Citrus Research and Development Foundation

Meeting with EPA – Office of Pesticide Programs

Thursday, February 22, 2018

9:15 am -- 11:30 am

Update on CRDF Research Initiatives on Therapeutic Treatments and Management of Huanglongbing Disease of Citrus

OPP Headquarters

Purpose of the Meeting

To provide the Office of Pesticide Programs an update on ongoing research efforts to provide therapeutic treatments for the causal agent Huanglongbing (HLB) Disease of Citrus. The discussions will include updates on funded research efforts to identify agents with activity against *Candidatus* Liberibacter asiaticus (*C*las); research on application methods to enhance movement of treatment agents into the vascular system of infected citrus trees; research on non-chemical management options including breeding (traditional and GMO), gene editing possibilities and RNAi tools using CTV as a delivery method. The recent joint venture with Bayer CropScience to identify biologicals with activity on the target organism.

CRDF Participants

Dr. Harold Browning, Chief Operating Officer
Dr. Lisa M. Weaver, Senior Project Manager
Dr. Stephanie Slinski, Project Manager – Bactericides
Daniel Botts, TPR, Inc.,
Michael Aerts, TPR, Inc.,

OPP Participants

Rick Keigwin, OPP
Cynthia Giles-Parker, OPP/RD
Hope Johnson, OPP/RD
Fatima Snu, OPP/RD
Tawanda Maigan, OPP/MUERB
Kevin Costello, OPP/PRD
Colwell Cook, OPP/BEAD
Kara Welch, OPP/BEAD
Monisha Kaul, OPP/BEAD

On Phone
Susan Jenning, OPP/
John Cowles, OPP/

Agenda

- **CRDF Overview / Update** — Dr. Browning
    - Approaches short-to long term
    - Cross-sectional view of HLB intervention topics and investment
- **Individual Research Project Updates**
    a. Antibiotics — Dr. Slinski
    b. Zinc bactericides — Dr. Slinski
    c. Other Molecules — Dr. Slinski
    d. Application Technologies
        i. Chemical delivery — Dr. Slinski
        ii. Nanotechnology — Dr. Slinski
        iii. CTV Vector delivery — Dr. Browning
    e. Bayer Joint Venture
        i. Antimicrobial microbes — Dr. Weaver
        ii. Plant Defense Modulators — Dr. Weaver
    f. RNAi — Dr. Weaver
    g. Breeding (GMO) — Dr. Browning
    h. Breeding (Non-GMO) — Dr. Browning
    i. Vector Exclusion — Dr. Browning
- **Q & A / Group Discussions**

Meeting Summary

The meeting followed the agenda as requested by EPA. The PowerPoint presentation used during the meeting is attached. The EPA participants at the meeting were interested in all of the options under investigation by CRDF and exhibited a working knowledge of the challenges and concerns arising from each option. Agency personnel at the meeting stressed the need for alternatives to traditional antibiotics as treatment options. After a brief discussion over the current Section 18 for Oxytetracycline and Streptomycin, the majority of the meeting was devoted to the other research topics described in the agenda. Another very important aspect of the meeting was the clear recognition by the EPA personnel of the difficulty measuring direct efficacy of any targeted treatment option against the pathogen involved. The meeting was very informal.

Among the major points discussed were:

- Efficacy and direct measurements of control of HLB
- Need for application methodologies to enhance introduction of control measures into the phloem stream of infected trees
- Interim measures to allow development of long-term host plant resistance
- Long-term viability of CTV mediated treatment methods

The group expressed interest in continuing the dialogue as the described research projects proceed and results become apparent. There was an implied willingness to work with CRDF and the industry as promising line of management are identified.

Attachments

Appendix 1. PowerPoint Presentation, HLB Intervention Technologies, Update to US EPA, February 22, 2018

Citrus Research and Development Foundation, Inc.

HLB Intervention Technologies

Update to US EPA

February 22, 2018

CRDF Staff and Consultants

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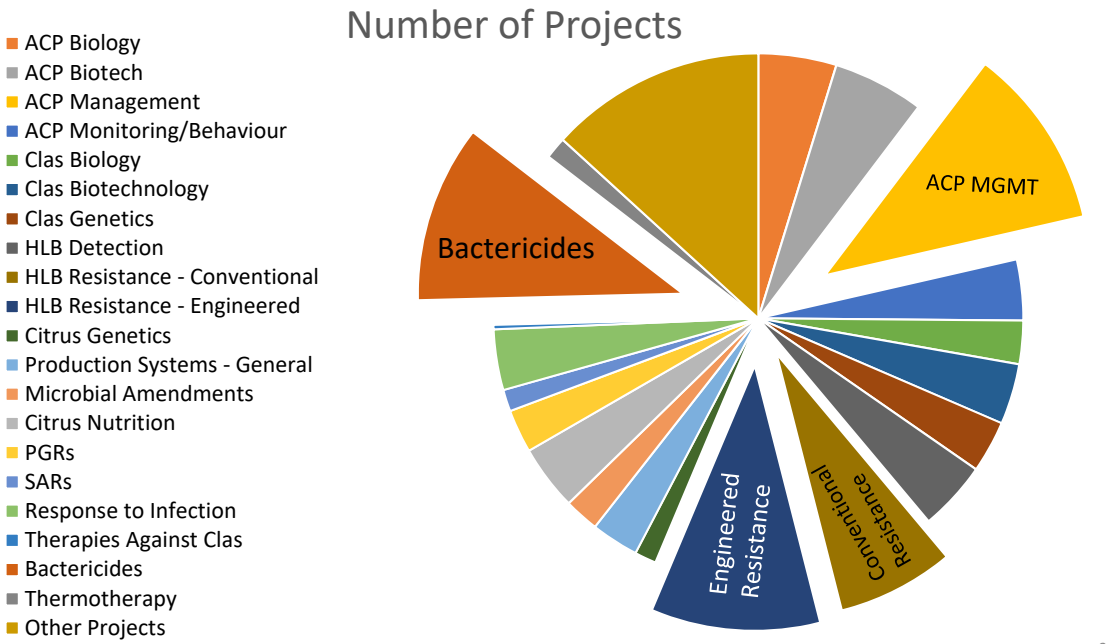
Overview of HLB Interventions/Solutions

- 12 years of U.S. Research on HLB: Knowledge and Solutions
- 400 projects ($120 million) funded by CRDF, majority on HLB, but also other diseases
- Full range of discovery to field testing, conventional to biotechnology
- Knowledge development informs pursuit of interventions
- Focus of intervention - all aspects of citrus HLB disease system:
  - Asian Citrus Psyllid ACP vector (1998)
  - *Candidatus* Liberibacter asiaticus (*C*Las) (<2005)
  - Citrus host plant and environment



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Antibiotic use in Citrus

What we have now…

- Antibiotics have been used in plant agriculture since the 1950s
- AIs currently in use in the US
  - Oxytetracycline
  - Streptomycin
  - Kasugamycin
- Screening *of* antibiotics
  - *L. crescens* (surrogate) assay
  - Plant assays

L. crescens Assay Results

Chemical	Concentration (ug/ml)	% Inhibition
Kasugamycin	500	75.34
	250	72.24
	125	69.28
Oxytetracycline	10	100.00
	5	100.00
	2.5	99.41
Streptomycin	10	100.00
	5	99.57
	2.5	99.05

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Antibiotic use in Citrus

- CRDF funded project to determine efficacy
  - AgroSource, Inc. and USDA ARS (CRADA)
  - 3-year study: Large Scale Laboratory/Greenhouse/Field Trial Evaluation of Citrus HLB Bactericidal Therapies
  - Reported yield increase and tree health improvements
  - Data supported emergency exemption and renewals (2016, 2017, 2018)
- Other studies
  - CRDF strip plot trials with Florida citrus growers (originally ~70)
  - Some evidence of improvement after one year, 2nd year in progress
- Questions:
  - Uncertainty about efficacy/value to growers
  - Long-term, broad-scale use

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Biopesticides

- Current/near-term Products
  - CRDF botanical oil trial (2yr)
  - Growers use
  - Available now, 25b minimum-risk
    - Efficacy?
      - Could delivery method improve efficacy?

