CRDF Commercial Product Delivery Sub-Project Progress Report FY 2018-19
Quarter Ending 30 Sept 2018

2. Asian Citrus Psyllid VECTOR INTERVENTION

2A. Asian citrus Psyllid Management

Narrative of Progress against Goals:

Obj. 1- Pursue actions that will support expanded tools for ACP management

C. Vincent's 16-020C project is on "Dyed kaolin to repel Asian citrus psyllid in field conditions." Both foliar insecticide and untreated control treatments were near 75% infected with HLB (up from 50% in June 2018) approximately 1 year after planting. Approximately 30% of undyed kaolin plants were infected (up from 19% in June), and 15% of red-dyed kaolin plants were infected—up from 0% in June. Plants in both white and red kaolin treatments continue to show higher growth rates than the insecticide and control treatments. All colors reduce leaf temperature. Red produces a ~30% reduction in light radiation while the other colors PAR more and reduced photosynthesis. White kaolin increased maximum photosynthesis, while red kaolin was not significantly different from control or white kaolin. Both red and white decreased midday depression. We are now planning an experiment to test whether red is improving water status under water limiting conditions.

L. Stelinski's 17-001C project is on "Insecticide resistance management in Florida citrus production." The objective of this study was to evaluate resistance of Asian citrus psyllid (ACP) to three insecticide modes of action. These were imidacloprid (neonicotinoid), fenpropathrin, binfenthrin (pyrethroids) and dimethoate (organophosphate). ACP were collected from May 22 to August 28, 2018 from 6 different geographical locations treated with different insecticide application schedules in commercial citrus groves in Florida. Biochemical assays were carried out to determine activity of 3 detoxifying enzymes in each sampled population and separately for both male and female psyllids. The results indicate that ACP exhibited low levels of insecticide resistance to imidacloprid for Lake Alfred and Arcadia field populations as compared to the susceptible population. However, there was no or a very low level of insecticide resistance in ACP field populations at this time for dimethoate, bifenthrin and fenpropathrin. We further examined whether certain detoxification enzymes (eg., GST activity) were involved in insecticide resistance. There were no differences between the field populations and laboratory susceptible population for mean GST activity. Our results suggest that bifenthrin and dimethoate can be used effectively in rotation for ACP management under a regular resistance monitoring program.

2b. **RNAi Molecules/Psyllid Shield**

T. Eyrich's 16-016C project is on the "Use of RNAi delivered by the Citrus Tristeza Virus Viral Vector to control the Asian Citrus Psyllid". 1. The second repetition of the experiment was conducted in late August through September 2018 over a period of five weeks. The protocol established was:

a. Selected trees were scouted for flush or pruned to induce flushing. b. Areas of flush were bagged and inoculated with 20 parent ACP. c. Parent ACP were removed from bagged flush after two weeks. d. First generation ACP were counted two weeks after removal of parent ACP. e. The presence of native ACP life stages also documented.

2. In September, all trial trees were sampled and tested using ELISA to detect the presence of CTV and gel electrophoresis to detect the presence and stability of CTVvv-RNAi,
3. All trial trees were also sampled and tested using qPCR for the presence of HLB.
4. Native ACP and lab-reared ACP were sampled and tested using qPCR for the presence of HLB.
5. Aphid scouting continues on a biweekly basis.

The presence of brown aphid has not been detected.

3. CITRUS HOST INTERVENTION

3B. Horticultural Practices and Impact on HLB

Project goal(s) for this project area for the next year:

1. Track ongoing research on horticultural aspects of HLB and tree health
2. Provide communication on project goals, progress and results to CPDC, CRDF and growers

Narrative of Progress against Goals:

The Horticultural Practices projects focus on the potential impacts of management practices on HLB status in existing trees. In the past, these have included thermotherapy, plant growth regulators, soil microbial amendments, nutritional treatments and overall grower practices. Most projects have been completed and reported in previous reports. There are ongoing projects on controlling HLB using soil and tree microbes, enzymatic techniques and tree nutrition as well as a project on evaluating grapefruit.

Microbes. N. Wang 15-042. End date 9-30-18 + extension; Final report. "Control of citrus Huanglongbing using endophytic microbes from survivor trees" This was a greenhouse and field project to understand the role of endophytic microbes from HLB survivor trees. We first investigated the role of endophytic microbes from survivor trees. We observed that HLB impaired the rhizosphere-to-rhizoplane enrichment process of the citrus root-associated microbiome by (1) decreasing the relative abundance of most rhizoplane enriched genera; (2) by reducing the relative abundance and/or expression activity of the functional attributes involved in microbe-plant interactions; and (3) by recruiting more functional features involved in autotrophic life cycle adoption, such as carbon fixation and nitrogen nitrification in the HLB rhizoplane microbiome. Our data showed that inoculation of Burkholderia strains isolated from the healthy citrus root-associated microbiome could trigger the expression of genes involved in induced systemic resistance in inoculated plants. Overall, we have identified multiple beneficial bacteria which promote citrus growth. However, the beneficial bacteria can only slow down the HLB disease progress but cannot reduce Las population in trees. The antimicrobials produced by certain isolates might have control effect against Las. The challenge is how to develop a practical and acceptable tool to use those antimicrobials.

