 4: This objective could not be initiated due to termination of the project

F. Gmitter 18-011 "Part A - The UF/CREC Core Citrus Improvement Program"
100% Completion of Objectives (January 2022 final)

This project (Part A) of the UF/CREC core citrus improvement program has 4 main reporting areas, and are as follows.
1. Develop new rootstocks that impart HLB-tolerance to scion cultivars. Seed from a first group of rootstock crosses was harvested and planted in the calcareous/Phytophthora soil as the first step in the gauntlet screen; parents included several previously selected but unreleased HLB-tolerant rootstocks, as well as some of the UFRs, HLB-tolerant pummelos, and US-897 and US-942. Fifty seedlings exhibiting tolerance of the poor soil challenge were selected, potted up, and rooted cuttings were produced for further gauntlet steps. Seed from a second group of crosses, using LB8-9 Sugar Belle® and other mandarins as seed parents with pollen from various hybrids of Poncirus trifoliata with citrus accessions, Citrus ichangensis (Ci), different Cleopatra mandarin x Ci hybrids, a Palestine sweet lime x Ci hybrid, and two C. latipes hybrids, were planted in a second round of gauntlet screening, to be completed spring 2022. In collaboration with researchers at IFAPA in Spain, new information has been generated regarding performance of selected UFRs and other unreleased rootstock hybrids from UF program in response to drought and flooding, Phytophthora, boron and salinity; UFR-6 has demonstrated good tolerance to all conditions except flooding and salinity, and 9 unreleased rootstock hybrids exhibiting tree size control and HLB tolerance in Florida likewise showed good tolerance of Phytophthora, with one of these showing best performance under all stress conditions. HLB ratings, fruit quality measures, or yield data were collected from 8 different rootstock field trials by the team during the reporting period.

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. Following extensive phenotyping of a replicated planting of hybrids between Monreal Clementine and an accession of Citrus latipes (perhaps the most HLB-tolerant citrus), at least two hybrids have remained PCR-negative after 6 years under high pressure in the field, produced large fruit somewhat resembling sweet orange.

Hundreds of seeds were collected from crosses made using their pollen onto low-acid breeding parents and planted. Seed from new early-maturing (first week of December) Vernia sweet orange clones, which had higher soluble solids than the other selected early-maturing Vernia clones, were collected and planted. Thirty new grapefruit hybrids generated using HLB and canker tolerant breeding parents were moved from embryo rescue into pots for subsequent field planting. One hundred twenty new EV protoclones, grafted on UFR-15 and US-802, were planted, in efforts to select a more robust early maturing Valencia clone. Three hundred ten new grapefruit hybrids or cybrids were planted at the CREC and in the Indian River area. Protoplast fusion experiments using W. Murcott suspension protoplasts with various leaf parents were caried out to create new tetraploid breeding parents that can be used for orange and mandarin improvement.

3. Screen germplasm collection for more tolerant types and evaluate fruit quality of candidate selections. Currently have more than seventy, 5-year-old-trees of `Marathon' mandarin on sour orange, that set large crops this last season. Although all trees have HLB, there are few to no obvious disease symptoms in fruit, leaves, or canopy, demonstrating a high degree of tolerance thus far. We have followed closely their performance, and individual trees yielded more than 300 pounds of fruit in 2 harvests in September and October. Fruit size distributions were determined, and post-harvest behavior and fruit quality data were collected and are now under analysis. Selections of apparently HLB-tolerant seedlings from breeding populations of oranges and orange-like hybrids, grapefruit and mandarins, and presented fruit and juice samples at displays in the CREC.

4. Conduct studies to unravel host responses to CLas and select targets for genetic manipulations leading to consumer-friendly new scion and rootstock cultivars. Several new genetic constructs have been developed using newly identified citrus specific promoters (phloem and root tissue), and new putative disease resistance genes, or downstream genes. Transgenic plants have been produced with some of these constructs, and additional transformation experiments are underway with several sweet oranges, grapefruit, and rootstocks. We assessed a diverse population of 459 hybrids from 30 crosses and 53 accessions for HLB tolerance using different morphophysiological traits and compared the relationship of these traits with a visual HLB severity score. Significant genetic effect on HLB tolerance have been documented which indicates opportunities for genetic improvement of HLB tolerance. Leaf area index (LAI) was the trait most highly correlated with HLB score. suggesting that LAI is a rapid, cost-effective, and reliable method in comparison to other existing HLB phenotyping measurements and can avoid cognitive bias in phenotyping trees for HLB tolerance.

Metabolomic studies have been completed metabolomic studies, in collaboration with Dr. Y. Wang, to gain insight into the underlying mechanisms of HLB-tolerance and sensitivity with reports being found at doi: 10.3389/fpls.2021.710598 and doi: 10.1021/acs.jafc.1c02875.

T. McNellis 18-016 "Testing grapefruit trees expressing an anti-NodT antibody for resistance to HLB" 98% Completion of Objectives (May 2022 final)

This is the final report for project 18-016. A final comprehensive report is being submitted along with this summary document. The final comprehensive report contains additional project details and relates the project to previous funding by CRDF.

The project objective is to test transgenic 'Duncan' grapefruit trees for resistance to citrus greening. Infections were done by grafting onto infected rough lemon rootstocks, caged infected psyllid feeding, and natural infection outdoors. Tree symptoms were visually assessed and 'Candidatus Liberibacter asiaticus' bacterial titers were determined by quantitative PCR (qPCR) in collaboration with Southern Gardens. All the tests proposed were performed and have now been completed. This quarter, we performed final data analysis and began to prepare a manuscript describing the tree citrus greening disease data. The manuscript is still at an early stage, but figures are nearing completion, which is the first major step. The text will be adapted from a M.S. student Chad Vosburg's thesis document.

Anticipated work: This is the final report. However, we are still working on a manuscript to publish the tree test data.

The project remains underspent in part due to support for graduate stipends and tuition waivers from the Plant Pathology & Environmental Microbiology Department of Penn State and the Penn State Graduate School.

We are reporting the project as 98% completed because we still need to submit the manuscript to a peer-reviewed journal and get it accepted for publication.

Z. Mou 18-017 "Establish early-stage field trials for new HLB-tolerant canker-resistant transgenic scions" – 100% Completion of Objectives (April 2022 final)

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

During the project period, Objectives 1 to 4 were accomplished. Objective 5 was delayed due to the Covid-19 pandemic. Field trial data will continue to be collected even though the project has been terminated.

1) Previously, three HLB-tolerant transgenic lines 'Hamlin' 13-3, 13-29, and Duncan '57-28' were generated,. The transgenic plants carried both the HLB-causing bacterium CLas and the flowering-promoting CTV vector. To remove CTV and CLas, the plants were treated in a growth chamber with alternating temperatures of 25°C and 42°C every 4 hours for a total of 60 to 90 days. New shoots formed on the treated plants were tested for the presence of CLas and CTV by quantitative PCR (qPCR) with specific primers. Alternating temperature treatment was highly effective for eliminating CTV and CLas. New shoots were free of both CTV and CLas.

2) & 3) Budwood from the new shoots were grafted onto Swingle rootstock. A total of 83 transgenic plants were produced using CTV- and CLas-free budwood of the above-mentioned transgenic lines

(28 for 13-3, 31 for 13-29, and 28 for 57-28). Moreover, 13 plants were produced from a new NPR1 transgenic line generated through mature tissue transformation, 17 plants from an EDS5 transgenic line, and 7 plants from an ELP3 transgenic line were also produced. All these transgenic lines showed HLB tolerance in the greenhouse. In addition, a total of 27 transgenic rootstock plants were produced. These transgenic plants include eight transgenic 'Carrizo' lines that express three different disease resistance genes. The transgenic rootstocks were replicated and grafted with 'Valencia'. All plants were grown and maintained in the greenhouse for two to three years.

4) The transgenic plants were planted in Picos Farm in 2019 and 2021 (no plants were planted in 2020 due to Covid-19). A total of 69, 98, and 27 plants were planted on May 9, 2019, May 20, 2021, and October 8, 2021, respectively.

5) This objective is still ongoing with funding from other resources. The transgenic plants transplanted on May 9, 2019 and May 20, 2021 were examined on April 24, 2022. The plants grew well in the field and one plant from the 2019 planting has shown HLB symptoms. Leaf tissues were collected on April 24, 2022 and analyzed for CLas titers. Transgenic plants in the field will continue to be monitored by periodically analyzing leaf samples in the coming years.

In addition to the proposed objectives, work continued on development of techniques to produce consumer acceptable citrus products. These techniques include CTV-delivered gene silencing, transgene-free CRISPR, and cisgenesis or intragenesis (cis/intragenesis). These CTV and CRISPR projects are supported by USDA. The cis/intragenesis project is partially supported with CRDF funds that created an intragenic vector. Unfortunately, the efficiency of the vector was extremely low. Therefore, the goal was to develop a strategy to significantly improve vector efficiency. The citrus 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) mutant (called EPSPS TIPS) that provides tolerance to glyphosate as a selective marker is being used to increase the intragenic vector efficiency. A strong citrus promoter is needed to drive the EPSPS TIPS gene. A system is being built to identify such citrus promoters.

Once the efficiency of the intragenic vector is improved, it can be used to either silence or overexpress a target gene. CTV-delivered gene silencing is being used to identify targets for silencing, the project supported by USDA. Genes are also being screened for overexpression through cis/intragenesis. two nicotinamide adenine dinucleotide (NAD)-binding receptors in the model plant Arabidopsis were recently discovered to increase resistance to bacterial pathogens when overexpressed. With the support of the CRDF funds, transgenic citrus expressing the Arabidopsis NAD receptors were generated. The transgenic citrus plants were inoculated with CLas-infected psyllids and are being maintained in the greenhouse to assay for HLB symptom development. The transgenic scions are being grafted onto sweet orange rootstocks for easier detection of HLB resistance or tolerance. Furthermore, the citrus genome encodes several putative NAD-binding receptors. NAD-binding activities of two of the putative receptors were tested. These citrus receptors will be overexpressed using the intragenic vector to create HLB tolerance.

Highly efficient citrus microRNA (miRNA) vectors were also developed. These vectors will be combined with the intragenic vector to specifically silence negative immune regulators to created HLB tolerance in citrus.

In summary, four of the five proposed objectives have been accomplished and the field trials under Objective 5) will be completed using funding from other sources.

E. Stover 18-022 "Delivery of Verified HLB-Resistant Transgenic Citrus Cultivars"
90% Completion of Objectives (November 2021 final)

Objective 1) Mthionin Constructs: Assessment of the Mthionin transgenic lines is ongoing. As the most proven of the transgenics, these lines are used as a reference in detached leaf assays, as well as studying them in established greenhouse and field trials. The first MThionin field trial (45 plants, WT or transgenic Carrizo with rough lemon scions) has shown transgenics maintaining higher average CLas CT values (2.5 CT higher @ 18 months), but with a high degree of variability. The larger second MThionin planting (205 total grafted plants of transgenic Hamlin scions, transgenic Carrizo rootstock, or WT/WT controls) is producing encouraging results; with the transgenic Hamlin on WT Carrizo having statistically better trunk diameter, tree height and canopy volume compared to controls. Leaf samples from the second planting are collected and undergoing CLas quantification. Results from the 2nd generation plantings detailed below also show the included mThionin lines with significantly improved growth and CLas titer numbers compared to WT when grafted to commercial scions.

The Mthionin construct has also been extensively transformed into additional varieties; with 10 confirmed transgenic lines of US-942 and 44 putative lines of Valencia and Ray Ruby undergoing expression analysis.

Objective 2) Citrus Chimera Constructs: Detached leaf assays, with CLas+ ACP feeding, have been conducted to screen citrus lines expressing chimera constructs TPK, PKT, CT-CII, TBL, BLT, LBP/'74', '73', and '188' (as well as scFv-InvA, scFv-TolC, Topaz and Onyx). Testing of all 35s driven Carrizo lines is complete and the analysis of phloem specific and scion-types is well underway. This work has already identified numerous lines with significant effects including increased ACP mortality (up to 95% from TBL, 96% from PKT, 60% from BLT and 70% from TPK) and decreased transmission of CLas into the leaf. Analysis of the ACP endosymbionts show a reduction in titer that may indicate a mode of action for ACP mortality (outlined in Objective 4). The best performing of these lines have been moved forward into greenhouse trials as described below.

Initial ACP-inoculated greenhouse trials on 8 lines of citrus Thionin-LBP chimeras ('73', and '74') showed a statistically significant reduction (13x) in CLas titer for '74' transgenics vs WT. However, many plants of both treatments remained CLas negative due to low inoculation efficiency. Two follow-up 3greenhouse studies are underway. The first is an improved ACP-inoculated trial with the best performing \`74' and \`188' lines. The second is a larger graft-inoculated study directly comparing the best performing 3rd generation chimeras (TPK and TBL) with the earlier 1st (Mthionin) and 2nd (\`74' and \`188') generation lines. For this, a total of 420 grafted plants (all on WT Carrizo rootstock for uniformity) were made and grafted with CLas+ RL for uniform transmission. In the earlier 6 and 9 month samplings, plants were beginning to test CLas positive and 12 month assessments are underway. An additional two rounds of rooted cuttings (totaling >1600 replicates) have been made from those same lines for paired ACP-inoculated greenhouse and field trials. ACP-inoculations will be done in free flying cages for a 2-month availability window, a protocol developed in a parallel project that has yielded up to a 100% infection rates. Both studies will begin as soon as the plants are large enough. The best performing PKT and LBT lines have also been replicated (>200 plants) for following the same greenhouse to field study pipeline, with graft inoculations as soon as the plants are of sufficient size.

Field trials of 2nd generation chimeras (`74', and `188') with included MThionin plants are ongoing; with 165 plants (WT Hamlin and Ray Ruby on transgenic Carrizo) and 70 plants (WT Valencia on transgenic Carrizo) moved to the field in August 2020 and May 2021 respectively. At the 1 year assessment, Hamlin plants with transgenic Carrizo rootstocks showed noticeably reduced CLas titer (in scion midribs) when compared to those on conventional rootstocks. WT Hamlin on `74' Carrizo had a 10x reduction in CLas titer, WT Hamlin on MThionin a 15x reduction and WT Hamlin on `188' a 144x reduction. Plants grafted on MThionin-Carrizo also showed better trunk diameter, tree height and canopy volume compared to WT/WT grafts. Because of the high ACP mortality seen in detached leaf assays, field plantings of all chimeras are also being surveyed to determine the rates of successful ACP colonization on new flush. An additional planting of transgenic scions (Hamlin) on WT rootstocks is also being prepared to complement these plantings.

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

In addition to the use of the Mthionin and its chimeric variants, new strategies have been implemented to develop HLB resistant citrus. These efforts include the expression of insecticidal peptides (to control ACP) and down-regulation of DMR6 genes (to enhance disease resistance). 54 independent transgenic lines of Carrizo, Hamlin and Ray Ruby expressing the insecticide peptide Topaz (a code name to protect IP), under constitutive and phloem specific (SCAmpP-3) promoters were evaluated by detached leaf assay. From these, 12 lines (4 event of each genotype) showed significant ACP mortality and were selected to move up in the screening pipeline for HLB/ACP tolerance. Also, 27 transgenic Carrizo and Hamlin lines highly expressing Onyx (a code name to protect IP), a peptide with both antimicrobial and insecticide activity, were evaluate by DLA. The 5 Onyx-Carrizo lines showing high ability to kill ACP (to 83% mortality) were selected for further evaluation. Strongly performing lines were replicated as rooted cuttings (250 Onyx and 189 Topaz plants) that will soon enter greenhouse trials. The available Onyx transgenic material is being further expanded through production of additional constitutive (13 Valencia and 6 Ray Ruby) and phloem specific lines (25 Carrizo, 5 Hamlin, 8 Valencia, and 13 Ray Ruby).

Down regulated DMR6 Carrizo, either by stable expression of specific hairpin RNA (for RNA interference) or by Cas9-sgRNA genome editing were generated, cloned, and are being assessed. Since DMR6 is a broad immune suppressor, down-regulated plants could be expected to have heightened immune response. To test this, they were first evaluated for potential Canker resistance which is both a quicker assay and a desirable trait. After Xanthomonas challenge on detached leaves, both hairpin and gene edited DMR6 lines showed reduced bacterial titers, statistically significant reductions in Canker symptoms and higher expression of some down-stream defense genes compared to WT controls. Several transgenic lines developed no disease symptoms whatsoever. A planting of 190 trees (including WT controls) from the best performing genome edited line (40 trees) and hairpin knockdown lines (150 trees) is being prepared; the plants have been replicated as rooted cuttings and will go into the field once regulatory requirements are met. An amendment to the current BRS permit has been submitted for their addition and is currently under review.

As an effort to accelerate development of non-transgenic HLB resistant plants using gene editing, we transformed early flower transgenic plants (carrying FT-Mcherry or FT-scFv gene) with the DMR6 targeting CRISPR construct. Cotyledons from FT-Mcherry line #3 seeds were transformed and 51 putative positive plants are now growing in soil. These plants are already showing strong early flowering phenotypes but must still be confirmed for presence of the CRISPR transgene. Seeds from 4 more FT-Mcherry lines are now being germinated for additional transformations. 50 additional plants from FT-scFv/CRISPR stacking transformations are also in soil awaiting confirmation. The early flowering trait will greatly decrease the time needed to produce an edited but non-transgenic offspring. A set of 30 confirmed positive plants from this gene stacking effort will be evaluated.

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

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

The detached leaf ACP-feeding assay (DLA) has undergone several small revisions to improve sensitivity and maintain consistent inoculation; adjusting feeding period and ACP numbers. Analysis of ACP bodies was expanded to include quantification of other major endosymbionts (Wolbachia, Carsonella and Profftella) to better investigate the activity of peptides causing CLas mortality. To further investigate the impact of transgenic or exogenous chimeral application, a DLA protocol to assess changes in ACP inoculability is also being developed. A manuscript detailing the protocol and findings is being prepared.

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

The best performing lines of Mthionin, chimeras `74', `188', TPK, TBL, DMR6 knockdowns and scFv transgenics have been submitted to Florida Department of Plant Industry for shoot-tip graft cleanup in preparation for future field studies. Hamlin/Mthionin transgenics (3 lines), Carrizo/Mthionin (2 lines), Carrizo/'74' (1 line), Carrizo/'188' (1 line) and Hamlin/'74' (1 line) have been returned certified clean.

Objective 5) Transgenic Product Characterization: Experiments are also underway to track the movement and distribution of transgene products using antibodies and affinity tagged protein variants. CLas+ RL have been grafted as scions onto MThionin expressing Carrizo as a platform to test peptide movement and effects across the graft union. Transgenic Carrizo lines expressing His6 and/or Flag tagged variants of chimeric proteins TBL (15 lines), BLT (15 lines), TPK (17 lines), PKT (20 lines), scFv-InvA (34 lines) and scFv-TolC (30 lines) have been generated and confirmed by RT-qPCR. Total protein samples have been extracted from His-tagged transgenic lines and sent to a collaborator for testing.

U. Albrecht 18-028C "Comparison of field performance of citrus trees on rootstocks propagated by seedlings, cuttings, and tissue culture" – 100% Completion of Objectives (June 2022 final)

The purpose of the project was to determine whether the method of rootstock propagation impacts growth and performance of grafted field-grown citrus trees.
Four field trials were established in different production regions within Florida. One field trial was established at SWFREC (Collier County) in 2017 and included different rootstock cultivars in combination with Valencia scion. The rootstocks had been propagated from seed and vegetatively by cuttings and tissue culture. Two more field trials were established with grower collaborators on a central ridge site in Polk County and on a flatwoods-type site in Hendry County in April 2018. These trials also consisted of Valencia scion in combination with different rootstocks generated by seed, cuttings, or tissue culture. An additional field trial was established in November 2019 in Indian River County. Detailed measurements were collected on above-ground tree traits using standard horticultural methods of evaluation. Roots structures were also measured in detail by excavating trees at the SWFREC and Indian River County location.
The overall objective of the project was to investigate effects of rootstock propagation method and the interaction with rootstock on root structure and tree performance during the early years of growth in the field. The project was designed to help growers and citrus nurseries resolve concerns about the quality of citrus trees propagated by methods other than by seed, specifically tissue culture.
After three years of field growth, no measureable differences were detected in tree growth, health, and productivity due to the rootstock propagation method that may raise concerns against using

cuttings or tissue culture propagated trees in commercial citrus production. In contrast to the propagation method, the rootstock cultivar had a considerable influence on tree growth and productivity, reiterating the importance of choosing the proper rootstock for each production site. Regardless of how the rootstocks are propagated, it is strongly recommended to purchase only high-quality trees from registered citrus nurseries, inspect root structures prior to transplanting, use proper planting practices, and good tree care when establishing a new grove.
Results from this project were disseminated in numerous extension presentations, trade journal articles, and peer-reviewed journal publications.
A comprehensive report reflecting the full results from the project has been submitted.

U. Albrecht 18-029C "Evaluation of citrus rootstock response to HLB in large-scale existing field trials using conventional and automated procedures" – 100% Completion of Objectives (December 2021 final)

The purpose of this 3-year project was to conduct evaluations of rootstocks in existing replicated field trials regarding their horticultural performance and ability to mitigate HLB-induced decline. The cooperative trials were planted in 2015 and contain more than 20,000 Valencia and Hamlin trees on 36 different replicated rootstock cultivars and 11 other non-replicated ones. Trials were under commercial management (Lykes Bros.) and planted near Basinger (Highlands County), a flatwoods-type location, and Lake Wales (Polk County), a central ridge-type location.

Annual evaluations were conducted in production seasons 2018/19, 2019/20, and 2020/21, only replicated rootstocks were included using 6 replications and each replication consisting of 8 linear trees. Plant assessments included annual measurements of tree height, canopy spread, trunk circumference, yield, and fruit quality. Tree health (canopy color, canopy density, foliar HLB symptoms) were rated on a scale of 1-5, with 1 indicating worst for canopy color and canopy density. All evaluations were conducted according to the Denver protocol. The complete set of rootstocks was included in year 3 evaluations. Leaves were collected annually for macro- and micronutrient analysis.

The rootstocks included UF/IFAS (CREC) released and experimental selections, California selections (available in FL), and selections from Spain. The Spanish selections are proprietary and not available for use in the USA currently but may be available in the future.

Overall conclusion from the 3-year study are as follows:

The results from this study show that rootstock performance varies by location, scion, and season. The most obvious and consistent differences among rootstocks were for their effects on tree size or vigor. Tetraploid rootstocks generally produced small trees with higher yield efficiency and higher-quality juice than the diploid rootstocks. However, the reduced yield on a per tree basis would likely require them to be planted at very high densities to be commercially feasible but they would facilitate mechanical harvesting. The strong HLB disease pressure and environmental factors may have diminished some of the positive attributes of these rootstocks.

Among the released UF/CREC rootstocks, UFR-5 was among the most promising rootstocks. Among the experimental rootstocks, White 1 also performed above average in terms of production, but results varied by location and scion.

The California rootstocks are commercially available in Florida and generally performed well, especially C-54, but like most of the other rootstocks, results varied by location, scion, and season. C-22 is the least vigorous of the four California rootstocks and is promising in terms of yield and fruit quality (especially in the early years); however, it was the least wind resistant of all rootstocks in these trials which may be of concern in a hurricane-prone state such as Florida.

X-639 was the most consistent rootstock in terms of tree size and apparent effect on tree health.

However, this did not always manifest as higher productivity, especially during the early production years. It was also among the lowest fruit quality inducing rootstocks. Interestingly, US-897, which is generally considered a semi-dwarfing rootstock, produced larger than average sized trees at the central ridge location. Its propensity for producing high-quality fruit did not clearly manifest in this study but fruit quality was low across all rootstocks.

The considerable differences in rootstock effects on tree size and production among the two locations may have been related to different microclimatic conditions, different disease pressures, and differences in trial management.

To extend the information from this study to the grower community, 11 industry and 3 conference presentations were given along with 1 trade journal article, and 4 journal series articles during the study period.

A full set of Excel files providing collected data has been given to CRDF.

R. Cave (previously R. Ferrarezi) 18-037C "Performance of newly released grapefruit cultivars and rootstocks in the Indian River Citrus District" – 100% Completion of Objectives (May 2022 final)

A large-scale trial of HLB-tolerant citrus cultivars addresses the need of Indian River growers to identify the best rootstocks and scions for growing fresh citrus fruit. This project had two objectives: (i) Assess the performance of new grapefruit cultivars with selected rootstocks; and (ii) Evaluate the influence of UFR and other rootstocks on grapefruit scion development in comparison to legacy/standard rootstocks. Trial 1 consisted of 18 grapefruit cultivars on three rootstocks (Sour orange, US-942, and X-639). Trial 2 assessed 31 rootstocks with `Ray Ruby' grapefruit as the scion. The total number of trees with grapefruit scions is 2,741. Control-release polycoated fertilizer was applied appropriately in May 2022. All trees were treated as needed with appropriate agrochemicals to manage canker, Asian citrus psyllids, mites, and citrus leafminers. No trees died due to the freezing temperatures experienced in February 2022.

Trunk diameter and canopy volume were measured on the three middle trees in each experimental plot in October 2021 to assess tree size. At the time, there were significant differences among scion/rootstock combinations. In Trial 1, `Pummelette UF-5-1-99-2' grapefruit on US-942 was 6X larger (395.5 ft3) than `US 1-83-179' grapefruit hybrid on US-942 (70.6 ft3). In Trial 2, grapefruit on UFR-15 was 3X larger (314.3 ft3) than on UFR-17 (123.6 ft3) for trees planted September 2019. Measurements were gathered from trees planted in June 2021 for the first time in May 2022. Phenology data collected monthly recorded flush initiation, flower initiation, full flowering, fruit set, fruit development, fruit color, and fruit maturity per tree. No fruit data were collected because a tree's first crop is not indicative of future production. Nearly all trees in Trials 1 and 2 have set fruit in May 2022. Fruit data will be collected in January 2023. Long-term evaluation of fruit yield and quality is needed to identify the most promising scions and rootstocks to determine their profitability and capability of meeting grower and market needs.

HLB is widespread in the study grove. Visual blotchy mottle symptoms and twig dieback canopy are present in a few treatments but not widespread. Leaf samples for quantifying CLas titer were collected in May 2022 and sent to Southern Gardens for analysis. Trees that are CLas-free (ct values >38) and CLas-infected and symptomatic (ct values of 26-32) can be found in the same plots, but many symptomatic trees are developing vigorous canopies.

The incidences of Asian citrus psyllid and citrus leafminer were frequent during flush periods. The incidences of aphids, root weevils, and orange dogs were sporadic. Canker damage was noticeable but not uniform; it was especially significant on `Ray Ruby' grapefruit trees. Tree growth has not been significantly affected by these pests due the timely applications of pesticides.

Results of the study were presented at the annual Florida Citrus Show in January 2022 in Fort Pierce, FL by graduate student Martin Zapien and in his Zapien's MSc thesis presented to the Graduate School in April 2022. The thesis and data collected to date are available upon request.

J. Grosser 18-039C "Part B - The UF/CREC Citrus Improvement Program's Field Trial Evaluations" – 95% Completion of Objectives (January 2022 final)

True sweet oranges: This was a very exciting quarter for identifying true sweet orange candidates to replace Hamlin. Multiple Vernia somaclone-derived seedling clones repeated for early maturity (first week of December) for the 3rd consecutive year. The best of these was MB-R25-T7, which had a 16.17 ratio and the highest brix (11.16), and significantly higher soluble solids than the Hamlin control. The Vernia somaclone-seedling population, planted in the Mathew Block at the Orie Lee Alligator Grove, has never had psyllid control. About 2 years ago, trees were supplemented with CRF to prevent decline. Trees were commercially harvested last week, and there was still no fruit drop observed. At this time the brix in the MB-R25-T7 was over 13. This new Vernia clone was entered into the PTP in January. A new early-maturing OLL clone was also discovered in the somaclone-derived seedling block of OLLs (located in the Lee Family Home Grove in St. Cloud). Clone FB-R7-T35 had a 14.12 ratio in mid-January with 12 brix, 5.7 lbs. solids/box (exceptionally high for a juvenile tree) with a 37.3 juice color score. This clone had an extended juvenility and did not have fruit last year. This new OLL clone will be entered into the PTP the first week of April, when new slots become available. Both clones are being propagated at the CREC to expedite further evaluation (with permission from DPI).

Promising orange-like hybrid: Triploid hybrid C7-11-7 [Sugar Belle x (Murcott + Succari sweet orange)] produces fruit morphologically indistinguishable from a true sweet orange, but with better external color. The original tree is exceptionally productive and appears to have HLB tolerance slightly better than standard sweet oranges. Juice from fruit of the original tree (with HLB for many years) had 13.87 brix, 15.1 ratio, 39.3 color score and 8.31 lbs. solids per box (double the state average for Hamlin!) in mid-January. This selection will also be entered into the PTP in early April.

Potential HLB tolerance/resistance from 'gauntlet' rootstock candidates: Rootstock sprouts recovered from 12 superior gauntlet trees that had the tops cut off to induce sprouting for rootstock recovery, all successfully grafted, were analyzed for CLas by PCR. Most interestingly, multiple grafted trees from two of these rootstock candidates had ct values above 36, indicating no CLas bacteria replication; and both hybrids were from the same cross (A+HBPxOrange 3-12-12 and A+HBPxOrange 3-12-10). Both hybrids appear to be more vigorous than either parent. Orange 3 is UFR-1, and both tetraploid parents in this cross produce fruit with high soluble solids (good rootstock pedigree). Further experiments are being designed to determine the value of strong rootstocks that do not support CLas replication in the root systems. Since recovered lines of these two rootstock candidates are pathogen free, TC propagation was initiated. A 3rd hybrid from this cross looks exceptional in the 'gauntlet' this spring, and it will also be cut to recover the rootstock.

CREC Trailer Park Trial: A good candidate for juice blending was identified: 18A-10-11. This selection is a low-seeded cybrid of 'Furr' that produces a large firm fruit, with exceptional quality juice (12.5 brix, 19.84 ratio and 46.1 color in mid-January). This selection was used in juice blends at both the CREC display and Gator Day at the State Capitol, and all were very well received. The exceptional juice flavor holds up well to pasteurization.
Rootstock candidate identified from Strang/Gapway trial. We were able to recover the x639 mutant (apparent deletion mutant) showing the exceptional HLB tolerance. One successfully grafted tree and 12 rooted cuttings have been obtained. PCR analysis showed a ct value of 40, indicating no CLas bacteria in the recovered rootstock. Additional propagation is underway as needed for a more robust field trial. (Also note that the Gapway property has been sold, so the trial has been terminated; however, the rootstock tree was dug up and successfully moved back to the CREC).

Field Trial Data Collection, etc.: various data (tree health, fruit quality, yield, etc.) was collected from the following trials: Tom Hammond, Greene River Citrus, IMG, Peace River Growers, Bryan Paul Citrus, Lee Family Groves, and Duda.

Data analysis and entry onto the Rootstock Data Website: new data posted on website included: Jackson Citrus, Lykes, GFC Citrus, Lee Family Groves, Banack Citrus and Duda. Trial data worked on, not yet added to website: Greene River Citrus, Peace River Growers, IMG, Bryan Paul Doe Hill, Citra trial, Cutrale, and the Serenoa trial. The Access Data Plan was also implemented on the website.

E. Stover 18-058C "Fort Pierce Field Test Site for Validating HLB and/or ACP Resistance" – 100% Completion of Objectives (April 2022 final)

The support period for the dedicated testing site at the Picos farm has concluded. Over the course of this and previous projects, Picos has served well as a transgenic capable field-testing site available to HLB and Citrus researchers. Full support for all associated projects was provided. The entire 10 acres was utilized, allowing government, university, and private sector researchers to make numerous plantings of both transgenic and conventional citrus varieties with a high probability of HLB tolerance/resistance. Several of the earlier established trials are wrapping up. However, due to the long timeframe of citrus field trials, there remains work to be done on many of the plantings. Several of these trials will be continued under two new NIFA funded projects; 2020-70029-33176 Therapeutic Molecule Evaluation and Field Delivery Pipeline for Solutions to HLB and 2020-70029-3319 Providing Practical Solutions for HLB Treatment and Prevention. For the projects not supported under dedicated grants or appropriate for USDA-ARS base funding, new sources of funding will need to be secured in order to allow for their continuation.

Recent quarters:

A significant USDA-funded infrastructure project has been completed, fully renovating the water management systems and significantly improving storm and flood protection. USDA has also acquired a topper hedger to facilitate canopy management and reflect the best practices of commercial farms. Site management and field trials are progressing well. The site remains available for access to all researchers and all regulatory protocols for the care and disposal of transgenic material are being observed. The trees have been hedged and topped to promote growth, open canopies and access to nutritional sprays. The foliar spray program was applied every two weeks and including standard treatments for commercial groves. Crosses have been made with transgenic pollen to help elucidate if sexual embryos can be rescued from polyembryonic females, making use of the transgenic markers to determine if sexual hybridization is successful. A third year of crossings has also been made with the early flowering (FT) transgenics, continuing the work described below.

An additional BRS transgenic release permit was approved (AUTH - 0000043620) for material with confidential business information (CBI) for a project led by R. Shatters. The primary BRS permit has also been renewed and amended to include a new construct from UF (Now AUTH - 0000206702). The annual site review from APHIS/BRS has been conducted successfully. Discussions have begun with APHIS-BRS to set conditions for new or expanded transgenic release permits in support of NIFA project 2020-70029-33176, Therapeutic Molecule Evaluation and Field Delivery Pipeline for Solutions to HLB, with field trials expected to begin later this year once all regulatory requirements are met.

Four new plantings from UF expressing resistance genes and two new plantings from USDA-CRADA partners expressing antimicrobial peptides and anti-CLas plantibodies have been made. With recent plantings, the transgenic site is operating at full capacity. Fall 2021 assessments were completed for USDA plantings as described below for trials #8, #9, #10, #11 and #15. Fruit have been harvested from the second year of controlled crosses using pollen from early flowering (FT) transgenics on traditional varieties maintained in the testing site. Seeds from these fruit and those of future crossings will be used to assess inheritability of the phenotype and for CRISPR gene stacking to combine genome editing with accelerated breeding traits. The UC Riverside-led trifoliate and trifoliate hybrid trial has concluded, a manuscript regarding identified HLB-tolerance is in preparation; and these trees can be removed as needed to make space available for future plantings. Dr. Stover analyzed data on canker incidence for this trial and a manuscript detailing these results has been published in HortScience DOI: 10.21273/HORTSCI15684-20.

Previously established at the site:

A number of trials are underway at the CRDF funded Picos Test Site. A detailed current status is outlined below this paragraph. Investigation of potential pollen flow from transgenic trees will continue to assess the possibility of reducing the isolation distances. Availability of the test site for planting continues to be announced to researchers.

Supplemental: Full details on trial plantings.

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

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

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

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

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

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

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

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

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

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

11) Multiple plantings with grafted trees of Hamlin, Valencia and grapefruit scions on transgenic rootstock expressing antimicrobial citrus-thionin and bacterial recognition domain fusion proteins (219 trees with controls) as a collaboration between USDA and the New Mexico Consortium.

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

13) Planting from Zhonglin Mou of UF that includes transgenic grapefruit (31 plants) and sweet orange (60 plants) scions expressing two different resistance genes and grafted on WT Swingle rootstocks; as well as non-transgenic controls.

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

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

N. Wang 18-064C "Evaluation of the control effect of bactericides against citrus Huanglongbing via trunk injection" – 100% Completion of Objectives (April 2022 final)

Objective 1. To illustrate whether application of bactericides via trunk injection could efficiently manage citrus HLB and how bactericides via trunk injection affects Las and HLB diseased trees.
1.1. Determination of the in planta minimum bactericidal concentrations (MBCs) of bactericides against Las
A new method was developed for evaluating the effects of oxytetracycline (OTC) treatment on CLas titers in infected plants and determined the relationship between OTC residue levels and control levels achieved for CLas using mathematical modeling in greenhouse and field experiments. In both greenhouse and field, OTC spray did not reduce the titers of CLas, and it produced undetectable or mild levels of OTC residue in leaves within 7 days post-application (DPA). In greenhouse, OTC injection at 0.05 g per tree decreased CLas titers to an undetectable level (cycle threshold value ≥ 36.0) from 7 to 30 DPA and produced a residue level of OTC at 0.68 to 0.73 µg/g of fresh tissue over this period. In the field, OTC injection at 0.50 g per tree resulted in the decline of CLas titers by 1.52

log reduction from 14 to 60 DPA, with residue levels of OTC at 0.27 to 0.33 µg/g of fresh tissue. In both trials, a first-order compart model of OTC residue dynamics in leaves of trunk-injected trees was specified for estimating the retention of effective concentrations. Furthermore, nonlinear modeling revealed significant positive correlations between OTC residue levels in leaves and the control levels for CLas achieved. The results suggested that the minimum concentrations of OTC required to suppress CLas populations in planta to below the detection limit are 0.68 and 0.86 µg/g and that the minimum concentrations of OTC required for initial inhibition of CLas growth in planta are ∼0.17 and ∼0.215 µg/g in leaf tissues under greenhouse and field conditions, respectively. This finding highlights that a minimum concentration of OTC should be guaranteed to be delivered to target CLas in infected plants for effective control of citrus HLB.
The in planta minimum bactericidal concentrations of streptomycin (STR) applied and its effect on CLas titers in planta was investigated by foliar spray and trunk injection of 3-year-old citrus trees that were naturally infected by CLas in the field. After foliar spray, STR levels in leaves peaked at 2 to 7 days post application (dpa) and gradually declined thereafter. The STR spray did not significantly affect CLas titers in leaves of treated plants as determined by quantitative PCR. After trunk injection, peak levels of STR were observed 7 to 14 dpa in the leaf and root tissues, and near-peak levels were sustained for another 14 days before significantly declining. At 12 months after injection, moderate to low or undetectable levels of STR were observed in the leaf, root, and fruit, depending on the doses of STR injected, with a residue level of 0.28 µg/g in harvested fruit at the highest injection concentration of 2.0 µg/tree. CLas titers in leaves were significantly reduced by trunk injection of STR at 1.0 or 2.0 g/tree, starting from 7 dpa and throughout the experimental period. The reduction of CLas titers was positively correlated with STR residue levels in leaves. The in planta minimum effective concentration of STR needed to suppress the CLas titer to an undetectable level (cycle threshold ≥36.0) was 1.92 µg/g fresh weight. Determination of the in planta minimum effective concentration of STR against CLas and its spatiotemporal residue levels in planta provides the guidance to use STR for HLB management.

1.2. Effect of bactericides via trunk injection on citrus HLB disease progression, tree health, yield and fruit quality in different aged trees with a different disease severity

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

The data demonstrate that for 6-year-old trees, 2.0 g OTC/tree, but not 1.0 g OTC /tree via trunk injection significantly reduced CLas titers. However, neither concentration reduced CLas titers for 22-year-old trees. Spray had no effect on CLas titers. STC at 1 g or 2 g/tree via trunk injection had no effect on CLas titers. OTC (2.0 g/tree) and STR (2.0 g/tree) but not at lower concentrations significantly increased fruit yield, but had no effect on quality.

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.
Residues of OTC or STR in fruit harvested from 22-year old Hamlin sweet orange trees injected with OTC or STR at different doses were determined. Injections were conducted in February-March 2021 and fruit was harvested in January 2022. Neither OTC or STR was detected at 1 g or 2 g/tree via trunk injection.
Residue of OTC or STR in fruit harvested from 6-year old Hamlin sweet orange trees injected with OTC or STR at different doses were determined. Injections were conducted in March 2021 and fruit was harvested in January 2022. The following residues were observed: OTC (1.0 g/tree): 0.08 ppm ± 0.03; OTC (2.0 g/tree): 0.19 ppm ± 0.07, STR (1.0 g/tree): 0.09 ppm ± 0.03; STR (2.0 g/tree): 0.17 ppm ± 0.08.

Objective 3. To determine whether trunk injection of bactericides could decrease Las acquisition by Asian citrus psyllids (ACP)
Twenty 1.5-year old citrus (Valencia sweet orange) plants were graft-inoculated by Las carrying buds in February 2020. These plants are being tested for Las infection and 4 plants were confirmed with Las infection (Ct values are between 34.0 and 35.0) at 4 months after grafting. They will be subjected to OTC or STR treatment by trunk injection and ACP acquisition access for 7 to 14 days. The time points to test OTC and STR treatment on ACP acquisition of Las was determined.
A test of how trunk injection of OTC affects ACP acquisition of Las was conducted. OTC significantly reduced ACP acquisition of Las.

Objective 4. To monitor resistance development in Las against bactericides and evaluate potential side effects of trunk injection of bactericides
Monitoring resistance development in Las against bactericides. Leaf samples for this test have been collected from 5 trees injected with OTC and 5 trees injected with STR at the highest doses in each of the three groves at 6 and 9 months after the injection, respectively. PCR-sequencing analysis on Las 16SrRNA gene showed there was no mutation compared with the reported sequence. However, accurate evaluation needs to be conducted once CLas is cultured.
No obvious side effects were observed at low OTC or STR concentrations. However, at some high concentrations, phytotoxicity was observed on leaves.

E. Stover 18-065C "High -Throughput Inoculation of Transgenic Citrus for HLB Resistance" – 100% Completion of Objectives (December 2021 final)

Project rationale and focus: The driving force for this three-year project was the need to evaluate citrus germplasm for resistance to HLB, including germplasm transformed to express proteins that might mitigate HLB, which requires citrus be inoculated with CLas. Citrus can be bud-inoculated, but since the disease is naturally spread by the Asian citrus psyllid, the use of psyllids for inoculations more closely resembles "natural infection", while bud-inoculations might overwhelm some defense responses.

CRDF funds supported high-throughput inoculations to evaluate HLB resistance in citrus germplasm developed by Drs. Ed Stover and Kim Bowman for the last 3 years, but also provided CLas+ ACP to other researchers. The funds cover the costs associated with establishing and maintaining colonies of infected psyllids; equipment such as insect cages; PCR supplies for assays on psyllid and plant samples from infected colonies; and two GS-7 USDA technicians. A career base-funded USDA technician also assigned ~30% of her time to the program to maintain colonies (including watering, setting up new cages, terminating old cages, cleaning growth chambers and cages). USDA provides greenhouses, walk-in chambers and laboratory space to accommodate rearing and inoculations.

Results: The purpose of this Service/Support Project was fully achieved in terms of maintenance of the infrastructure and implementation of a high-throughput inoculation with Asian citrus psyllid (ACP) to evaluate citrus scion and rootstock germplasm for resistance or tolerance to HLB. A total of 24 ACP colonies were continuously maintained in two walking-in environmental chambers and during these 3 years (2019 to 2021). A total of 62,885 CLas infected psyllids were supplied to accomplish the following objectives: (1) evaluation of rootstock breeding material, (2) evaluation of transgenic lines expressing antimicrobial and insecticide natural peptides, and (3) supplement of Clas+ ACPs for research involving CLas control in citrus performed by USDA and University researchers. Besides that, thousands of infected psyllids were used for renewal and rehabilitation of colony cages to allow continuous ACP supply for all research needs, even during the Covid-19 pandemic period. Also, approximately 360 ACPs were tested monthly for monitoring the colony quality by qPCR (approx. 13,000 ACPs tested in 3 years).

1) Evaluation of rootstock breeding material – The Bowman lab utilized ACPs from the positive colonies to inoculate grafted sweet orange on 18 different rootstocks with variable resistance to HLB, grouped as seven separate experiments during 2019-21. Each experiment used 1,260 ACPs, for a total of 8,820 ACPs from the positive colonies for the seven experiments. In each replicated experiment, seven trees on nine different rootstocks were inoculated by exposure to ACPs for 4 weeks, and then compared by periodic evaluation of tree health, growth, and CLas titer via PCR conducted on the inoculated trees. In the early experiments, significant differences between rootstocks following infection were observed for tree growth, health, and HLB symptom development, suggesting significant differences in disease tolerance were detected. The later in the series of experiments are less than 12 months from inoculation, and evaluation is ongoing. Conclusions will be drawn from the experiments after the full series is completed. A positive feature of this testing method for rootstocks is that a high frequency of uniform infection of trees was obtained. Negative features of this method were the difficulty in maintaining high titer of CLas in healthy ACP colonies, and the necessity to use very small trees that could fit into the small cages for the inoculation step.

2) Transgenic events evaluation – The Stover Lab evaluated hundreds of citrus transgenic lines expressing antimicrobial peptides and defensive proteins targeting CLas, as well as natural

insecticide peptides to control ACP, the CLas vector. Breeding lines in different stages of development, were inoculated with CLas infected psyllids using two different methodologies to evaluate HLB tolerance/resistance. The Detached leaf assay (DLA) is the first step of evaluating newly produced transgenic plants to CLas response or ACP mortality. In this assay, detached leaves are inoculated with 10 infected ACPs for 7 days, producing data that support the selection of promising transgenic lines that can be moved to the next levels of analysis.
In the second method, which is performed with more developed transgenic plants previously selected by DLA and propagated in greenhouse, whole transgenic trees are cage inoculated with 20 CLas+ ACPs for 2 weeks. Greenhouse evaluation to confirm HLB tolerance is performed to select plants to be established in the field.

The Stover lab utilized 35,180 CLas infected ACPS to perform the DLA in 3,518 individual leaves belonging to 495 transgenic citrus lines (Carrizo, Hamlin and Ray Ruby) expressing the peptides Onyx and Topaz, and the antimicrobial thionin chimeras designated TS, ST, BT, TB, 74 and 188, under constitutive and SCAmp-P3 phloem specific promoters. This assay helped to eliminate nonperforming lines, and facilitated selection of approximately 45 lines, that were replicated for next level in the evaluation pipeline (CLas bud and ACP inoculation). The best performing lines produced from 70-80 to more than 90% ACP mortality (SCAmp-P3-ST). Many of these lines produced a decrease in CLas in midribs, compared to the control (qPCR) and decrease in different types of ACP symbionts, what may partially explain the ACP mortality.

Evaluation of the microbiome of ACPs fed on transgenics represents an important modification implemented into the usual DLAs since it furnishes insights on the mode of action of peptides in controlling psyllids. A research paper has been prepared (Rapid in vivo screening for huanglongbing resistance in genetically modified citrus by detached leaf assay- J. Krystel, M. Grando, Q. Shi, E. Cochrane, E. Stover). In addition, to evaluate the effect of new peptides in controlling HLB bacteria, 260 CLas + ACPs were used in the psyllid homogenate assay developed by the Stover Lab (Assay described in the paper Krystel et al., 2019. An in vitro protocol for rapidly assessing the effects of antimicrobial compounds on the unculturable bacterial plant pathogen, Candidatus Liberibacter asiaticus. Plant Methods. 15:85.

For more advanced transgenic lines, a total of 354 whole trees were cage inoculated with 7,080 CLas+ ACPs during the 3-year period, allowing reevaluation of the HLB tolerance or insect control of the transgenic lines. Twenty-one transgenic lines (more than 100 plants) selected from this assay were transplanted into the field in 2021 for phenotypic and symptomatic studies, under special permit.

3) Supplying CLas infected ACP for USDA and University researchers- This service project contributed to many research investigations focusing on HLB by supplying the scientific community with 11,545 CLas-infected ACP for various research purposes. Recipients included researchers with USDA in Fort Pierce (Dr. Yongping Duan, Dr. Randy Niedz, Dr. Bob Shatters) and Ithaca (Dr. Michelle Heck), UF in Gainesville (Dr. Dean Gabriel), and Florida International University, for Jessica Dominguez, a Ph.D. student, who is developing a thesis on alternative compounds to control CLas bacteria.

Major shortcomings, unfinished business:

As a result of this 3-year project, the identified superior material moved from Lab to greenhouse, then to the field and numerous transgenic lines were moved up in the pipeline. Encouraging results were obtained in using this method for the evaluation of rootstocks in graft combination with sweet orange scion. The availability of the CLas infected ACPs made it possible to evaluate all expressing transgenic lines, even the ones with medium and low gene expression levels. Frequently high gene expressers transgenic may fail in deliver the expected phenotype because of post transcription modification and epigenetic phenomena. This project helped to move germplasm (both rootstocks and scions) with improved tolerance/resistance to HLB through the research process and into further field studies to help the citrus industry, by providing the means to perform uniform methods of HLB challenge to evaluate new material tolerance/resistance. This project also supported many other projects within the research community to find other potential solutions to HLB. The major shortcoming faced during this 3 year periods was the general slowdown of activities and the hardship in keeping the hot colonies due to the pCOVID-19 pandemic. The researchers involved in this project maintained the activities at the maximum time allowed by USDA regulations.
The efforts funded by this project will contribute to release of new rootstocks and new transgenic lines entering field evaluation and will ultimately provide citrus growers with new HLB resistant/tolerant citrus material.

Publications from this project:

1. Krystel, J., Q. Shi, J. Shaw, G. Gupta, D. Hall, and E. Stover. 2019. An in vitro protocol for rapidly assessing the effects of antimicrobial compounds on the unculturable bacterial plant pathogen, Candidatus Liberibacter asiaticus. Plant Methods 15:85
2. Krystel, J.*, H. Liu, J. Hartung, and E. Stover. 2021. Novel plantibodies show promise to protect citrus from greening disease. J. Amer. Soc. Hort. Sci. 146:377-386.
3. Gupta, G., S. Basu, L. Huynh, S. Zhang, R. Rabara, H. Nguyen, J. Valesquez, G. Hao, G.Miles, Q. Shi, and E. Stover. 2021. Two Candidatus Liberibacter asiaticus effectors combine to suppress critical innate immune defenses and facilitate huanglongbing pathogenesis in citrus. Scienific Reports (submitted).
4. Krystel J., M. Grando, Q. Shi, E. Cochrane, E. Stover. 2022. Rapid in vivo screening for huanglongbing resistance in genetically modified citrus by detached leaf assay- In preparation

N. Killiny 19-015 "Evaluation of the tolerance of newly developed citrus cultivars, on different rootstocks, to Huanglongbing" – 100% Completion of Objectives (April 2022 final)

The project has three objectives:

1. Evaluate the tolerance of newly released/developed citrus cultivars to `Ca. L. asiaticus' pathogen.
2. Evaluate the tolerance of newly released/developed citrus cultivars to Diaphorina citri.
3. Determine the mechanism underpinning the tolerance of the newly developed cultivar to HLB.

This study aimed to provide growers with a list of newly developed cultivars that are potentially tolerant to HLB and give insights about the mechanism behind their tolerance. The study focused on leaf chemical analyses because this is the site of landing, feeding, and reproduction for Diaphorina citri, the Asian citrus psyllid (ACP) and subsequent `Ca. Liberibacter asiaticus' inoculations. Comparisons were made of the newer varieties to established varieties to determine any differences in their potential for ACP resistance and `Ca. L. asiaticus' tolerance.

Obj. 1. To evaluate the tolerance of newly released/developed citrus cultivars to `Ca. L. asiaticus'. To complete this objective, `Ca. L. asiaticus'-infected D. citri were used to inoculate several of the new varieties including Sugar Belle, Nova×Osceola, and its hybrid called Lucky. qPCR analysis was preformed one year after ACP-inoculation and found that infection rates of 20%, 60%, and 80% for Sugar Belle, Nova and Lucky respectively. Unfortunately, the hybrid called Lucky was did not show resistance to the HLB pathogen `Ca. L. asiaticus' in this small study. However, Sugar Belle had a low infection rate and has shown HLB tolerance in the field. Lucky has not been released, and it may yet prove to be tolerant in the field, if it retains vigorous growth and production similar to its parent, Sugar Belle.

Obj. 2. To evaluate the tolerance of newly released/developed citrus cultivars to D. citri.

1. The first work completed was an evaluation of three new hybrids of Pummelo x Citrus latipes. We investigated the ACP attraction of three new hybrids produced from the cross metabolomic profiles of C2-5-12 Pummelo (Citrus maxima (L.) Osbeck) × pollen from C. latipes. The hybrids were selected based on leaf morphology and seedling vigor. The selected hybrids exhibited compact and upright tree architecture as seen in C. latipes. To examine the effect of released volatiles on the attraction of D. citri, A preference assay was conducted using the parents and their offspring. The preference assay was repeated ten times in ten different designs to exclude the effect of orientation of host plants. Hybrid II was the most attractive to D. citri (40% landing) followed by hybrid III (22%) and C. latipes (14%). Pummelo and hybrid I were the least attractive to D. citri and the number of psyllids attracted to each of these two hosts was not significantly different from the number of psyllids that did not make any choice. This work was published: Metabolic profiling of hybrids generated from pummelo and Citrus latipes in relation to their attraction to Diaphorina citri, the vector of huanglongbing (Killiny et al., 2020).

2. In the small study involving a new mandarin hybrid called Lucky (two parents are Sugar Belle and Nova×Osceola) we performed two three-way choice challenges with ACP, one in the winter of 2020/21 and again in the summer of 2021. Survival of ACPs on the three varieties was not good in either experiment, the first due to cold weather (January 2021 outside experiment) and the second (growth room experiment in July and August 2021) may have been due to residual pesticide treatment for mites. In both cases, however, ACP survival and reproduction was the highest on the hybrid `Lucky' compared to the two parental varieties. Therefore, we can suggest a correlation may exist between the lack of inheriting thymol and several other deterrent compounds from its parents (see Obj. 3) and the higher ACP counts, so maybe it is not very `Lucky' after all.

3. Sugar Belle response to ACP infestation. This study was focused on the leaf volatile organic compounds (VOC) response of Sugar Belle mandarin hybrid to infestation with the Asian citrus psyllid. Since Sugar Belle has been widely accepted as `tolerant' of HLB, the goal was to examine the effects of D. citri infestation on the secondary metabolites produced in the leaves using gas chromatography-mass spectrometry (GC-MS). Previous work determined the leaves contain thymol, which has known antimicrobial activity and is thought to be one reason behind its tolerance to the HLB pathogen. In this study it was found that Sugar Belle had a robust volatile response one month after D. citri infestation, producing increased amounts of VOCs in a rootstock-dependent manner. This work was published: Influence of rootstock on the leaf volatile organic compounds of citrus

scion is more pronounced after the infestation with Diaphorina citri. Plants 10 (Jones & Killing, 2021).

Obj. 3. To determine the mechanisms underpinning the tolerance of the newly developed cultivars to HLB. The majority of the work completed was in this area of objective 3. Extensive biochemical evaluation was conducted on the following sets of data: Vernia, Valquarius, Valencia, CUPS Mandarins, CUPS Grapefruit, UFR Rootstocks, USDA Rootstocks.

Evaluation of Vernia and Valquarius - Two newer releases, Vernia and Valquarius were evaluated compared to traditional Valencia. Qualitatively, the three sweet orange varieties are very similar, but the total VOC content was highest in Valencia, followed by Valquarius, then Vernia. We identified 47 VOCs in the hexane-extracted leaves of the three varieties. No unique compounds were detected among the three varieties. Many differences (37/47) were statistically significant because of the high content found in the traditional Valencia variety as compared to the two newer varieties. Higher levels of VOCs were previously associated with ACP attraction, so there may be a slight advantage to the newer varieties in having a lower VOC content. Quantitatively, Valencia had the largest total VOC content 4252.51 µg/g fresh weight compared to 3350 and 2900 µg/g respectively for Valquarius and Vernia. The compounds found in the highest concentrations included phytol, sabinene, neral, geranial, β-myrcene, δ-carene, linalool, trans-β-ocimene, and limonene. Due to the high similarity with Valencia, ACP biology with these new varieties. was not pursued.

Evaluation of Lucky mandarin hybrid - We sampled the mature leaves and did two different extractions - 1) hexane for leaf volatiles such as limonene, linalool and caryophyllene; and 2) methanol- chloroform-water for non-volatile metabolites such as sugars, amino acids and organic acids. Volatile organic compound (VOC) analysis was completed to determine if Lucky will have any of the chemical properties of Sugar Belle, which is considered tolerant to HLB. Overall, 54 volatile compounds were detected among the leaf extracts of the three varieties. Lucky and Nova produced sabinene and β-ocimene as their major monoterpenes, whereas in Sugar Belle the major monoterpenes are β-ocimene and γ-terpinene. Linalool appears at similar levels in all three, and limonene is relatively low in all three (compared to sweet orange varieties, where it is the dominant monoterpene). The dominant sesquiterpenes are caryophyllene and β-and γ-elemene. Lucky was found to have slightly more total VOCs than either of the parents, and about the same as Valencia sweet orange, which we used for a comparison. Like other mandarins, it was low in d-limonene and almost completely lacked neral and geranial, the aldehydes that dominate Valencia sweet orange leaves. Most of the compounds detected were in common between the three varieties, but a few were unique to each variety. As reported previously, the characteristic VOCs for Sugar Belle included thymol and thymol methyl ester. Neither Lucky nor Nova produced thymol, but Nova produced traces of the thymol methyl ester. In addition, the monoterpenes para-cymenene and para-methatriene were detected in Sugar Belle, which was not found in the hybrid Lucky offspring or Nova. Overall, the VOC profile of Lucky showed contributions from both parents but did not express all of the possible compounds (44/54). There are many that are found uniquely or in two of the three varieties. These include para-cymene, para-menthatriene, and thymol (in SB only), thymol methyl ether (SB, Nova), germacrene C (Nova only), β-and γ-elemene (SB, Nova), 7-epi-thujene (Nova, Lucky), β-cubebene (Lucky), 4-epicubedol (new, in Nova), and β-sesquiphellandrene (Nova, Lucky).

A comprehensive final report has been received which outlines results of all studies.

Y. Wang 19-024 "Near-term approaches of using alternative HLB-tolerant cultivars for increased production and improved juice quality" –100% Completion of Objectives (October 2021 final report)

This project was completed in October 2021. The project had two objectives and the progress on each objective is outlined below.

Objective 1. Evaluation of blended juice using released HLB-tolerant sweet orange/mandarin cultivars via analyses of sensory and consumer acceptance. All the sensory studies have been completed to verity the potential of blending Sugar Belle juice with early variety (Hamlin) and late variety (Valencia). From the results obtained, 50/50 Sugar Belle blend was preferred by most panel members. Ten percentage of Sugar Belle blended with Hamlin could improve the juice appearance and flavor significantly, while 10% of Sugar Belle blended with Valencia didn't improve the juice quality significantly. In addition, a shelf-life study for the Sugar Belle juice has been conducted. In a 90-day shelf-life study, Sugar Belle juice was stored at 4 °C and samples were tested at day 0, 30, 60 and 90. A sensory panel was used to determine changes of sweetness, bitterness and overall flavor. For the samples of the first year, no significant changes ($p \le 0.05$) were observed over time for the storage intervals, while for the second year, samples at day 0 were different from other intervals indicating the thawing process may affect the samples at day 0.

Objective 2. Identify more tolerant cultivars resembling the quality of Valencia for the juice market and identify a chemistry definition of consumer accepted orange flavor. Fourteen citrus cultivars and selections were used in a comprehensive study to determine how each of those selections compared to Valencia orange. A publication entitled "Identification of key flavor compounds in citrus fruits: a flavoromics approach" has been completed and now published in Journal of ACS Food Science & Technology: https://pubs.acs.org/doi/10.1021/acsfoodscitech.1c00304 .

A comprehensive, 9 page report has been submitted to fully identify and highlight all findings and options for future use of the platform used in the study to compare varieties to standard Valencia.

U. Albrecht 19-030C "Use of compost and interaction with low- and high-vigor rootstocks to accelerate young sweet orange tree establishment and enhance productivity" – 90% Completion of Objectives (July 2022 quarterly)

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

Progress this quarter: Another round of compost was applied at the usual rate.

Flush ratings and soil moisture measurements continued. Soil moisture in the compost treated plots continues to be significantly higher than in the control plots and there is significantly more (two to three-fold) weed pressure in the compost treated plots. Soil and leaf samples were collected for macro- and micronutrient analysis. Many of the soil nutrients continue to be present in higher concentrations in the compost treated plots than in the control plots except for copper which was reduced by the compost. However, higher nutrient concentrations in the soil did not always translate to higher concentrations in the leaves. Cation exchange capacity and soil pH were also significantly higher in compost plots than in control plots.

Fibrous root respiration was measured. Roots from the compost plots have a higher respiration rate than roots from the control plots. Significant differences were also found among rootstocks with the citrandarins having higher respiration rates than US-802. This was also reflected in the specific root length which was lowest for US-802 and highest for US-897. Specific root length appears to be moderately correlated with pounds soluble solids.

A manuscript for publication was submitted and is under review.
A Citrus Industry magazine article on this study was published in the June 2022 issue.

Work anticipated for next quarter:
Flush ratings and soil moisture measurements will continue. Root respiration and specific root length will be measured. Tree ratings and tree size measurements will be conducted. Leaves will be collected for CLas determination. The weed biomass will be determined. Data analyses will continue.

M. Dutt 20-014 "Understanding the role of systemic acquired resistance (SAR) in enhancing tolerance to HLB in the Parson Brown sweet orange" – 100% Completion of Objectives (February 2022 final)

Conclusions from the one-year preliminary study:
1) \`Parson Brown' trees, irrespective of location, clone or rootstock had enhanced SAR activity as indicated by higher PR1 and PR2 gene expression. Statistically significant differences were always observed in the different locations during the March and December samplings.

2) PP2 gene downregulation indirectly suggests an active protection mechanism in the phloem of \`Parson Brown'. Targeted RNA seq and metabolomics of the phloem would confirm these results and provide more insight into the exact mechanism of HLB tolerance.

3) All trees (\`Parson Brown' and \`Hamlin') evaluated in this study were HLB positive with similar CLas titer. \`Parson Brown' trees maintained a thicker canopy and greater fruit retention than comparable \`Hamlin' trees.

4) % Oil content ranged from 0.007 to 0.0011 in \`Hamlin' juice whereas it ranged from 0.020 to 0.042 in \`Parson Brown' juice, depending on the location from which the fruit was harvested. This could be due to clonal differences between trees grown in different locations.

5) Lbs. Solids Per Box were comparable between `Parson Brown' and `Hamlin.' However, `Hamlin' has produced good Lbs. Solids Per Box in 2021, and samples will need be evaluated for multiple seasons to understand whether there are differences between the two early season sweet oranges.

6) Limonin levels were less than 5 ppm in both `Parson Brown' and `Hamlin' and within acceptable levels.

7) It was not possible to identify specific clones based on the data generated. Whole genome sequencing can shed insights into clonal differences by looking at the overall pattern of SNPs and INDELS among the specific selections.

8) `Parson Brown' trees at the Premier Citrus location in Lorida had the best growth, canopy coverage, followed by trees at the groves managed by Pat and Marty McKenna and the Premier Citrus location in Ft. Pierce. There were no significant differences in the oil content from fruit from these locations. It may be worthwhile to reintroduce a couple of these superior lines into the DPI's parent tree program for the benefit of the citrus stakeholders.

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

D. Manker 16-026C "Establishment and application of tools to allow a systematic approach to identify and characterize hits with confirmed in planta HLB activity" – (June 2022)

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

The first three years of the project were funded by CRDF, as well as additional funding during a two month gap until USDA NIFA approved the project for which funding began on September 1, 2020 and has confirmed continued funding has been approved through August 2023.

The objectives of the grant are:

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

An 82-slide power point presentation from the NIFA June 2022 Stakeholder meeting can be accessed at the following link:

https://app.box.com/s/uaju7a7s57fh6cxitp3g9b37v4ku4ize

F. Gmitter 18-010 "Upgrading Citrus Genome Sequence Resources: Providing the Most Complete Tools Necessary for Genome Editing Strategies to Create HLB Resistant Cultivars" – 70% Completion of Objectives (April 2022 quarterly)

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

Original plans for transcriptome sequencing, necessary to annotate the genome assembles produced, were to rely on Illumina short reads; but it was decided to also include long PacBio Sequel II3 reads to capture full length transcripts. The first transcriptome data for two target genomes, were found to be inadequate. So, a new vendor for this service was identified. New samples have been collected for RNA preparation.

the first two genomes for which PacBio long read assemblies coupled with Hi-C sequencing using Hi-Rise software were focused on for the best quality chromosome scale assemblies. For these two citrus types, there was access to a collection of re-sequenced genomes of related mutants and closely related accessions which is enabling exploration of additional potential HLB tolerant or resistant rootstocks. Because these assemblies have much improved contiguity (i.e., completeness), the MITE sequence diversity (MITEs are a type of mobile DNA that inserts into different locations and contributes to genetic and phenotypic diversity) have enabled better characterization of the locus that controls nucellar embryony, a very important and widespread trait in commercial citrus cultivars. And, as these two genomes contain contributions from several biological citrus species, species specific genes can be investigated.

The new Hi-C assemblies have now been completed by Dovetail Genomics and have been transferred. Next, the long process of analyzing the assembly output can begin for detection of possible Type 2 errors, attempting to anchor several still unanchored sequence contigs, etc. to polish and produce the most complete and accurate assemblies.

The current inventory of sequence resources is being assessed to identify additional needs to successfully complete all 9 genome sequence assemblies to a chromosome scale and with the greatest accuracy and contiguity technically possible. Sufficient PacBio long read coverage is still lacking for Ruby Red grapefruit and Shekwasha. New HMW DNA preparations have been made and are pending sequencing. The collection of RNA transcripts needs to be expanded, both Illumina short reads and PacBio long reads, and with broader collections of tissue types to maximize the number of expressed genes for annotation of the assemblies. Samples have been collected of tender flush, mature leaves, flowers, young and nearly mature fruit, bark, and for some accessions leaf tissue with and without symptoms of CLas infection and citrus canker. RNA processing and sequencing of the samples are pending.

Seven of the nine genomes have been assembled using both the PacBio and Hi-C sequencing and assembly using Hi-Rise. Quality control and assessment is underway, to properly phase chromosomes, to identify and anchor unanchored sequence contigs, to find haplotype swaps via 2 different approaches, to define centromeres, and to polish telomeres and resolve highly repetitive sequences in these regions. Phasing of the LB8-9 Sugar Belle® mandarin hybrid genome has been a challenge because of several runs of homozygosity (ROH) throughout the genome. This issue is being addressed by using sequence information already available from Dancy tangerine and Duncan grapefruit but using Minneola (the pollen parent) would be better so plans are in place to re-sequence Sugar Belle®. Comparing the Dovetail assemblies, the highest quality was found to come from Carrizo citrange, not surprising given that the parents represent the greatest possible genetic diversity.

E. Rogers 18-019 "Phloem specific responses to CLas for the identification of novel HLB Resistance Genes" – 75% Completion of Objectives (May 2022 quarterly)

The project is examining phloem gene expression changes in response to CLas infection in HLB-susceptible sweet orange and HLB-resistant Poncirus and Carrizo (a sweet orange - Poncirus cross). A recently developed methodology for woody crops that allows gene expression profiling of phloem tissues is being used. The method leverages a translating ribosome affinity purification strategy (called TRAP) to isolate and characterize translating mRNAs from phloem specific tissues. The approach is unlike other gene expression profiling methods in that it only samples gene transcripts that are actively being transcribed into proteins and is thus a better representation of active cellular processes than total cellular mRNA. Sweet orange, and HLB-resistant Poncirus and Carrizo (sweet orange x Poncirus) will be transformed to express the tagged ribosomal proteins under the control of characterized phloem-specific promoters; tagged ribosomal proteins under control of the nearly ubiquitous CaMV 35S promoter will be used as a control. Transgenic plants will be exposed to CLas+ or Clas- ACP and leaves sampled 30, 60, 90, and 120 days later. Ribosome-associated mRNA will be sequenced and analyzed to identify differentially regulated genes at each time point and between each citrus cultivar. Comparisons of susceptible and resistant phloem cell responses to Clas will identify those genes that are differentially regulated during these host responses. Identified genes will represent unique phloem specific targets for CRISPR knockout or overexpression, permitting the generation of HLB-resistant variants of major citrus cultivars.

During this most recent quarter, the 2nd quarter of the 1st 6 month no cost extension (the original end date was 11/30/2021; one 6 month and one 3 month no cost extension was granted; the current end date is 08/31/2022), the Stover lab sent the last of the transgenic lines needed for the project to the Rogers lab. Now at least 4 high expressing lines for each of the 9 promoter/genotype combinations are in the containment greenhouse facility at Ft. Detrick and are being prepared for no-choice psyllid inoculation experiments.

The Rogers lab has continued no-choice psyllid inoculation experiments on the rooted cuttings available and ribosome pull-downs from the tissue collected. Work has progressed more quickly since the ARS 25% occupancy cap was lifted on Monday, March 28th. A qualified and interested post-doc candidate has not been identified to hire with less than 3 month left on the grant. As many translatome RNA samples as possible will be sent for sequencing in July, leaving the month of August for data analysis.

N. Wang 18-025 "Optimization of the CRISPR technology for citrus genome editing" – 98% Completion of Objectives (April 2022 quarterly)

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

Objective 1. Expanding the toolbox of citrus genome editing.

In this study, StCas9, NmCas9, AsCpf1 (from Acidaminococcus), FnCpf1 (from Francisella novicida) and LbCpf1 (from Lachnospiraceae) were adapted for genome modification of citrus.

CRISPR-LbCas12a (LbCpf1), which is derived from Lachnospiraceae bacterium ND2006, was employed to edit a citrus genome for the first time. the study showed that CRISPR-LbCas12a can readily be used as a powerful tool for citrus genome editing. One manuscript entitled CRISPR-LbCas12a-mediated modification of citrus has been published on Plant Biotechnol J. LbCas12a-crRNA-mediated genome editing has been further optimized to make homologous biallelic mutations.

AsCpf1 and FnCpf1 were tested for their application in citrus genome editing and generating homologous biallelic mutations. Both homozygous and biallelic mutations were generated in the EBE region of LOB1 gene in pumelo. This work has been submitted for publication.

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

Objective 2. Optimization of the CRISPR-Cas mediated genome editing of citrus.

In this study, different promoters were tested including INCURVATA2 promoter, the cell division-specific YAO promoter, and the germ-line-specific SPOROCYTELESS promoter, and ubiquitin promoter for driving the expression of Cas9 and Cpf1 orthologs.

To optimize the expression of sgRNA and crRNA, multiple citrus U6 promoters and two citrus U6 promoters showed higher efficacy in driving gene expression in citrus than 35S promoter and Arabidopsis U6 promoter. The mutation efficacy was increased to 50%.

The CRISPR/Cas9 system was further optimized. Currently, the biallelic mutation rate reaches 89% for Carrizo citrange and 79% for Hamlin sweet orange.

One homozygous line in the promoter region of canker susceptibility genes was generated in Hamlin. One biallelic mutant of grapefruit that is canker resistant has been generated. multiple biallelic and homozygous mutant lines of sweet orange were generated that are canker resistant.

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 was conducted in citrus protoplast. Citrus genome editing using Cpf1/crRNA plasmids and ribonucleoprotein complex was also conducted in citrus protoplast. The plasmid-transformed protoplast has 1.7% editing efficiency, and the RNP-transformed protoplast has approximately 3.4% efficiency. Regeneration of non-transgenic genome-edited plants has been achieved. One patent has been filed on the CRISPR-Cas mediated genome editing of citrus.

Citrus protoplast isolation and manipulation has been optimized to more than 98% viability of the isolated protoplasts. A transfection efficiency of approximately 66% or above is routinely obtained. ErCas12a has been used for non-transgenic gene editing of embryogenic Hamlin sweet orange protoplast cells. Genome editing of the critical genes in HLB disease development has been conducted using the optimized method.

Adenine base editors (ABE) have been adapted to modify the TATA box in the promoter region of the canker susceptibility gene LOB1 from TATA to CACA in grapefruit and Hamlin sweet orange. Inoculation of the TATA-edited plants with the canker pathogen Xanthomonas citri subsp. citri (Xcc) demonstrated that the TATA-edited plants were resistant to Xcc. In addition, cytosine base editors (CBE) were used to edit the acetolactate synthase (ALS) gene of Carrizo citrange. Editing the ALS genes conferred resistance of Carrizo to the herbicide chlorsulfuron. Two ALS-edited Carrizo plants did not show green florescence although the starting construct for transformation contains a GFP expression cassette. PCR amplification for Cas9 gene in the mutant plants was performed to confirm Cas9 gene was undetectable in the herbicide resistant citrus plants. This indicates that the ALS edited plants are transgene-free, representing the first transgene-free gene-edited citrus using the CRISPR technology. In summary, the base editors have been successfully adapted for precise citrus gene editing. The CBE base editor has been used to generate transgene-free citrus via transient expression.

M. Leslie 20-015C "Vismax™: A novel peptide-based therapeutic for mitigation of citrus diseases, including HLB" – 33% Completion of Objectives (May 2022 quarterly)

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

During this quarter, a fourth canker test has been done, this time testing different surfactants. No data was presented.

Plans for phytophthora tests are planned for the second year.

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H. Jin 21-003 "Test of SAMP efficacy in the field and greenhouse on important citrus varieties in Florida" – 30% Completion of Objectives (June 2022 quarterly)

1. Field test #1 of small trees is underway by Megan Dewdney. No results of the test are provided.

2. Field test #2 of 4 year old trees is being planned by Megan Dewdney.

3. Greenhouse tests have been changed from Greg McCullum, USDA Fort Pierce, to Kris Godfrey at UC Davis. There is no explanation for this change. Experiments apparently are under way and no results are reported for further greenhouse tests.

4. Funds ($200,000) were totally to purchase more finger lime peptide for these tests. Costs for doing the experiments are covered by a USDA grant.

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" – 100% Completion of Objectives (October 2021 final report)

The goal is to understand how citrus interacts with Candidatus Liberibacter asiaticus (Las) infection and develop improved and long term HLB management strategies.
Objective 1. Identification of the receptors for Las PAMPs in susceptible and tolerant citrus varieties
Potential PAMPs from Las (either homologous to known PAMPs or pilin genes) LasFlaA (flagellin), LasEF-Tu, LasCSP (cold shock protein), LasSSBP (single strand binding protein) and pilin assembly genes were cloned under 35S promoter and the Arabidopsis phloem specific promoter SUC2 and introduced into Agrobacterium. Multiple receptors for the aforementioned PAMPs were identified in tobacco and citrus.
Las outer membrane proteins were hypothesized to directly induce plant immune response in the phloem sieve elements because Las lives in the phloem. 21 outer membrane proteins have been cloned and the putative targets in citrus were identified using Yeast 2 hybrid (Y2H) system. Two outer membrane proteins showed positive interactions with citrus proteins based on Y2H assays which were confirmed using GST pull-down assays.
In addition, multiple PAMPs have been tested for their effects in inducing plant defense against Las in the greenhouse and at least four different PAMPs showed significant effect in manipulating plant immunity.
RNA-seq analyses were conducted on HLB-susceptible Valencia sweet orange and HLB-tolerant Sugar Belle mandarin in winter, spring, summer and fall. Significant variations in differentially expressed genes (DEGs) related to HLB were observed among the four seasons. For both varieties, spring had the highest number of DEGs. CLas infection stimulates the expression of immune-related genes such as NBS-LRR, RLK, RLCK, CDPK, MAPK pathway, ROS, and PR genes in both varieties. The immune responses of both varieties to CLas result in oxidative stress with induced expression of RBOH genes and suppressed expression of many genes encoding antioxidant enzymes, such as superoxide dismutase, ascorbate peroxidase, catalase, thioredoxins and glutaredoxins. HLB-positive Sugar Belle trees contained higher concentrations of maltose and sucrose, which are known to scavenge ROS. In addition, Sugar Belle showed higher expression of genes involved in phloem

regeneration that might contribute to its tolerance to HLB. This study sheds light on the pathogenicity mechanism of the HLB pathosystem and the tolerance mechanism against HLB, providing valuable insights into HLB management.
The control effects of different PAMPs against HLB were evaluated. Three PAMPs showed strong activity in inducing plant defenses.
The flagellar genes of Las and other Rhizobiaceae were analyzed, and two characteristics are unique to the flagellar proteins of Las: (i) a shorter primary structure of the rod capping protein FlgJ than other Rhizobiaceae bacteria and (ii) Las contains only one flagellin-encoding gene flaA (CLIBASIA_02090), whereas other Rhizobiaceae species carry at least three flagellin-encoding genes. Only flgJAtu but not flgJLas restored the swimming motility of Agrobacterium tumefaciens flgJ mutant. Pull-down assays demonstrated that FlgJLas interacts with FlgB but not with FliE. Ectopic expression of flaALas in A. tumefaciens mutants restored the swimming motility of ΔflaA mutant and ΔflaAD mutant, but not that of the null mutant ΔflaABCD. No flagellum was observed for Las in citrus and dodder. The expression of flagellar genes was higher in psyllids than in planta. In addition, western blotting using flagellin-specific antibody indicates that Las expresses flagellin protein in psyllids, but not in planta. The flagellar features of Las in planta suggest that Las movement in the phloem is not mediated by flagella. Movement of Las was also characterized after psyllid transmission into young flush. The data support a model that Las remains in young flush after psyllid transmission and before the flush matures. The delayed movement of Las out of young flush after psyllid transmission provides opportunities for targeted treatment of young flush for HLB control.
The type IVc tight adherence (Tad) pilus locus is a putative PAMP encoded by Las. The Tad loci are conserved among members of Rhizobiaceae, including 'Ca. L. asiaticus' and Agrobacterium spp. Ectopic expression of the 'Ca. L. asiaticus' cpaF gene, an ATPase essential for the biogenesis and secretion of the Tad pilus, restored the adherence phenotype in cpaF mutant of A. tumefaciens, indicating CpaF of 'Ca. L. asiaticus' was functional and critical for bacterial adherence mediated by Tad pilus. Quantitative reverse transcription PCR (qRT-PCR) analysis revealed that 'Ca. L. asiaticus' Tad pilus-encoding genes and 'Ca. L. asiaticus' pilin gene flp3 were upregulated in psyllids compared with in planta. A bacterial one-hybrid assay showed that 'Ca. L. asiaticus' VisN and VisR, members of the LuxR transcriptional factor family, were bound to the flp3 promoter. VisNR regulates flp3. Negative regulation of the flp3 promoter by both VisN and VisR was demonstrated using a shuttle strategy, with analysis of the phenotypes and immunoblotting together with quantification of the expression of the flp3 promoter fused to the β-galactosidase reporter gene. Comparative expression analysis confirmed that 'Ca. L. asiaticus' visNR was less expressed in the psyllid than in the plant host. Further, motility and biofilm phenotypes of the visNR mutant of A. tumefaciens were fully complemented by expressing 'Ca. L. asiaticus' visNR together. The physical interaction between VisN and VisR was confirmed by pull-down and stability assays. The interaction of the flp3 promoter with VisR was verified by electrophoretic mobility shift assay. Taken together, the results revealed the contribution of the Tad pilus apparatus in the colonization of the insect vector by 'Ca. L. asiaticus' and sheds light on the involvement of VisNR in regulation of the Tad locus.

Objective 2. Generate transgenic/cisgenic citrus expressing PAMP receptors recognizing Las
Putative receptors or targets of Las PAMPs (identified in Poncirus) were transgenically expressed in Valencia sweet orange or Duncan grapefruit. They are driven by 35S promoter and phloem specific promoter AtSuc2.

6 constructs to express PAMP receptors were made individually or in combinations. Three overexpression lines were generated.

Objective 3. Investigate the roles of effectors in HLB disease development
30 putative Las effectors were screened and 4 of them repressed plant defense.
Y2H assays for the four defense-suppressing effectors were completed and their targets identified in Valencia sweet orange. Meanwhile, CTV-mediated gene silencing of 15 putative HLB susceptibility genes was conducted in collaboration with Dawson lab. Sweet orange plants carrying the CTV constructs were inoculated with Las via grafting. Interestingly, gene silencing of one of the putative HLB susceptible genes led to significant HLB tolerance. The plants showed mild HLB symptoms, similar growth as non-inoculated plants whereas the growth of inoculated control plants was significantly reduced and plants showed severe HLB symptoms. In addition, the HLB S gene was overexpressed in Valencia sweet orange to further understand the mechanism and plants will be inoculated with Las once they are one year old.

V. Orbovic 18-066C "Support role of the Citrus Core Transformation Facility remains crucial for research" – 95% Completion of Objectives (December 2021 final report)

The objective of this project was to support operation of Citrus Transformation Facility (CTF) that provides service for production of transgenic citrus plants to researchers working towards solution against huanglongbing (HLB) and citrus canker. The CTF has been a part of the multipronged approach to fight citrus diseases by producing genetically transformed citrus plants that were used to gain important knowledge about possible introduction of tolerance/resistance traits. This knowledge represented a foundation for understanding of effects that introduced gene(s) could have on the growth of transgenic plants and on their ability to sustain and survive diseases. Within the scientific community working in this field there are laboratories without transformation capabilities, and for those research groups CTF was the place where projects were supported through of the production of transgenic plants.

Major accomplishments:
The major accomplishment of this project is that it facilitated the existence of the CTF throughout the last three years. The CTF has undergone the transition into the Educational Business Activity (EBA) unit within the University of Florida that overhauled the way it provides service and operates as business. This transition together with the wide-ranging effects of COVID-19 pandemic have negatively affected operation of the CTF. CRDF funding together with the funds provided by the University of Florida have kept the CTF open.

Within the last three years, the CTF produced 388 transgenic plants (Table 1). Number of plants produced in 2019 was 246, in 2020 was 108, and in 2021 was 36. Without exception, all the transgenic plants were created under the research directed towards finding a solution to HLB and citrus canker diseases. Some transgenic plants including selected Duncan grapefruits and all pummelos exhibited increased resistance to citrus canker. The other plants produced have the potential to be either tolerant or resistant to HLB, or in the case of Indian curry leaf plants (Murraya koeinigii), they produce chemicals that can kill Asian Citrus psyllids. The CTF also produced transgenic Mexican lime, Valencia sweet orange, Kumquats, Pineapple sweet oranges, and Indian curry leaf plants. All of these plants stayed in the state of Florida and were used in tests to determine the efficacy of introduced transgenes on desired traits.

Table 1. Transgenic plants produced by CTF from 2019 to 2021

Cultivar	Number of plants produced
Duncan grapefruit	257
Mexican lime	32
Valencia sweet orange	51
Pummelo	22
Kumquat	4
Pineapple sweet orange	10
M. koenigii	12
Total	388

The number of orders placed at the CTF was 57. In 2019, there were 25 orders, in 2020 there were seven, and in 2021 there were 25 orders. The work done on these orders was performed within 266 experiments that included 358 co-incubations of seedling explants with appropriate bacterial strains. Altogether, 314,000 explants were processed in these experiments. Approximately, 170,000 explants were inspected under the microscope equipped with the blue light source for the presence of GFP-fluorescing shoots and buds. Close to 3,000 PCR reactions were done in search of shoots either carrying or expressing a transgene of interest. Also, 1,750 GUS histochemical assays were performed to confirm the transgenic nature of shoot and plants. In average, the transformation rate was about 1% and that number included both chimeric and fully transformed shoots. The percentage of shoots that were successfully grafted was low in the range of 50%.

With the help of staff from CREC's Business office, during the year 2021 the CTF was able to organize the system of payments for the work performed by the CTF as an EBA and get first payments processed.

J. Zale 18-067C "Continued Funding for the Mature Citrus Facility to Produce Disease Tolerant, Transgenic Citrus" – 100% Completion of Objectives (December 2021 final report)

The objectives of this project were to produce disease resistant, commercially and agronomically acceptable, mature citrus transgenics, intragenics, and GMO/non-GMO edited trees using Agrobacterium as a service for research and commercialization. The research focus of this project was to improve transformation efficiencies, so that the mature citrus protocols become more productive, decrease prices for scientists, and contribute more to financial self-sufficiency of the Mature Transformation Facility. A biolistic transformation protocol was also developed to overexpress all-citrus intragenic sequences using a citrus gene for selection, which is useful for food crops. A final comprehensive report will be submitted soon to CRDF.

The efficiency of the mature citrus Agrobacterium transformation protocol was increased significantly, primarily due to the introduction of new cultivars developed by the Plant Improvement Team and determining which ones were not recalcitrant to Agrobacterium. The mature citrus Agrobacterium protocol was originally developed for Pineapple sweet orange, but Pineapple is not commercially important to the Florida juice industry. Instead, we have found several new sweet orange cultivars that are readily transformed with Agrobacterium.

Currently the focus is on producing non-GMO edited mature citrus and have seen some encouraging results. In addition, production continues for all-citrus intragenics using biolistic and Agrobacterium transformation. The plants produced using these technologies might not be as strictly regulated compared to plants produced by transformation with genes from foreign organisms. The USDA APHIS, EPA and FDA will work with each scientist on a case-by-case basis to deregulate superior intragenic trees. Intragenic trees can likely be provided to the growers relatively rapidly. During the last quarter, ~82 transgenics were produced using Agrobacterium transformation of mature rootstocks and ~87 Cas9 transgenic edited scions were produced for another scientist. These are two potential projects involving scientists from other states and countries.

A manuscript was published describing a protocol for a new liquid selection system in mature rootstock, which showed significantly higher Agrobacterium transformation efficiency in liquid than on solid medium. In addition, Agrobacterium transformation efficiency also increased in mature scions using liquid selection and another manuscript is in preparation. Currently, a manuscript is in review reporting a the new citrus, intragenic selectable marker.

It is the responsibility of the scientists to field test their trees and at least two to three projects were being field tested in USDA Ft. Pierce Picos Farm during this last granting cycle.

The Mature Transformation Facility transitioned to an Educational Business Account for earning salaries and operating funds, however it is difficult to earn enough money for salaries by selling plants. Prices are being increased again in 2022 when funding is no longer received from CRDF funding.

d. Horticultural Practices

E. Triplett 18-024 "Foliar phosphate fertilization: a simple, inexpensive, and unregulated approach to control HLB" – 100% Completion of Objectives (February 2022 final)

The final data analyses were done for this paper during this quarter. This included the qPCR of CLas titer in all trees for the last two samplings of the project. Analysis of CLas titer after the six-month mark did not show a decline. Yields and canopy cover also did not improve. CLas titer was determined in two labs using two different primer sets. This was done to give us more confidence in the results. Both labs gave essentially the same results.

During this period a manuscript describing this work is nearly completed. The current draft is attached along with this report. The sense of this work is that this approach still has promise. Greenhouse results were very encouraging. Foliar applied phosphate did reduce citrate levels in phloem. CLas titers did decline in graft-infected citrus saplings in the greenhouse upon foliar phosphate application. Citrus was also found to get all its phosphate needs through foliar fertilization.

However, the field experiments were harder. We were not able to identify plots with low infection rates at the beginning of the work. The heavily infected plants may have been too weak to have the full benefits of foliar phosphate fertilization. In the first 3-6 months of the field experiments, declines in CLas titer were observed with the lowest level of foliar phosphate application but the plants did not recover in subsequent quarters.

More work on this would require larger groves with more experimentation on the frequency of foliar phosphate application. We choose application every two months and used that frequency throughout. More frequent application at lower doses may improve the efficacy of foliar phosphate treatments. This treatment is also more likely to work on younger trees with less infection. Younger trees were used at the Immokalee site but those trees were highly infected. The trees in Hamilton were more than 25 years old.

I remain confident that nutritional approaches can help this problem. It will just require the correct nutrients applied at the right time. The upcoming manuscript is entitled "A systems biology approach suggests a simple strategy to alleviate citrus greening disease" in the APS Journal, Plant Health Reports, is expected by May 2022.

Budget status: There was no overspend on this project.

F. Alferez 18-032C "Preventing young trees from psyllids and infection with CLas through use of protective netting" – 100% Completion of Objectives (November 2021 final report)

Objective 1. Assessing tree growth and absence of psyllids and HLB disease symptoms (including CLas bacteria titer) under protective covering (i.e., IPC). The most striking finding in this objective has been the finding that fruit retention was significantly improved in trees that were covered by IPCs, resulting in larger yields and improved internal quality. As a reminder, covers were removed in August 2020. These trees bloomed and set fruit for the first time in February 2020; therefore, this represented the first crop. Fruit drop was non-existent in trees that had been protected by the IPCs, whereas non-covered trees showed 60% fruit drop. Fruit quality was also dramatically improved by use of IPCs: Brix was 10.9 in IPC trees whereas in non-covered trees Brix was 7.5. Even though the project has ended, we are going to harvest the second crop in about one month to see if this effect is maintained during time. So far, no fruit drop was observed in the uncovered trees that were initially covered by IPCs and the size of this fruit is larger than the fruit non-covered from the beginning. Fruit drop is starting in the control non-covered fruit. With regard to the trials with mandarin varieties, SugarBelle trees are showing very good growth and fruit set with no differences in growth between IPC or no-IPC conditions. In contrast, Tango trees are growing significantly larger under the IPCs, as compared to non-covered trees. Finally, Early Pride trees are not performing well under the IPCs. Typical twig dieback in this variety is exacerbated inside the covers, and trees are significantly smaller. Based on these observations, IPCs are not recommended for Early Pride mandarin trees.

Objective 2. Assessment of alternative netting approaches including 'targeted', 'alternated' and 'patterned' setup of IPC in groves for more cost-effective protection. Lower bacterial levels (higher Ct values) continue to be detected in internal rows of non-covered trees planted in an alternate pattern, which suggests that internal rows in a grove may have some protection if external tree rows are covered by IPCs.

Objective 3. Monitoring the transition from vegetative to reproductive stage in the covered and non-covered trees. In both mandarins and Valencia oranges no significant differences were detected in fruit set under the IPCs as compared to non-covered trees. Fruits inside IPCs were larger.

Objective 4. Comparing IPC with CUPS-like systems. Fruit growth inside the CUPS is being monitored to later compare with IPCs. In the first season of evaluation, fruit quality inside IPCs was comparable to CUPS in terms of yield, internal quality and packout (Paper in preparation).
Budget status: All the budgeted funds for salary were spent.

L. Duncan 18-036C "Cover crops and nematicides: comprehensive nematode IPM across the grove landscape" – 100% Completion of Objectives (May 2022 final)

The effect of six nematicide treatments on tree size and fruit yield was described in the previous report (4/02/2022). Here we describe the effects of 3 annual applications of aldicarb on trees at a second grove (Avon Park site) that was harvested on April 4, 2022. After 3 years of treatment, we found no significant effects of the aldicarb on trunk girth (2943 vs 2971 cm2, untreated vs treated), tree height (4.97 vs 4.96 ft.), or cumulative fruit drop measured on 4 occasions (48.7 vs 45.2 fruit). Aldicarb-treated trees had 16% greater weight of harvested fruit per tree (3.8 vs. 4.4 lbs, untreated vs treated), but the results were not significant (P=0.63; r2=0.0%). The most notable aspect of this 3rd trial year was the virtual absence of fruit production - fewer than 9 boxes per acre. Both groves, this location, and that with the multiple nematicide trial, are noteworthy for their low amount of fruit production. The manager of this grove has agreed to pull some of the trees to determine if nematode damage is evident on roots deep in the soil, which might account for the poor tree performance despite the absence of nematodes in soil samples taken to a depth of 12 inches. This project is operating on a no-cost-extension to allow harvest data to be reported. An overview, final report of the project will be submitted in June. A manuscript summarizing the results of the trial is also being prepared.

D. Kadyampakeni 18-042C "Development of Root Nutrient and Fertilization Guidelines for Huanglongbing (HLB)-Affected Orange and Grapefruit" – 100% Completion of Objectives (October 2021 final report)

The purpose of the project was to develop new guidelines for restoring root health and improving overall tree nutrition for Florida oranges and grapefruits. The objectives of the project were 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.
Summary Description of the Project:
The project was conducted at three sites: Citrus Research and Education Center (CREC), Southern Gardens Citrus near Clewiston, FL and Indian River Research and Education Center (IRREC). Data collection on root, leaf and soil nutrient evaluations, root scanning, canopy size determinations and soil sampling on the central Ridge and southwest Flatwoods along with fertilizer treatment applications were done throughout the project. Mini-rhizotrons were installed at the beginning of the experiment and root images were taken monthly and root density data were measured half-yearly. Fruit yield and juice quality and bacteria titer data were measured yearly. We compared

standard fertilization with elevated (1x and 2x of current recommendations) macronutrients (potassium, magnesium and calcium) along with elevated (1x, 2x and 4x of current recommendations) micronutrient blends (iron, zinc, boron and manganese). At the UF/IFAS IRREC in Fort Pierce, FL the research was conducted on flatwoods soils in a randomized complete block design field study on 'Ruby Red' grapefruit. Micronutrients (B, Fe, Mn and Zn) were applied using three different concentrations (1x, 2x, and 4x current UF/IFAS guidelines) in the form of either dry granular water-soluble fertilizer, controlled-release fertilizer, or liquid fertilizer. A total of 600 trees divided in 40 experimental units were employed. Leaf and root nutrient concentrations, canopy volume and tree height were collected twice a year. Mini-rhizotrons were installed at the beginning of the experiment and root images were taken four times a year.

Major accomplishments of the project:
One key finding in HLB-affected oranges is that it is not beneficial to double macronutrients such as Ca, Mg and K, but rather increasing micronutrients such as Fe, Mn, B and Zn by 1x to 4x because fruit yield, root health and canopy volume were optimized where micronutrients were elevated compared to current guidelines. For HLB-affected grapefruit, the results of this study may show evidence that rehabilitation of HLB-affected trees may be limited by the age of the tree and number of years it has been HLB-affected and should start early at the establishment of a grove. Two graduate students completed graduate degrees in Soil and Water Sciences and Horticultural Sciences. There were 8 grower presentations, and publication of two MS theses, 5 citrus industry articles and 3 refereed journal articles. Next steps Additional 5 articles will be published in refereed journals in the next few months upon completion of data analysis. This will also be followed by extension bulletins based on the current results and follow-up studies to follow on the inconsistent results on yield, canopy size, root growth and root density patterns and bacteria titers. In the next phase of the research, the plan is to use tools learned from growers and other projects such as using real-time artificial intelligence to measure nutrient deficiency, soil amendments and using variable rate fertilization tools, besides fertigation and controlled release fertilizers.

Please state budget status (underspend or overspend, and why):
The project milestones were completed and about 99.5% of the budget spent. We are grateful to the Florida Citrus Growers and CRDF for their financial support.

J. Qureshi 18-052C "Sustainable Management of Asian citrus psyllid (ACP) and Citrus Production"
– 100% Completion of Objectives (December 2021 final report)

For developing tools for sustainable management of Asian citrus psyllid (ACP), four IPM programs i) conventional and organic insecticides plus biological control ii) organic insecticides and HMO plus biological control, iii) conventional insecticides plus biological control, iv) HMO plus biological control and (v) only biological control program were evaluated in a 15-acre block of mature Valencia on Swingle oranges. Objectives were to 1) evaluate effectiveness of IPM programs to reduce ACP populations and resistance to commonly used classes of insecticides. 2) evaluate naturally occurring and commercially available predators and parasitoids in all programs, 3) evaluate Clas titers in trees and yield in all programs and 4) disseminate findings and knowledge to clientele

ACP were sampled biweekly using the tap sampling method. A treatment threshold of 0.1 ACP adults per tap sample was used for insecticidal sprays. The highest number of ACP, an average of 0.12 individuals per sample, was observed in the biological control only program (v), which relied on natural enemies (control). ACP populations reached the treatment threshold 22 times in the control (v), 15 times in the program with HMO plus biological control (iv), 14 times in the program with organic insecticides and HMO plus biological control (ii), 11 times in the program with conventional and organic insecticides plus biological control (i), and 8 times in the program with conventional insecticides plus biological control (iii). There was relatively more suppression of ACP in the programs in which conventional insecticides were used and programs using organic insecticides and HMO required additional applications. Overall, all four programs provided significant reduction in ACP compared with control and there were no differences in the ACP populations among the programs. Colonies of the psyllids established from adults from the different IPM programs were used to test F1 generation adults of these populations against dimethoate, fenpropathrin, and imidacloprid. Independent of IPM programs the susceptibility of ACP adults to dimethoate, fenpropathrin, and imidacloprid was similar.

Among naturally occurring predator's spiders and lacewings were dominant groups found in all programs. Ceraeochrysa cubana, Ceraeochrysa claveri, and Chrysoperla rufilabris were the three species of the lacewings observed in all programs and dominated by C. cubana, 70% of all lacewings collected from any program. These species were tested for their development on ACP and their tolerance to imidacloprid. C. cubana and C. claveri completed development exclusively on the nymphs of ACP in the field. C. cubana appear to be more tolerant to imidacloprid than C. claveri and D. citri.

The commercially available predators Hippodamia convergens, Amblyseius swirskii, Sympherobius barberi, and Rhyzobius lophanthae, were released and tested in all programs against ACP. Rhyzobius lophanthae and S. barberi were tested for their potential to control ACP by testing their consumption rate and different pest densities. The consumption rate averaged 12.70 ± 0.63 nymphs per R. lophanthae and 11.80 ± 0.95 nymphs per S. barberi. Prey consumption by each predator increased with the increasing density of the prey, however, there was no significant difference in the consumption rate between the two highest densities (20 and 40 nymphs per shoots) for either species. We also tested three release rates of both predators. In the field, citrus shoots infested with ACP nymphs were enclosed with 1, 3, or 5 individuals of R. lophanthae or S. barberi in the sleeve cages. The consumption rate increased with the increasing density of the predator averaging 28-64% for the R. lophanthae and 56-84% for the S. barberi. Both predators were recovered multiple times after releases in the programs. Findings on R. lophanthae or S. barberi suggest that both species are good predators of ACP nymphs Density of Amblyseius swirskii predatory mite to be released for the control of ACP was investigated. ACP eggs were used for experiments because in pre-tests eggs were determined as the most preferred prey for this mite. Among the densities tested in laboratories, 1:1 (mite: ACP eggs) was the best, followed by 1:2 and 1:4. A replication of this experiment was conducted in the green house using 1:2, 1:1, and 2:1 densities (mites: ACP eggs), which showed that 2:1 was the best density to use for ACP control. Furthermore, these mites were released in all programs, and most (40% trees containing mites) were recovered in the control which relied on natural enemies and the lowest percentage of trees with A. swirskii (20%) was found in the program with conventional pesticides plus biological control. Success with the recovery of H.

convergens was very limited. Tamarixia radiata were also released in all programs on regular basis. Controlled experiments concluded that parasitism rates were better under light conditions than dark and in the afternoon than morning.

Exclusion experiments conducted in all programs demonstrated the effectiveness of natural enemies in reducing the ACP populations. It seems that the naturally occurring predators as well as releases of the two commercial predators and T. radiata were negatively impacting ACP populations. To avoid direct and residual effects of the insecticides, releases of the beneficial organism's need be timed one week after the sprays. The percentage of HLB positive plants was extremely high in all programs, and the yield as well as the number of dropped fruit were similar among the programs. Findings from these studies were disseminated to stakeholders through seven presentations. Three manuscripts are under preparation for publication in peer reviewed journals and information will also be used in Citrus Industry publications.

J. Qureshi 18-055C "Optimizing Benefits of UV Reflective Mulch in Solid Block Citrus Plantings" – 80% Completion of Objectives (December 2021 final report)

This project evaluated young tree protection from ACP/HLB with approaches such as ground cover using UV reflective mulch, insecticides, and irrigation management at three locations 1) Southwest Florida Research and Education Center (SWFREC), Immokalee, FL, 2) Citrus Research and Education Center (CREC), Lake Alfred, FL, and 3) Florida Research Center for Agricultural Sustainability, Vero Beach, FL. Objectives were to evaluate (1) ACP populations and HLB incidence, (2) plant growth parameters including yield, (3) input costs, and (4) outreach results to clientele.
Project was set up to evaluate two psyllid management programs 1) soil-applied neonicotinoids interspersed with sprays of a different mode of action insecticides on a calendar basis, and 2) rotation of insecticide modes of action sprayed twice on each major flush, implemented to the trees on UV reflective mulch and bare ground. The irrigation deficit treatments to manage flush were also functional at the Immokalee and Lake Alfred locations to trees on UV reflective mulch and bare ground to synchronize flush to target spray applications on major flushes. All the trees were drip-irrigated with two emitters. A separate irrigation treatment using a microsprinkler was also evaluated at the Lake Alfred location.
Significant effects of ground cover on psyllid populations were observed at all three locations. The adult numbers on the plants with mulch compared to those on the bare ground were reduced by 60% in 2020 and 44% in 2021. The overall reduction at the Immokalee location was 56%. An even larger reduction, averaging 81%, was observed at the Lake Alfred location. Such difference was not apparent at the Vero Beach location, but mulched trees had a 65% reduction in the flush infestation rate. The trees with mulch produced 7% to 14% more flush than the trees on the bare ground, thus there were more opportunities for the adults to reproduce on those trees, which may have played a role in reducing the impact on the infestation rate compared to huge reductions observed in the adult numbers at Immokalee location. The flush infestation also varied with the sampling date and was significantly reduced by a large magnitude with mulch on several dates. In September 2020, HLB incidence determined using qPCR was 58% in the trees sampled on the bare ground and 25% in the

trees on mulch at the Immokalee location. The numbers increased to 95% on the bare ground and 77% on mulch in March 2021.
The trees on irrigation-deficit treatments produced less flush compared to those on the full or conventional treatment; this trend persisted on the mulch or bare ground. A significant reduction of 21% to 30% in ACP adults in the flush-timed spray program compared with the calendar-based program was observed at Immokalee. However, only a 13% reduction in the flush infestation was observed. The preliminary findings of reduced adults or flush infestation among locations suggest that the flush-timed spray tactic is sufficient for ACP population reduction. Further modification of the deficit-irrigation treatments will help improve flush management and application of spray treatments.
Soil analysis from the Immokalee location showed higher soil moisture and nutrient concentrations in the mulch plots compared with the bare ground except for magnesium, potassium, and boron. The soil characteristics at CREC indicated similar results that, in general, most of the nutrients showed a higher concentration at mulch plots except potassium which was higher in bare ground plots with conventional drip or microsprinkler. These findings suggest better nutrient distribution within the root zone and minimal leaching threat in trees on mulch. Trees on UV-reflective mulch showed significant increase in the growth indicators at all three locations such as rootstock and scion growth, and tree canopy density and the later increased by 40% at the Lake Alfred location and 30% at the Immokalee location. Similar effects were observed at Vero Beach location.
The start of this project was significantly delayed due to the logistics involved in setting up three large trials in three regions of the state and later negatively impacted by COVID-19 restrictions. Unfortunately, project end date was not extended to compensate for that, therefore, data on the yield and cost comparisons are not available because trees are not bearing fruit yet.
A detailed article describing project progress and accomplishments was published in a trade journal article in `Citrus Industry' in November 2021. Findings were shared with the clientele through several quarterly progress reports and presentations.
A NCE requested on the project was denied.

S. Strauss 18-059C "Citrus row middle management to improve soil and root health" – 100% Completion of Objectives (April 2022 final)

The overall goal of the project was to provide a detailed assessment of cover crop impacts on soil nutrient cycling and microbial communities, weed growth, tree performance, and economic considerations. Treatments in this trial included two different mixtures of cover crops planted in the row middles: legumes + non-legumes (LG + NL) and non-legumes only (NL). These were compared to a grower standard control (GSC) that primarily consisted of weeds. Cover crops were planted twice during the year: June and late October/early November to correspond with the rainy season. Trials were conducted in two different locations in Southwest Florida (labeled North and South groves).

Cover crops were found to have a significant impact on components of soil health for this project. Both cover crop mixtures increased soil organic matter, though this increase was only found at the North grove location. The increase in soil organic matter was linked with increased nitrogen availability in the row middle soils where cover crops were planted and significant changes to the soil microbial community. The mixtures of cover crops planted (LG + NL compared to NL only) also significantly impacted the abundance and types of microbes in the soil, and their roles in soil nitrogen availability. For example, there were more bacteria capable of fixing nitrogen in soils under LG + NL, as well as completely different types of bacteria involved in other aspects of the nitrogen cycle. While these changes in soil organic matter, microbial communities, and nitrogen availability in the row middles did not translate to changes in root or tree growth or production, they do indicate changes to soil health are possible. The trees in both locations of this study were 25+ years old, and therefore more time may be necessary to observe changes in production. Based on a survey of citrus growers about the use of cover crops conducted as part of this project, growers were on average willing to wait 4.4 years for cover crops to benefit citrus production. It is possible that changes in production due improved soil health from cover crops may also be evident more quickly in younger trees.

In addition to changes to soil parameters, cover crops also significantly suppressed weed growth. In particular, the cover crop mixtures in this study substantially suppressed the germination and growth of grasses and sedges. Based on the economic analysis conducted in this project, there is an estimated short-term savings of $75.47 per acre due to not frequently mowing the row middles where cover crops are planted. The overall net cost of adopting cover crops was estimated to be $144.32 per acre, on average.

Based on the results from this project, cover crops can significantly impact components of soil health, reduce costs related to weed suppression, and have the potential to provide greater impacts to soil nutrient availability. There have been nine publications from this project to date, with at least five more in preparation. Publications include both articles for the Citrus Industry magazine and peer-reviewed scientific journals. In addition, PIs on this project have presented results at multiple grower meetings. A grower/extension seminar with presentations of the results of this project by all PIs is scheduled to be held at the UF/IFAS SWFREC on June 23.

T. Vashisth 18-061C "Evaluating sustainability of yield and fruit quality of sweet oranges with use of controlled release fertilizer and micronutrients" – 100% Completion of Objectives (November 2021 final)

This project is an continuation of an objective of existing CRDF funded project (# 00124558 ; ended in March 2019, final report submitted to CRDF) with some added treatments to be evaluated in comparison to control (dry conventional fertilizer with foliar micronutrients). Altogether currently there are 25 treatments of citrus nutrition that are being compared to control.

In this quarter the project has been completed. A comprehensive final report has been submitted. Overall, the 5 years yield and fruit quality results suggest that soil applied micronutrient in form of Tiger-Sul product are beneficial for the trees. Foliar micronutrient should not be used as sole source of any nutrient. The soil-applied program takes time to show its effect and therefore, fertilizer programs should be evaluated with patience over the years. It is to be noted that fertilizer program should be site specific as results of one site cannot be replicated at other site. Therefore, the fertilizer program should be tweaked to address the specific needs of each unique site. Altogether, the best treatments (trts # 4, 6,7,10 in Arcadia) and (trts # 9 in Fort Meade) yielded about a box per tree more fruit than the control - suggesting a substantial increase in profit/acre. In addition total soluble solids were improved with use of Tiger micronutrients. These data support the data and conclusions from field experiments that micronutrients delivered to the roots can improve health and productivity of HLB-affected trees.

L. Rossi (R. Ferrarezi) 20-003 "Nitrogen fertilization of 4 to 7-year-old trees planted in high density" – 100% Completion of Objectives (December 2021 final)

This project was initially awarded to Dr. Ferrarezi at the Indian River Research & Education Center (IRREC) in Ft. Pierce. Following Dr. Ferrarezi departure from UF/IFAS the project was transferred to Dr. Rossi.

The project was early terminated by CRDF at the end of the first year (December 2021) and money were returned to CRDF in January 2022. Since the project was terminated early, only limited data was collected prior to the notice of termination.

The purpose of this project was to assess nitrogen (N) fertilization rates for high-density plantings of different scion and rootstock combinations in four commercial citrus growers.

The overall approach was to: 1) determine the appropriate N rates needed to support tree growth and productivity, 2) compare data obtained with existing UF/IFAS N recommendations, and 3) revise best management practices (BMPs) for N application in young trees planted in high-density plantings affected by Huanglongbing (HLB).

The Ph.D. student and Research Assistant dedicated to the project were hired in Spring 2021.

Grower collaborators participating in the project were contacted in Spring 2021. The 4 growers for the 3-year project were as follows: 1) IMG Citrus (Brian Randolph), 2) Peace River Citrus (Larry Black Jr.), 3) Agromillora Florida/ Lost Lake Groves (Dr. Clay Pedersen), and 4) Graves Brothers (David Howard).

The graduate student and the research assistant scheduled and performed site visits to all locations in Spring 2021 and Summer 2021. The planting densities and variety/scion combinations were chosen, field maps generated, experimental units identified with permanent signs, and the current information regarding N fertilization were requested to growers in Spring 2021.

Dr. Ferrarezi contacted Yara (Bill Easterwood) and received fertilizer donations for the first year (20 tons or 40k lbs). Unfortunately, the delivery was delayed due to COVID-19 logistics finding a local distributor, blending the four different formulations, and delivering in Fort Pierce. Calcium nitrate and ammonium nitrate were procured despite the challenges of sourcing these fertilizers. That was only possible because of the partnership with Yara.

Treatments were applied all locations in Summer 2021 and initial tree size and leaf area index data collection was performed as expected in Summer 2021.

Raw data collected during the first year of the study were generated by the graduate student located at the UF/IFAS IRREC and all data resides with Dr. Rossi.

D. Kadyampakeni (previously E. Johnson) 20-004 "Organic acids compared to conventional acidification for improved nutrient uptake and root physiology" – 50% Completion of Objectives (June 2022 quarterly)

Objective 1: Determine effects of lowered soil pH on CLas populations and root physiology including internal root apoplast and vascular tissue pH. Due to a collapse of the inoculum trees, trees for these experiments are being re-inoculated. Because some trees did not turn out positive for CLas in April 2022 the greenhouse portion of the study will be re-started at the end of July 2022. All protocols are developed and pretesting for the study is finalized.
Objective 2: Field test multiple acidification materials including organic acids for tree response CLas suppression, nutrient uptake, and root and vascular pH changes. In this quarter, soil and leaf tissue samples were collected which show sufficiency in all treatments. Evaluation of root density, and PCR of selected trees is underway. All acids and elemental S were applied in the appropriate treatments and canopy changes and soil trends are being monitored. Pre-treatment fruit harvest and fruit quality evaluations were completed in April 2022.
Acids and elemental S will be applied in the next quarter. The greenhouse study on effects of pH on root physiology and CLas populations will also be conducted and completed in the next quarter.
The budget is on track and the project milestones are being met.

T. Vashisth 20-011 "Right Leaf Sampling-The first and most critical step to good nutrition program" – 65% Completion of Objectives (June 2022 quarterly)

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

In this quarter a lot of the activities were similar to previous quarter. The trees were fertilized for spring based on spring and summer flush nutrient analysis. Another set of nutrient analysis was performed in this quarter. In addition, leaf samples were collected from fruiting and non-fruiting branches for comparison.
The analysis shows that spring flush leaves are deficient in immobile nutrients as compared to random leaves therefore, suggesting that method of sampling can affect the results significantly. In addition, fruiting branches show consistent accumulation of low levels of micronutrients in the leaves whereas the non-fruiting branches decrease in micronutrients from summer to fall. This suggests a higher metabolism of nutrients.

'Tip of the week' was published in June based on the fruiting versus nonfruiting branch nutrient analysis data generated from this project.
Work is anticipated for next quarter:
1. Data analysis and interpretation
2. Collecting samples for nutrient analysis
3. Applying fertilizer treatments based on leaf nutrient analysis results
The budget is being spent as per the plan where major funds have been used for nutrient analyses.
60% completion.
In this quarter the summer flush was tagged on all the trees of both varieties and locations. In addition, the trees were fertilized for summer based on spring and summer flush nutrient analysis. Another set of nutrient analysis were performed in this quarter. In addition, leaf samples were collected from fruiting and non-fruiting branches for comparison.
The preliminary analysis shows that the mild HLB trees are responding to the fertilizer treatments that were based on summer flush nutrient analysis. Mild trees getting additional fertilizer are not losing as much canopy density as the control trees. It is interesting that the response to fertilizer is not the same in mild and severe trees. This observation supports the variability seen in groves with nutritional treatments.
Work is anticipated for next quarter:
1. Data analysis and interpretation
2. Collecting samples for nutrient analysis
3. Applying fertilizer treatments based on leaf nutrient analysis results

The budget is being spent as per the plan where major funds have been used for nutrient analysis.

C. Pederson, Agromillora 21-001 Trees for Phase 3 Rootstock Trials – Contract signed; 50% deposit, balance on delivery of rootstock trees, expected late summer/fall 2022.

Two of the experimental varieties have repeatedly shown a physiological problem where stems become brittle and snap. This suggests that the usefulness of these varieties will be severely limited.

Additional delays have been encountered propagating the Blue 1 and the UFR-05 varieties. Consequently, grower cooperators will the option of staggering the planting of these varieties to include them when the trees are ready.

T. Wood, CRAFT 21-004C "Large Scale Field Trials Cycle III" – 90%

The contract executed between CRAFT and CRDF was effective September, 2021, however work began on Cycle III projects beginning in July 2021 and deliverables executed during that period will be reported herein.
CRAFT PARTICIPATION APPLICATION PROCESS:
The CRAFT Technical Working Group, USDA/ARS and CRAFT staff were all involved with preparing the CRAFT application content for the Cycle III online application experience. The application period was open to Florida citrus growers from July-October, 2021. CRAFT received 84 applications

representing more than 3,300 acres, including 2,470 acres for solid set plantings and 878 acres for reset plantings. The geographic distribution represented 10 counties throughout Florida's citrus producing regions in.

Based upon the contractual requirement stating 50 percent of the funding be allocated to small growers (less than 2,500 acres) and 50 percent be allocated to large growers, applications have been sorted into their respective categories. There was a total of 72 applications within the small grower category accounting for 2,399 acres and 12 applications within the large grower category accounting for 949 acres.

PROJECT SELECTION & DEVELOPMENT:

The Technical Working Group continues to review all projects submitted, working with growers to establish proposed experimental designs, and evaluating the relative value of each project.

Based upon the projects presented, applications have been organized within subgroups of similar projects (for purposes of replication) and assigned a group leader to help develop the project design. The groups are as follows:

1. Rootstock/Scion
2. Soil/Tree Fertility
3. Pest Management
4. Biostimulants
5. Resets

As of June 30, 2022 a total of 48 projects, representing 1,236 solid set acres and 585 reset acres, had been presented to the Board of Directors for inclusion in Cycle III. Staff continues to work on contract development for each of the projects.

In addition to review and execution of contracts, CRAFT staff continued communications and outreach efforts including, articles in multiple industry and general publications; updating of the CRAFT website (craftfdn.org); public meetings of the Technical Working Group and Board of Directors; and more. A full list of communications efforts is available upon request.

4. OTHER CITRUS DISEASES

a. Post-Bloom Fruit Drop

F. Alferez 21-007 "Reducing fruit drop by altering hormonal responses within the tree through nutritional and hormonal therapies: a mechanistic affordable approach" – 12% Completion of Objectives (July 2022 quarterly)

Specific objectives are:

1) To determine the right timing for Zn and K treatments to minimize fruit drop; 2) To determine effects of GA3 and 2,4D applications on fruit retention when applied at different times during fruit development; 3) To develop a strong and proactive outreach program.

Objective 1) The first set of treatments were applied on the first week of June, and the second set of treatments, by the end of July. Every set of treatments is performed in an independent block, so treatments are applied only once, to assess the best timing to achieve the optimum result. We have trees under Zn and Zn+K treatments are observed to have greener canopies. Leaf chlorophyll was analyzed after noticing this clear difference. In August control trees had some off-blooms but they were not observed in the treated trees. Off bloom trees have been tagged to record fruit drop.

Objective 2) GA3 and 2,4D treatments have been applied as planned.

Objective 3) Outreach -Boakye, D, Alferez, F. The interplay between Zn, K, and IAA biosynthesis and signaling during the abscission process on Huanglongbing (HLB)-affected Hamlin trees. ASHS 2022 Annual Conference. July 2022. Chicago. Oral presentation.

2. Work is anticipated for next quarter: In next quarter, treatments for both objectives will be continued and fruit drop will begin to be assessed in Hamlin but not yet in Valencia. Analysis of samples collected to date will begin.

3. Budget status (underspend or overspend, and why): Underspending on student salary and tuition is still happening as the student Divya Aryal did not join UF yet. This will be rectified this month, as she is already accepted in the HOS doctoral program and her visa is finally approved.

J. Curtis 22-006 "CRDF Study on Preharvest Fruit Drop Prevention Using Plant Growth Regulators (PGRs)" – This Project started July 1,2022.

b. Citrus Black Spot

M. Dewdney 18-006 "Understanding the underlying biology of citrus black spot for improved disease management" – 100% Completion of Objectives (April 2022 final)

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, as it will affect management practices. Additionally, the existing asexual population may be more diverse than currently measured. If multiple clonal linages exist, then there may be different sensitivities to fungicides or other phenotypic traits. It also needs to determine whether P. paracitricarpa or P. paracapitalensis are present in Florida for regulatory concerns due to misidentification. The plan is to survey for the MAT-1-1 mating type, unique clonal lineages, and two closely related Phyllosticta spp.

In this project, a two-year skirting and fungicide timing trial was conducted in a grove with a moderate severity of citrus black spot. Skirting was found to have a non-significant effect on CBS incidence and severity. The fungicide timing programs did improve the CBS management but only in the second year when the disease intensity was higher. In conclusion, skirting did not provide additional benefit to a fungicide program and the fungicide program as currently recommended is sufficient for CBS management in Florida. The optimal timing of fungicide programs had not previously been confirmed in Florida and was based on best estimates from the literature from other countries, particularly Brazil.

Azoxystrobin mixed with difenoconazole was confirmed to be a good product combination for CBS management in Florida and South Africa. Similar results were obtained with febuconazole although the activity was inconsistent between seasons. The reason is not easily determined. The mixture of fluopyram and tebuconazole did not perform well in either country. Promising results were obtained with zinc polyoxin-D rotated with pyraclostrobin in Florida but was not able to be trialed in South Africa. Good results were also obtained with the adjuvant Goodspray One with pyraclostrobin rotated with copper hydroxide and thyme oil rotated with copper hydroxide in the 2nd year. The product results with only one year of data will need confirmation in another season. The results with two years of data will be used to make chemical recommendations in the Florida Citrus Production Guide.

The collection P. citricarpa isolates was screened and no MAT1-1 isolates were found, meaning that there appears to be only one mating type in Florida at this time. Also, the new pathogen P. paracitricarpa was not found in the collection. In screening the collection, a species previously not associated with citrus, P. hymenocallidicola, was identified which was not pathogenic on citrus. It appears to be an endophyte on citrus but this was not confirmed. The species is poorly described in the literature, and it was unclear as to whether it was a pathogen on the host it was originally described from Hymenocallis littoralis. P. hymenocallidicola was confirmed to be a pathogen on H. littoralis, causing leaf spots.

Colleagues from Cuba were assisted in the characterization of their P. citricarpa isolates. It was found that their isolates were very similar to those in Florida and only had the MAT1-2 mating type idiomorph. It appears that these populations may be linked, but how, is uncertain. This work was published in 2022. Further work from the South African group showed that whole genome analysis of isolates from Florida could detect isolate differences even among this clonal population. This allows for better differentiation among populations in larger population studies. This work was published in 2021.

Further work on the global populations of CBS confirmed that Florida is a clonal population based on the number of genetic differences and that the Cuban population was indeed very close to the Floridian one. The next closest population is from eSwatini, but it is quite distant. Again China and Australia were identified to have the most population variation. The remaining populations were regions within South Africa, Argentina, Brazil. These populations had less diversity than China and Australia but had more diversity than the North American population. The populations outside of North America and China were interconnected and relatively close to each other. This work shows that more collections of isolates need to be screened, especially from Southeast Asia to better understand how P. citricarpa has been moved globally, likely in plant materials.

c. Citrus Canker

J. Jones 18-013 "Using a Multipronged Approach to Engineer Citrus for Canker Resistance" – 90% Completion of Objectives (November 2021 final)

This project is a continuation of previously funded CRDF grants to TWO BLADES focused on utilizing multiple strategies to produce canker-resistant citrus plants plus the addition of a new strategy using gene editing. The project has focused on transforming Duncan grapefruit with genes that express EFR or a gene construct designated ProBs314EBE:avrGf2 that is activated by citrus canker bacteria virulence factors. The process of testing citrus that has been transformed is underway to modify the bs5 gene to enhance resistance to the citrus canker bacterium.

Objective 1. To determine if Bs3-generated transgenic grapefruit plants are resistant to diverse strains of the citrus canker bacterium in greenhouse experiments and to the citrus canker bacterium in field experiments in Fort Pierce. In late March, 2019, in the field at Fort Pierce in collaboration Dr. Ed Stover, the transgenic material was planted. Citrus canker has developed on plants in the field and the trees were rated for disease in November 20, 2021and there was moderate disease on Duncan grapefruit trees but none on JJ5. The plots were rated again on July 28 (not June as mentioned in May report) 2021 and there were similar trends as in November 2020 although disease was lower given significant defoliation in the plots. JJ5 has also been analyzed for response to strains from Dr. Nian Wang to determine if those strains with unusual characteristics in terms of targeting the susceptibility gene could overcome the JJ5 resistance. Interestingly unique phenotypes were noted in plants inoculated with these strains although they were not typical disease reactions but more of a water-soaked appearance. It was noted that they grew to higher populations in inoculated tissue, although the disease phenotype was very weak and not typical of canker. A 5' Race kit was also used to determine the transcription start in the JJ5 construct and observed that all strains tested activated transcription. As for developing a different transgenic with ProBs314EBE:avrGf2, the construct was placed in a different vector that is acceptable for future transgenic purposes. The previous constructs contain an additional selectable marker that allowed for identifying putative transgenics with a higher success rate. Given that there was concern about the additional marker, the new construct contains only NPT as a selectable marker. A second construct has been created for Vladimir Orbovic given the first attempt was not successful. The construct was transferred to Vladimir Orbovic, who was tasked with creating additional transformants in sweet orange. He identified several putative transgenic trees that were grown and tested for disease reaction. Three of the putative transgenics were shown to elicit a hypersensitive reaction when infiltrated with a bacterial suspension of a *Xanthomonas citri* strain. Currently, the transgenic plants are being maintained in the greenhouse. It is hoped that these transgenic trees will be of use in the near future given that these particular ones do not express GFP. Publication of this work is in process.

Objective 2. To determine if EFR-generated transgenic grapefruit plants are resistant to the citrus canker bacterium in field experiments in Fort Pierce. The two most promising EFR transgenic plants (based on ROS activity) were grafted onto two rootstocks (812 and Sour Orange) and planted in the field at Fort Pierce in collaboration Dr. Ed Stover. They were planted in the field in late March and were recently rated in late July. The trees were rated for disease in November, 2020 and there was considerable disease on all EFR plants with disease being more severe than on susceptible Duncan control. The plots were rated again on July 28 (not June as mentioned in May report) 2021 and there were similar trends as in November 2020 although disease was lower given significant defoliation in the plots. This research project was completed in 2021. Greenhouse experiments were also conducted in which plantswere inoculated by spray inoculation and by pin-prick inoculation. No differences in disease reactions of any of the transgenic trees were observed for different transformation events, when compared to reactions in wild-type Duncan Grapefruit. These results are in the process of being writen up forpublication.

Objective 3. To determine if bs5-generated transgenic Carrizo plants are resistant to X. citri and to generate transgenic grapefruit carrying the pepper bs5. The budwood from UC Berkeley was from two transgenic events and a third was from a tree that was run through the transformation process, but that was negative for the gene. The latter served as the negative control as it had undergone the transformation process. The buds were grafted and several have developed into branches. In initial tests, one of the transgenic trees when inoculated resulted in reduced canker symptoms, but no differences in bacterial populations when compared to the wild-type Carrizo plants. The other transgenic tree reacted similarly as the wild-type Duncan grapefruit in symptom development as well as in bacterial populations. The material was cut back several times to stimulate plant growth, but for unknown reasons it was not possible to get significant regrowth to confirm earlier results and therefore unable to make enough progress prior to the grant being terminated to make substantive assessment as to the responses of the transgenic events.

d. Lebbeck Mealybug

L. Diepenbrock 20-002C "Developing near and long-term management strategies for Lebbeck mealybug (Nipaecoccus viridis) in Florida citrus" – 70% Completion of Objectives (September 2022 quarterly)

The PI and team are working on a number of objectives to develop management strategies for Lebbeck mealybug in commercial groves in Florida.
1. Near term field management strategies:
    (a) Develop methods to time management actions
Working with 6 commercial citrus growers throughout central Florida in developing a robust dataset to describe the seasonal phenology ("life cycle") of Lebbeck mealybug populations.
Findings as of October through December 2021: Sampling continues every other week to describe the phenology of Lebbeck mealybug. Starting in late September/early October, documented an increase in population was detected that was like that seen in the mid-late spring. This is likely due to temperatures becoming more ideal for population growth and suggests that management will need to be done in the late spring/early winter to reduce the population before spring growth starts on trees.
    (b) Expand laboratory insecticide and adjuvant screening.
No activity this quarter- will resume in spring.

(c) Evaluate promising materials in open grove setting

No new data to report this term. Seeking sites for management program comparisons for 2022 pending continuation of funding.

(d) Fire ant management as part of Lebbeck mealybug management

Project has continued as previously reported with management for fire ants, sampling of ants associated with mealybug clusters, and cluster collection to determine impact of management on establishment of predators. While the team is still exploring the data and awaiting development of predators from field-collected ovisacs, a clear impact of fire ant removal has been noted whereby a higher abundance of predators are found in ovisacs and mealybug clusters without fire ants present than cluster where fire ants are actively tending clusters. The same association with any other ant species present in the research site has not been seen, which suggest the importance of fire ant management for control of this pest.

II. Long term management

a. Assessment of predator- what is currently in the system, can they be enhanced, how to implement use of predators alongside insecticide use for ACP and mealybugs

In addition to predators previously found in fields, at least one species of parasitoid has been detected that is promising for management. The detected species has been sent to a taxonomic specialist to get a definitive identification of this predator and determine if it can be establish a laboratory colony to better understand the potential of this predator for control in citrus groves.

b. Determine how to implement mealybug management concurrent with other pest management programs

No new data to report this quarter

c. Determine what insecticide chemistries inhibit feeding

A successful protocol has been developed to analyze Lebbeck mealybug feeding pathways using the electrical penetration graph (EPG). To date, several known feeding interactions have been documented including stylet tip puncture of cell membrane, salivation into the sieve element, and ingestion from phloem. Additionally, a new waveform has been noted that appears consistent with oviposition (egg laying). Thorough documentation and analysis of these steps is key to developing assays to determine what insecticides may (1) inhibit feeding, and (2) halt offspring production. Documentation will continue throughout spring and summer, with continued funding insecticide assays should begin in summer 2022.

d. Develop tools to minimize spread

Data have been submitted for publication, recommendations using isopropanol and steam to treat infested materials will be ready to share in spring 2022.

As this pest is now becoming established in residential plantings, which can serve as a reservoir to reinfest managed fields, the team will be determining parameters for sanitation for solarization and killing via freezing this spring and summer.

Manuscripts Published

Olabiyi, D.O., Duren, E.B., Price, T., Avery, P.B., Hahn, P.G., Stelinski, L.L., and Diepenbrock, L.M. 2021. Suitability of formulated entomopathogenic fungi against hibiscus mealybug, Nipaecoccus viridis (Hemipter: Pseudoccidae), deployed within mesh covers intended to protect citrus from Huanglongbing. Journal of Economic Entomology. DOI: 10.1093/jee/toab243

II. Next quarter:
1a. Test management plan based on pest phenology data
1b &c. Field Program note: postdoctoral researcher previously working on this project left in May for a faculty position and the new hire started in mid-August, thus some objectives are behind schedule.

I. Research progress June 2022-August 2022
1. Near term field management
(a) Develop methods to time management actions.
In the previous report, discussion on developing a relative scouting method for lebbeck mealybug detection was offered. To determine if one could use an element of host attraction to monitor for lebbeck mealybugs, damage was induced to infested trees on peripheral limbs and wrapped these locations in corrugated cardboard. All damage “traps” were paired with undamaged limbs in similar locations on infested trees. After 2 weeks, more juvenile lebbeck mealybugs were counted in the damaged locations than in other traps. It is now suspect that one may be able to use the odors produced by trees in response to damage to attract mealybugs.

To move this concept forward, work on identifying odors associated with various tree parts (mature leaves, flush, flower buds, and various stages of fruit damage) to determine what odors are common to things it is suspected that the mealybugs are attracted to from this work so far: immature/setting fruit and damage. Once the analysis of the odors is completed, work will begin on determining what odor blends may be used as an attractant using lab studies.

(b) Expand laboratory insecticide and adjuvant screening.

No new data to report in this quarter.

(c) Evaluate promising materials in open grove setting.

Spray trials are currently underway to determine optimal insecticides for managing lebbeck mealybug. While early season control is key to protecting young trees, populations can and do establish throughout the year which may require management, in particular in CUPS, IPCs, and fruit intended for fresh market. Currently a range of contact and systemic insecticides are being tested in the CREC research groves. These tests include efficacy testing at 0, 7, 14, 21, and 28 days after treatment to better understand the potential of residual to management population build up. Recent rains have delayed the second round of trials, which are anticipated to start again the week of September 19. These are tests that are not part of routine insecticide evaluations but are important to help growers understand which materials may be better options to incorporate into their programs.

(d) Fire ant management as part of lebbeck mealybug management

No new data this quarter- still working on publishing previously collected data.

(e) Evaluate management options for IPCs

No work done this quarter to report on.

II. Long term management

a. Assessment of predator- what is currently in the system, can they be enhanced, how to implement use of predators alongside insecticide use for ACP and mealybugs.

Data presented in previous quarter has encouraged a classical biological control researcher within UF to start working on the basic research needed to determine if any of the predators identified by the work could be targets for mass rearing and release for management. Their work is currently funded using their UF faculty start up package while they seek funding opportunities.

b. Determine how to implement mealybug management concurrent with other pest management programs

No new data to report this quarter.

c. Determine what insecticide chemistries inhibit feeding.

Feeding interactions between lebbeck mealybug and citrus hosts is currently being documented, with the goal of starting insecticide assays in mid fall 2022. The new postdoc has taken up the data amassed in the past year to decipher the various interactions in the overall feeding process and compare those with other hemipteran pests. Once these behaviors are fully documented, optimal insecticide rates can be determined for killing feeding lebbeck mealybugs, which will reduce population growth and can be used to help develop optimal rates for use in IPCs.

d. Develop tools to minimize spread

No new data to report this quarter.

II. Next quarter:

1b, c. and 2b Test of promising insecticide will be continued using field aging to determine duration of efficacy post-application. Adjuvant + Delegate trial was not completed as planned in the previous quarter in the absence of the postdoc, so that will be completed in the upcoming quarter.

2c. The feeding interaction documentation will be completed and move into insecticidal drench assays to look at impacts of insecticide on the feeding interaction.

2d. Develop protocol for sanitation using solarization and freezing (not completed last quarter as planned).

III. Budget status

A request has been submitted to move funds into the materials budget to cover increased gas/travel costs to the Lake Placid research site and for additional trees to maintain lebbeck mealybug colonies on. The mealybugs destroy plants at a faster rate than anticipated.

e. Phytophthora

M. Dewdney (previously E. Johnson) 19-010 “Determining new cost-benefit guided Phytophthora propagule treatment thresholds for HLB-affected citrus” – 79% Completion of Objectives (September 2022 quarterly)

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

Objective 1) Determine if labelled Phytophthora management maintains efficacy in the field on HLB-affected trees for reducing fibrous root loss and improving yield.
Soil cores from Hamlin and Valencia were taken from the south Florida location in early to mid-August. They were evaluated for root density and phytophthora levels. In the Hamlin block, both P. palmivora and P. nicotianae were detected. In most treatments, there was more P. palmivora than P. nicotianae. The total Phytophthora counts ranged between 7.6 to 42.8 CFU/cm2 of soil. Only two of the treatments, Phosphite alone and Phosphite alternated with Ridomil had levels above the treatment threshold. The root densities were comparable among treatments. In the Valencia block, there was less P. palmivora than P. nicotianae. The overall Phytophthora counts ranged from 2.8 to 37.6 CFU/cm2 of soil with the greatest number of propagules being found in Phosphite alone and Phosphite alternated with Orondis. Fall treatments were applied in mid-September but were complicated and slowed by animal damage to the polytubing that had occurred since the last treatment. Nearly every line had be severed and needed repair.

At the second site in the Wauchula area, the Prophyte treatments were applied in mid-July. Since soil cores had been done in May, it was decided to wait until the fall to take the next round of soil samples. The Pi are working with the grower to get last year's harvest data (yields) since it was not possible to get pre-treatment data by plots.

Objective 2) Determine benefit-cost thresholds for Phytophthora treatment on HLB-affected trees
Waiting for more data.
PI reports a no-cost extension will be applied for.

f. Nematodes

L. Duncan 19-016 "How do subterranean pests and diseases affect root health of trees with and without HLB?" – 80% Completion of Objectives (April 2022 final)

The commercial and potential rootstock seedlings to be screened for tolerance to burrowing nematode were moved from the laboratory to the rebuilt greenhouse in early April and inoculated with Radopholus similis in late May. A new screenhouse was constructed and the Swingle citrumelo and Carrizo rootstock Valencia trees were moved from the laboratory, pruned to initiate new root and shoot growth. They will be inoculated with citrus nematode and graft-inoculated with CLas in the first week of June. A trial comparing the effects of burrowing nematode and CLas on rootstocks susceptible and resistant to burrowing nematode was terminated. Neither pathogen significantly affected the shoot weight of either rootstock; however burrowing nematode increased the root weights ($P=0.001$) and the effects differed for the two rootstocks. On the susceptible Carrizo citrange the nematode increased root weight by 12% (17.2g vs 19.2g) and on the resistant Kuharske Carrizo by 24% (54.1g vs 66.8g). Unlike the adverse effect of CLas on citrus nematode populations (July 2021 report), nearly twice as many burrowing nematodes per gram of root occurred on Carrizo seedlings infected by CLas than on seedlings only infected with burrowing nematode (65.9 vs 34.3), but the effect was not significant. There were no burrowing nematodes recovered from the resistant rootstock Kuharske. The repeat experiment is ongoing.

The project ended on 30 April. Covid-related delays related in the NCE request and the unannounced 3-month closure of the greenhouse have delayed the completion of several experiments that include a repeat of the trials evaluating interactions between CLas and burrowing nematode described above and citrus nematode in which the nematodes increased the virulence of Las in citrus plants, whereas Las-infected plants were less suited to nematode population growth than non-infected plants. These and the trial screening rootstock resistance to the burrowing nematode are ongoing and will be completed and reported in a supplemental report in 2022.

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