L. crescens Assay

Product	Concentration (ug/ml)	% Inhibition
ThymeGuard	100	100
Other Botanical Oil Products	50-500	100

2018 Yield (kg)

Product	Mean	SD	
Thymeguard	28.19	11.15	AB
Ecotrol	36.09	15.46	A
Onguard EO	31.21	16.56	AB
Xplode	29.03	18.09	AB
UTC	32.35	15.19	AB

P = 0.0001

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Biopesticides

- Antimicrobial microbes (AM)
  - Projects funded by multiple groups (CRDF, USDA, CRB)
    - Microbes from “survivor trees”
    - Microbial products on the market
    - Microbe libraries
- Shorter registration time for biopesticides, but evidence of efficacy against HLB?
  - Basis for CRDF/Bayer partnership

Zinc Bactericides

What may come next…

- Zinkicide (Santra, UCF)
  - Novel nanotech-enabled ZnO bactericide
    - Systemic, ~size of phloem proteins (4-6 nm)
    - Efficacy studies funded in 2014 -2016 (CRDF)
    - 2015 - 2020 NIFA SCRI funded studies
      - Formulation improvement
      - Field application optimization and efficacy
      - Residues inc. non-target toxicity
      - Economics

Zinc Bactericides

- Zinkicide
  - Current Knowledge
    - More active against surrogate bacteria *in vitro* (vs. Cu)
    - Improved citrus canker (*X. citri ssp. citri*) protection compared with Cu
    - HLB study: Improved yield reported in 2018
  - Nano-ZnO is a new active, next steps:
    - Environmental fate studies
    - Residue study
    - Other studies…
    - Product Development
    - Registration
  - What is the time to market?

Zinc Bactericides

- T-sol (Santra, CRDF funded)
  - Non-nano Zinkicide
    - Shorter regulatory path?
  - Systemic
  - Good activity against canker
  - No HLB efficacy data
  - MS3T, T-sol + clay film (NIFA SCRI)
  - Early in development

Other Molecules

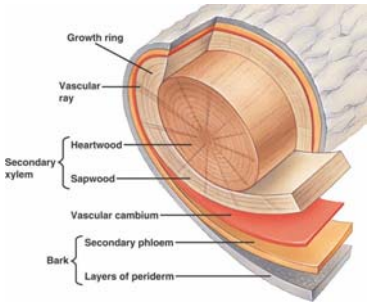
- Novel chemistries (Many groups)
  - Cu & Mg chemistries
  - Chemistries inactivating critical *C*Las genes
  - Various timelines for both R&D and commercialization
- Antimicrobial peptides
  - Small, host-defense peptides
  - Disrupt membrane integrity or other cell functions
  - Usually effective against only one group of microorganisms
  - Next-generation bactericide (or transgenic candidate)
- Delivery?
  - All available bactericides may benefit from alternative application methods

Application Technologies

- Most chemicals will inhibit *L. crescens in vitro* (≈ *C*Las)
- Points of contact
- Challenge for delivery

Vector

Host



Application Technologies

- Delivery to phloem
  - Conventional Foliar applications
    - Low % absorbed
    - Low % in contact with pathogen
      - Movement within plant
    - High contact with environment

Application Technologies

- Mechanical Delivery
  - Trunk injection
  - Infusion
  - Other novel methods

- Scale (~400,000 bearing acres in FL)
  - Time and money
    - Efficacy (# of injections)
- Residues
  - Fruit
  - Off-target

Small-scale

Medium-scale

Application Technologies

- Nanotechnology (several groups)
  - Smaller particles may have better access to phloem
    - Nano-particles
    - Nano-emulsions
      - Lipid delivery
    - Regulatory considerations
      - Environmental fate..
    - Time-to-market
      - Nano = longer timeline

Application Technologies – CTV Vector Delivery

- Citrus tristeza virus, among largest known plant viruses
- Vectored by *Toxoptera citricida* and other aphids.
- Phloem-limited closterovirus
- Causes catastrophic citrus disease worldwide in susceptible rootstocks
- Research tool developed by UF (WO Dawson) as transient expression vector, based on prior work with tobacco mosaic virus (TMV)
- Full length sequence clone of Florida strain allows multiple large inserts of non-native DNA to be incorporated and inoculated into citrus plants
- When inserts rejected, reverts to native virus type
- Insertion of GFP gene allowed study of virus dynamics in citrus phloem, which may be predictive for HLB

Application Technologies – CTV Vector Delivery

- Proven test bed for screening constructs (genes, AMPs, RNAs, markers)
- Proven to foster longer term expression and stability than expected (years)
- Can insert more than one gene or construct of interest
- Significant screening since 2008-09 of a range of constructs of interest
- Laboratory and greenhouse results allow selection of performing candidates
- Next steps may involve transformation of candidates into citrus trees
- Somewhat stable expression allows for field evaluation of candidates using CTV delivery mechanism – graft inoculation of virus-infected material in greenhouse (or field)
- USDA, APHIS BRS engaged in permitting of field testing using CTV vector
  - Regulation as a plant pest

Bayer Project Summary

- Large company with demonstrated experience registering products
- Extensive capability in entire pathway to create a product: discovery, development, commercialization
- Project is 3 years, discovery/early development, aimed at two technical approaches – microbial (biological) and plant defense modulator that induces plant defense – PDM (smaller synthetic molecule)

Bayer Project: Microbial- West Sacramento, CA

- Technical Strategy
  - From sequenced strain collection a "high hit likelihood" training set selected
  - "hits" coming from the training sets are used for screen validation
    - Screens utilize model microbes since *C*Las is not culturable
  - Broths from leads fractionated for active fractions – scale up and optimization
- Early assessments
  - Toxicology / Human pathogen activity
  - Stability of strain
- Current Status
  - Primary screen initiated
  - Validation of screens in process
  - A few potential hits have been generated but not validated

Bayer Project: Plant Defense Modulators– Lyon, France

- Technical Strategy
  - Utilize model plant reporter system to screen libraries of synthetic compounds that induce pathways involved in plant defense
  - Possible hits move on growth chamber, model plants and model pathogens screen
- Early assessments
  - Phytotox and Toxicology/environmental fate screening
  - Counter screen - compounds with direct antimicrobial activity against model pathogens are not further considered
- Current Status
  - Primary Screen established
  - Validation of screens in process
  - A few potential hits have been generated but not validated

RNAi approaches

- RNAi primarily focused on vector intervention - Asian Citrus Psyllid (ACP)
- Used as a tool:
  - Target validation (several projects)
    - Silence genes to validate function, phenotype, or suitability to monitor etc.
    - Introduced primarily via artificial diet or injection
- Used as the outcome of a project (discovery stage research)
  - Silence essential targets for ACP life or fertility
  - Silence targets involved in acquisition or transmission of *C*Las
  - RNAi in these projects introduced by diet, feeding on plants generating RNAi molecules, or attempts to engineer psyllids

RNAi approaches

- Currently two projects utilize RNAi approaches
  - *Implementing Transgenic Tools to Produce Commercial Scion Cultivars Resistant to HLB and Canker (year 3 of 3)*
    - *RNAi against psyllid targets as one of several approaches*
    - *Plants have been generated and are in testing*
  - *Use of RNAi delivered by CTV vectors to control ACP (year 2 of 3)*
    - *Small scale replicated field trial to test effects on ACP feeding on trees expressing psyllid targets that performed well in growth chamber experiments.*

Citrus Breeding – Biotechnology Assisted

- Genetics of citrus complex and perhaps is limited in providing sources for HLB or ACP resistance
- Alternative strategies have been pursued using various biotechnologies
- Capacity and tool-building have been major elements of research here
  - Genetics, genome mapping, metabolomics seeking targets in host and pathogen
  - Juvenile and mature citrus transformation systems for rootstocks and scions
  - Early flowering through gene manipulation
  - Gene editing tools, including CRISPR-Cas9
  - Use of CTV for "quick" screening prior to transformation
  - Screening methods, including inoculation of plants containing candidate genes
  - Transition to field evaluation of candidate plants

Citrus Breeding – Biotechnology Assisted

- Approaches being evaluated include the following:
  - Antimicrobial peptides, Cell-penetrating peptides
  - Known plant disease resistance genes (NPR1, etc.)
  - Pathogenicity site targets
  - Variable chain fragment length antibodies (*C*Las epitopes)
  - RNA interference
- Status of deployment of biotechnology-assisted HLB resistance
  - Majority of transformants in early stages of lab/greenhouse evaluation
  - Limited field plantings of event outcomes in USDA permitted sites
  - Progress in adapting CRISPR editing tools to citrus
  - Some licensing of technologies advancing
  - Mature transformation lab is putting candidates into commercial genotypes
  - Farthest along are the private efforts of Southern Gardens Citrus

Citrus Breeding - Conventional

- Significant efforts by USDA, ARS and UF breeding programs
- In-field trials have become HLB screening trials
- More than 80 rootstock and 80 scion trials are in the field in Florida
- Tolerance (better performance) is being observed, but there are limited data to indicate relative performance against industry standards. Tolerant plants continue to decline but at slower rates. Genotypes include limes, Parson Brown oranges, some pommelos, sugar bell tangelo, etc.
- Long evaluation periods mean released materials have limited data
- No indication of resistance or immunity in citrus germplasm or relatives
- Crosses are incorporating tolerant candidates, but early to determine outcomes
- Searches for "survivor trees" in Florida continue. May find mutation. Genetic and microbiome evaluation of some survivors.

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Citrus Breeding - Conventional

- Field trials of field-located grapefruit seedlings that grew up within the row and appear to be more tolerant than are parent trees.
- Is this due to seedling nature or is there genetic association?
- Grafted trees are being tested compared to seedling trees.
- Federal funding (NIFA, MAC) are promoting the cross-state exchange of plant germplasm to test for HLB susceptibility (Florida) as well as adaptability (all citrus states).
- Florida growers at present do not have clear choices in replanting that indicate better success compared to long-term standards.
- Annual production continues to drop as infected trees show increasing decline.

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ACP Suppression and Transmission Management

- Biology and Ecology of ACP
- Seasonal Phenology and Movement
- Attraction and Repellency – Behavioral Interactions with citrus plant
- Non-Citrus Hosts and Reservoirs for acquisition/transmission
- Genetics of ACP and endosymbionts
- Search to locate/create transmission incompetent ACP population
- Applied Management Tactics
  - Conventional pesticides across most AI groups
  - Soil applied neonicotinoids have been core to replanting and first 2-3 years of grove
  - Biopesticides – plant essential oils, others with some suppressive activity
  - Kaolin clay, other physical approaches, including reflective mulches
  - Increased ACP population are occurring, applying more control pressure

ACP Suppression and Transmission Management

- New product evaluations
- Biological control of limited impact in Florida
  - Parasitoid *Tamarixia radiata* introduced in 1999
  - *Isaria fumosarosea* fungus active, but delivery and stability challenges
- Windbreaks and other physical barriers can affect ACP dispersion
- CUPS (Citrus under protective screen) is being demonstrated
- Pesticidal product stewardship
  - Neonicotinoid use and avoidance of bloom
  - Target resistance monitoring and field research
  - Recommendations for product rotations

Thank you!

CRDF is proud to provide support to the Florida citrus industry