E. Triplett's 16-009 project is on "Developing second generation antimicrobial treatments for citrus greening disease". We are continuing to improve the defined medium (Cruz-Munoz et al. 2018) for the culture of Liberibacter crescens, the closest cultured relative of the citrus greening pathogen. Laboratory tests have shown that low levels of glyphosate (5 mM; Roundup) inhibit L. crescens. Both the L. crescens and CLas genomes possess ESPS synthase, the target of glyphosate. The predicted proteins in both genomes are expected to be sensitive to glyphosate based on amino acid sequence of the codon involved in glyphosate sensitivity. So, in collaboration with Michael Rogers at the CREC, we are now interested in testing whether glyphosate can control citrus greening disease. Even if glyphosate adversely affects citrus, we can

engineer it in a non-transgenic way to make it resistant. A provisional patent on the idea has been filed by UF. Our next step is to get field efficacy data.

Enzymatic techniques. N. Wang's 15-028 project (end 9-30-18, Final) is on "Control citrus Huanglongbing (HLB) by counteracting the SA hydroxylase of Candidatus Liberibacter asiaticus". Trunk injection of SA showed significant control effect against HLB. The data for trunk injection has been collected and a manuscript has been submitted for publication. After four applications over two consecutive growing seasons we found that the combination of compost, urea, and plant defense elicitors β-aminobutyric acid (BABA), plus Ascorbic acid (AA) and potassium phosphite with or without salicylic acid (SA), slowed down the progression of HLB and reduced disease severity by approximately 18%, on mildly-diseased trees compared to the untreated control. This treatment resulted in a higher yield compared to the untreated control. It seems application of SA and analogs and other plant defense inducers can partially overcome Las SA hydroxylase. Thus, the application of SA and analogs and other plant defense inducers is a viable approach to mitigate HLB at an early stage of infection.

N. Wang's 16-005 project is on "GFP labeling of Candidatus Liberibacter asiaticus in vivo and its applications." The goal of this project is to generate green fluorescence protein (GFP) labeled Ca. Liberibacter asiaticus (Las), test its application in study of Las movement and its distribution in planta, and investigate the control effect of different measurements including heat treatment and antimicrobial treatment. We are testing co-culturing Las with citrus tissue culture. We have used two approaches to label L. crescens. Preliminary data showed one approach works for Las in vitro. We are testing whether we can label Las in vivo and observe its movement. We have conducted Las movement and multiplication in planta based on qPCR method. We have tested approaches to prevent Las movement in planta. We have been testing the effect of different control approaches including application of bactericides. The manuscript entitled: "Control of Citrus Huanglongbing via Trunk Injection of Plant Defense Activators and Antibiotics" has been published by Phytopathology. We have developed a method for targeted early detection of Las before HLB symptom development. This manuscript has been submitted to Phytopathology.

Nutrition. J. Grosser's 15-013 project is on "Interactions of Rootstocks and Constant Nutrition to Enhance Profitability of Citrus Plantings in HLB-Endemic Areas". Valencia on WGFT+50-7 rootstock showed significantly reduced Clas populations with Treatment #5 (Harrell's 12-3-9- St. Helena mix). This rootstock is also showing good HLB tolerance in several field plantings and is being considered for commercial release. Evidence is accumulating that nutrition can indeed suppress CLas titers. Higher rates of constantly applied soil micronutrients seem to improve the fruit yields and quality whether as frequent application of conventional fertilizer or adding some amount of CRF or fertigation is supported by data. There are positive results showing a therapeutic affect from overdoses of manganese against HLB.

A. Schumann's 15-023 project on "Citrus nutrition studies for improved survival of HLB-affected trees". End date extended to 12-31-18 to allow data analysis in hurricane damaged field sites. Purpose is to find the reasons for inconsistent responses of HLB-affected citrus to Enhanced Nutrient (EN) programs and to develop feasible and economical remedies that can consistently replicate successful HLB mitigation with ENs in all Florida groves. Objective 1: Leaf nutrient thresholds. Final analyses of three years of leaf nutrient data for Diagnosis and Recommendation Integrated System (DRIS) are underway. This will allow investigation of cross-correlation of

variables and environmental effects. Objective 2: Determine soil conditions that favor root hair and VAM proliferation. Nine Carrizo seedlings have been established in each of 3 hydroponic tanks for use. Tank 1, is a control fertilizer mix, including all nutrients and pH adjusted to approximately 7.0. Tank 2, pH 7.2, has reduced P fertilizer and Triple Calcium Phosphate added as the sole source of phosphate. Tank 3, pH 7.8, has added CaCO3 to increase the pH and to reduce the amount of available phosphorus. All 3 tanks receive an extra dose, 100 ppm, of calcium from calcium chloride. The added calcium and the pH at approximately 7, should drive the chemical reactions in solution towards the removal of available phosphorus, keeping the available phosphorus in the solution at the concentration that favors root hair development, while still providing enough phosphorus to keep the plants nutrition balanced. The Carrizo seedlings from all 3 tanks are healthy, showing no visual signs of nutrient deficiency. Roots from Tanks 1 and 2 have a high density of root hairs while Tank 3 has few root hairs. Those results should be available soon.

Y. Duan's 16-007 project is on "Field evaluation of the selected variants of Ruby Red grapefruit volunteer seedlings for greater HLB resistance/tolerance". Plot Plans were revised and "Ground-Truthed" for all entries for the three grapefruit plantings and field maps will be submitted to both the CRDF Project Manager and Grower Cooperator (Daniel Scott). The USHRL farm crew continues to maintain the 3 grapefruit seedling planted previously on the Picos Farm. Greenhouse materials were inventoried that belong to the Grower Cooperator including the number of rootstocks on which they were budded. As of October 1, 696 of the 750 trees have been budded or grafted onto 3 rootstocks; More rootstock trees have been acquired to complete the budding and grafting operation for the planting in the Scott Grove; A revised budget of Project #16-007 was submitted it to CRDF. Project advisor met with both RMC and CRDF BOD to answer any questions relative to project and to provide a verbal report of progress.

Significant Meetings or Conferences:
A research update presentation on this leaf nutrition work by Schumann was given as part of CRDF's continuing communication seminars series following the July 24th CRDF BOD meeting.

Grosser's yield data was presented at the OJ Break on May 15, 2018 and at SWFREC. Detailed results of years 1 and 2 of this experiment was presented at Citrus Expo Aug 15, 2018 and at the annual ASHS meeting in August in Washington DC; a manuscript is in preparation.

Obstacles Encountered and Breakthroughs:
Other Information:

Service & Support Projects

M. Irey's 17-002C project is for "Continued Support for the Southern Gardens Diagnostic Laboratory". This project is a continuation of funding that has been provided to Southern Gardens Citrus (SGC) to provide growers and researchers with a facility to do testing to detect Candidatus Liberibacter asiaticus. As has been the trend for the last couple of years, almost all of the plant samples appear to come from research trials. Customers are asking more

frequently for copy number determination, and the number of samples coming from individual trials is increasing. These trends seem to indicate that more formal/structured trials (i.e. replicated trials) are being conducted instead of "demo" types of trials. To date through the first quarter of year two funding, a total of 33,395 samples have been processed for the first 5 quarters of the funding. If the trend continues, it is likely that the lab will process less than the 60,000 samples that were budgeted for the funding and cost and billing will be adjusted as necessary based on the final sample volume.

PLANT IMPROVEMENT

3c. Deployment of Disease Resistant or Tolerant Citrus Rootstocks and Scions

Narrative of Progress against Goals:

Obj. 1- Track ongoing research projects evaluating emerging scion and rootstock genotypes for tolerance or resistance to HLB, citrus canker, and other diseases.

a) F. Gmitter 15-010 – Project reports progress towards objectives, however, it continues to be a challenge to track individual projects.
b) D. Hall 15-016 – Publication reporting a 77% average infection rate of germplasm by the psyllid inoculation protocol for resistance /tolerance screening purposes.
c) E. Stover 15-025 – Negotiations with USDA-APHIS Biotechnology Regulatory Services (BRS) have resulted in conditions that permit testing of transgenic plants from several labs alongside other research needs for the USDA Ft Pierce Picos Farm.
d) V. Orbovic 15-033c and J. Zale 15-045c – The transformation laboratories continue to produce transgenic plants for researchers. Various events have putative resistance or tolerance towards HLB and citrus canker. CPDC met and recommended that the labs undergo a review of their operations by a panel composed of CPDC, CRDF staff and external experts in production of transgenic fruit crops.
e) Z. Mou 15-020 – The three independent transgenic lines (NPR1-Ham-13-3, NPR1-Ham-13-29, and NPR1-Dun-57-25) that have shown robust tolerance to HLB have been treated with the CTV vectored early flowering gene to produce mature flowering plants. The plants are being treated to remove CTV and the CLas bacteria. The resulting clean plants will be propagated for field trials. Transgenic lines are tolerant in that they are CLas infected but do not produce symptoms. However, the transgenes are unable to prevent psyllids from being infected by CLas. The three transgenic lines that are highly tolerant to HLB will be planted into the field.
f) Y. Li 16-001 – A vector containing a Cas9 gene and a sgRNA gene has been developed that targets a citrus gene for endogenous chemical resistance. The gene may be useful to reduce wt and mutant chimeras when Agrobacterium-mediated transient expression of the Cas9 and sgRNA are used to produce transgene-free edited plants.

Obj. 2- Cooperate in in-depth evaluation and planning exercises related to Florida (and the US) citrus breeding to better focus on HLB solutions and rapid evaluation and deployment of rootstocks and scions.

On October 22, 2018, Florida citrus stakeholders met to discuss ways the citrus-producing states could work together in the fight against HLB, as well as the idea of creating a "centralized coordinating body to set up and manage the infrastructure needed to support collaboration among citrus scientists/breeders" as recently put forth by the California Citrus Research Board. The recommendation sent to Dr. Tom Bewick, National Program Leader for

NIFA, Division of Plant Systems-Production is to support a Multistate Research Program, an existing program overseen by the USDA Secretary of Agriculture, which leverages the regional State Agricultural Experiment Stations (SAES) to cooperate on technical opportunities and complex problem-solving activities that are beyond the scope of a single SAES.

Obj. 3- Develop and implement plans for expanded management of tolerant and resistant citrus

Obj. 4- Facilitate identification of best performing candidate rootstocks that appear to have HLB tolerance or resistance from Florida (and other) breeding programs

Obj. 5- Implement and evaluate Phase I and II grower field trials of most promising candidate HLB tolerant rootstocks using standard varieties as scions.

Significant Meetings:

Citrus Regulatory Working Group met October 1-2, 2018 in Denver. Catherine Hatcher represented CRDF and Florida in this collaborative group working to develop guidelines for citrus germplasm movement between citrus producing states. The goal is to streamline State and Federal regulations to accelerate research while protecting the Citrus Industry from movement of pests and diseases.

HLB-MAC met Novemeber 28-29, 2018 in Wash DC. Rick Dantzler and Jim Graham represented CRDF in the evaluation of 28 projects with possible shovel ready solutions to HLB were considered. HLB-MAC will update website information and send out notices to stakeholders to announce suggestions for the next round of funding in February.

4. Other Citrus Diseases (CBS, PFD)

CBS. M. Dewdney's 15-005 project is on "Asexual inoculum production of Guignardia citricarpa, the causal agent of citrus black spot". This project is 1) to determine the temperature and relative humidity optima for CBS infection and production pycnidiospores on citrus twigs, leaf litter, and fruit; 2) To determine whether CBS can survive and reproduce on citrus debris on grove equipment. Pycnidia form rapidly at 32C but may be sterile and the greatest number of spores occur around 24C. Data from 20C and 28C treatments remain to be completed. Work on the field sample qPCR has continued. DNA quantities tend to be lower than 10^2 fg in most samples but can increase to 10^3 fg. It is expected that the fg/mg of tissue will become more consistent among samples. We now have samples from Sept 2016 to end of May 2017 and while DNA quantities of P. citricarpa are low, the fungus is always present. In the conidia suspension samples, the DNA quantities on average were higher than the amount of DNA on the twigs on the same date. The amount of DNA tended to have peaks at three-month intervals; data are being analyzed in terms of environmental variables to determine the number of spores and quantity of DNA. Data on spore survival in the presence of citrus debris and disinfectants was organized. Materials and Methods for a manuscript were written and data analysis is proceeding.

PFD. M. Dewdney's 16-010C project is on "Enhancement of Postbloom fruit drop control measures" including fungicide treatments, Luna Sensation to protect early flowers and to determine if the period flowering of trees affected by HLB can be narrowed to eliminate the offseason bloom and PFD inoculum. Fruit data were collected in June and there were no statistical differences among the treatments. This again supports the earlier conclusion that the Brazilian model is more conservative and accurate to not indicate sprays when conditions are unsuitable for PFD development. It is hoped that there will be at least one season of where disease occurs to ensure that the model predicts disease as well as it predicts no disease. A presentation was given on the citrus advisory system (CAS) at Citrus Expo in August on the PGR trials, the trees were re-flagged, data were collected on canopy volume (2018) and the treatments were started with applications for Fall 2018. We met to discuss the function of the CAS and if there are any further improvements that are immediately needed. Much of the discussion centered around leaf wetness measurements used in the model and a small project was initiated to evaluate historical data on how well leaf wetness probes perform compared to models and if models would be sufficient in all situations.

Obj. 2- Engage registrants and regulatory entities in need for label modifications

Obj. 3- Continue participation in pesticide stewardship activities

Obj. 4- Continue to support CHMA implementation of ACP and other HLB management tools

Obj. 5- Communicate progress and results of project to CPDC, CRDF and growers

Significant Meetings or Conferences:
Obstacles Encountered and Breakthroughs:
Other Information: