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Ensure a sustainable California citrus industry for the benefit of growers by prioritizing, investing in and promoting sound science.

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CALENDAR OF EVENTS 2019-20

2019

October 4

Lindcove Citrus Gala
Exeter, California. For more information, visit *www.lindcovecitrus.com*

October 24

California Citrus Quality Council (CCQC) Board Meeting
Doubletree Hotel, Bakersfield, California. For more information, visit *http://ccqc.org*

November 13

Citrus Pest and Disease Prevention Committee (CPDPC) Meeting
Ventura, California. For more information, visit *www.cdfa.ca.gov/citruscommittee*

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2020

January 15

Citrus Pest and Disease Prevention Committee (CPDPC) Meeting
Visalia, California. For more information, visit *www.cdfa.ca.gov/citruscommittee*

February 11-13

World Ag Expo
International Agri-Center Tulare, California. For more information, visit *https://www.worldagexpo.com*

March 11

Citrus Pest and Disease Prevention Committee (CPDPC) Meeting
Riverside/San Bernardino, California. For more information, visit *www.cdfa.ca.gov/citruscommittee*

November 8-13

International Citrus Congress
Mersin, Turkey. For more information, visit *www.icc2020.org*

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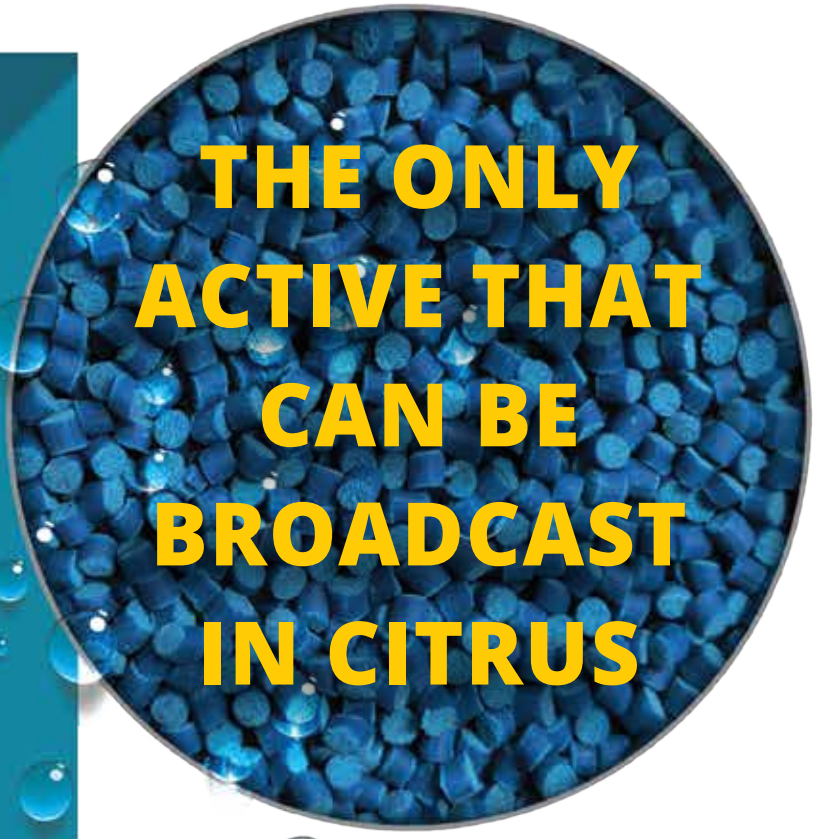

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A grower inspecting flush for Asian citrus psyllid nymphs.

Newly Developed Best Practices for Growers' Response to HLB

Jim Gorden

As detections of huanglongbing (HLB) in California continue to rise, it has become evident that our state's citrus industry needs a cohesive set of best practices to use in response to HLB – and I've heard growers echoing this need.

Because of this, the Citrus Pest & Disease Prevention Committee (CPDPC) recently endorsed a set of best practices for growers to voluntarily employ in response to HLB in California. These endorsed practices provide growers with a strong toolbox of science-supported strategies and tactics to protect their orchards and their neighbors' orchards from the disease.

Symptoms of huanglongbing include yellow, blotchy leaves. Look for this symptom when inspecting your orchard for the disease.

These recommendations – which were developed based on a grower's proximity to an HLB detection – represent the most effective tools known to the California citrus industry at this time and are meant to supplement the California Department of Food and Agriculture's required regulatory response. The best practices were developed by a task force of growers from various regions across the state and also scientists, all of whom were nominated by the CPDPC.

Voluntary best practices were developed for growers in the four following scenarios:

- ***Scenario 1***: Orchards outside of a five-mile HLB quarantine area.
- ***Scenario 2***: Orchards located between one and five miles of an HLB detection (within an HLB quarantine area).
- ***Scenario 3***: Orchards within one mile of an HLB detection, but not known to be infected.
- ***Scenario 4***: Orchards with HLB.

In each scenario, the best practices vary, but all address:

- awareness,
- scouting for the Asian citrus psyllid (ACP),
- controlling ACP with treatments,
- protecting young trees and replants,
- employing barriers or repellents,
- visually surveying for HLB symptoms,
- testing ACP and plant material for the HLB pathogen using a direct testing method like polymerase chain reaction (PCR) and
- tending to trees' overall health.

The CPDPC encourages growers to use as many methods as are feasible for their operation to limit the spread of ACP and HLB. It's critical to remember that the cost to manage the Asian citrus psyllid is far less than the potential long-term costs or loss to the industry should HLB be allowed to spread throughout our state.

Voluntary best practices for each scenario can be found in the At a Glance table on page 14. Visit *CitrusInsider.org/BestPractices* for more information. The full voluntary best practices report, including the At a Glance table, is available for downloading and printing at the web site. The report also contains a section that explains the scientific basis behind the recommendations.

Jim Gorden is the chairperson of the Citrus Pest & Disease Prevention Committee. He also is a board member and past president of the Citrus Research Board. For more information, contact **jim@gordenag.com**

AT A GLANCE:

BEST PRACTICES IN RESPONSE TO HUANGLONGBING IN CALIFORNIA CITRUS | UPDATED JUNE 10, 2019

CPDPP

	SCENARIO 1 *Orchards outside a 5-mile HLB quarantine*	SCENARIO 2 *Orchards between 1 and 5 miles from HLB detection*	SCENARIO 3 *Orchards within 1 mile of HLB, but not known to be infected*	SCENARIO 4 *Orchards with HLB*
AWARENESS	• Stay informed: communicate with others, such as Grower Liaisons, Cooperative Extension, or Pest Control Advisors, and attend meetings. • Get to know your neighbors. • Sign up for alerts on CitrusInsider.org.	All actions from Scenario 1, plus: • Help educate your neighbors about the seriousness of HLB. • Be prepared to help with communications and spray applications.	All actions from Scenario 2, plus: • Offer to lead your psyllid management area's communication network.	All actions from Scenario 3, plus: • Help connect your neighbors to organizations that assist homeowners with citrus tree removal.
SCOUT FOR ACP	• Deploy trained scouts every 2 weeks. • If ACP are found, treat before they reach 0.5 nymphs/flush.	All actions from Scenario 1.	All actions from Scenario 1, plus: • Pay special attention to vigorously flushing trees or areas under high ACP pressure, such as edges that border residences, or where ACP have previously been found.	All actions from Scenario 3.
CONTROL ACP WITH INSECTICIDES	• Try to eliminate psyllids. • Apply extra treatments within label limits if ACP populations start to increase before a scheduled areawide treatment. • In mature orchards, a perimeter-only treatment can be applied if the center is free of psyllids. • Treat the orchard border before the center. • Make applications at night when psyllids are inactive. • When treating for other pests, utilize insecticides known to have efficacy against ACP.	All actions from Scenario 1, plus: • Treat the entire orchard at least 3 times per year with an ACP-effective, long-residual insecticide. Coordinate with your liaison, PCD, and/or local task force for timing. If psyllids exceed 0.5 nymphs/flush between the 3 applications, treat again, if an additional treatment is within label limits.	All actions from Scenario 1, plus: • Treat the entire orchard at least 3 times per year with an ACP-effective, long-residual insecticide. Coordinate with your liaison, PCD, and/or local task force for timing. • Treat the orchard border before the center. • If psyllids exceed 0.5 nymphs/flush between the 3 applications, treat the entire orchard again if an additional treatment is within label limits. • Make applications at night . • Use ACP-effective insecticides when treating for other pests.	All actions from Scenario 3.
YOUNG TREES / REPLANT PROTECTION	• Consider additional protectants for young trees and replants, such as psyllid-proof mesh covers, kaolin, or insecticides.	All actions from Scenario 1, plus: • Treat orchards in their entirety (do not use border treatments).	All actions from Scenario 2, plus: • Replant with tolerant/ resistant trees as they become available.	All actions from Scenario 3. • Infection of unprotected replants is highly likely if ACP are present.
BARRIERS/ REPELLENTS	• Create barriers and/or apply repellents to limit ACP establishing on the perimeter of the orchard.	All actions from Scenario 1.	All actions from Scenario 1.	All actions from Scenario 1.
VISUAL SURVEY FOR HLB	• Conduct a survey for HLB symptoms in the orchard perimeter and the uppermost part of the canopy once a year.	• Conduct a survey for HLB symptoms in the border rows/trees and in the uppermost part of the canopy **twice a year.**	• Conduct a survey for HLB symptoms in the **entire** orchard, including the uppermost part of the canopy **twice a year**.	All actions from Scenario 3.
DIRECT CLAS DETECTION PROTOCOL	N/A	• Test foliage and psyllids from 10 trees in each corner of the block (40 trees total) using direct methods of bacterium detection (such as PCR).	• Test foliage and psyllids from all perimeter trees using a direct method of bacterium detection (such as PCR). • Test additional trees through a laboratory or commercial kit.	All actions from Scenario 3.
TREE HEALTH	• Ensure appropriate nutrient and water applications to tend to your grove's root health.	All actions from Scenario 1.	All actions from Scenario 1.	All actions from Scenario 1.

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California Citrus Welcomes Three New Experts

Melinda Klein and Ivy Leventhal

The California citrus industry is pleased to welcome three new highly qualified experts – Danelle Seymour, Ph.D., Ashraf El-kereamy, Ph.D., and Peter Ako Larbi, Ph.D. All three talented additions have begun working in key positions within the University of California system.

Danelle Seymour, Ph.D.

This July, Seymour joined the Department of Botany and Plant Sciences at the University of California, Riverside (UCR) as an assistant professor of genetics. Her responsibilities include enhancing UCR's citrus research program to address challenges posed by climate change, including exotic pests and diseases. She also will work toward improving the health benefits of citrus, including the application of modern genetic and genomic approaches to facilitate the development of commercially successful citrus cultivars.

Seymour is looking forward to making a difference. "Now is such an exciting time to begin working as a citrus breeder," she said. "Recent advances, particularly in genomics, make it possible to pinpoint the genetic basis of traits important to citrus growers at an unprecedented scale. In my opinion, this will greatly accelerate the development of commercially successful citrus cultivars for the California citrus industry."

The researcher comes to UCR from the National Science Foundation, where she was a post-doctoral research fellow at the University of California, Irvine. Seymour received her Ph.D. in Biology from the Max Planck Institute for Developmental Biology (University of Tübingen) in Tübingen, Germany. Her M.S. in Genetics and B.S. in Genetics both were earned at the University of California, Davis.

Ashraf El-kereamy, Ph.D.

In February, El-kereamy joined the UCR-Lindcove Research and Extension Center (LREC) staff as a citrus horticulture cooperative extension specialist. His charge is to lead the Citrus Horticulture Extension Program, including development and implementation of a comprehensive local and statewide research and extension education program

to help the citrus industry maximize production and improve efficiency as challenges emerge. El-kereamy also is working with collaborators and industry partners from California and other states to establish a nationally-recognized outreach and citrus horticulture research program.

"It is a great opportunity to work at the heart of the industry in a location like UCR-LREC," El-kereamy said, "which is a great place to interact with the citrus industry and to be able to design the research, extension and outreach program around their needs. The Center, with all of its citrus varieties and facilities, is a perfect resource to develop and implement an excellent applied research and extension program."

The researcher most recently served as a viticulture and small fruit advisor to the University of California Cooperative Extension at Kern County, where he established a research and extension program serving the San Joaquin Valley table grape industry. In prior positions, El-kereamy worked as a post-doctoral researcher and research associate at the University of Guelph in Canada, where he first studied genotype variation in nitrogen use efficiency and plant heat stress tolerance and later studied plant drought and heat stress tolerance. Previously, he was an assistant/associate professor in the Department of Horticulture at Ain Shams University in Egypt. He holds a Ph.D. in Agriculture with emphasis in Grapevine Physiology and Molecular Biology from Toulouse University in France. His M.S. in Pomology and B.Sc. in Horticulture were earned at Ain Shams University.

Peter Ako Larbi, Ph.D.

Last summer, Larbi joined the California citrus industry as an assistant cooperative extension specialist in agricultural application engineering at the University of California, Division of Agriculture and Natural Resources (UCANR), Kearney Agricultural Research and Extension Center in Parlier, California. He is charged with providing regional leadership in agricultural engineering extension and applied research with a focus on spray application engineering. His primary goal is to improve agricultural productivity while reducing the impact of pesticides and other agricultural chemicals on the environment.

"I am delighted to join UCANR to contribute by providing engineering solutions to the California agricultural industry, particularly pertaining to pesticide spray application in citrus and other specialty crops," said Larbi, who added that he looks forward to engaging with key industry players to promote best practices for safe, economical and environmentally sound pesticide spray applications.

Most recently, Larbi had been an assistant professor of agricultural systems technology at Arkansas State University in Jonesboro, Arkansas. The position was a joint appointment with the Division of Agriculture at the University of Arkansas. He previously served as a post-doctoral research associate at the Center for Precision and Automated Agricultural Systems at Washington State University and as a post-doctoral researcher at the University of Florida's Citrus Research and Education Center. Larbi holds a Ph.D. in Agricultural and Biological Engineering from the University of Florida, as well as an M.Sc. and B.Sc. with Honors in Agricultural Engineering from Kwame Nkrumah University of Science and Technology in Ghana.

***Melinda Klein, Ph.D., is the chief research scientist for the Citrus Research Board in Visalia, California, where she also serves as the science editor for* Citrograph. *Ivy Leventhal is the managing editor of* Citrograph. *For more information, contact* melinda@citrusresearch.org *or* ivy@citrographmag.com**

University of California Cooperative Extension Farm Advisor Greg Douhan, Ph.D., provided a regional update to attendees at the Exeter, California seminar.

Grower Seminars Focus on Multiple Approaches to HLB Control

Carolina Evangelo, Sonia Rios, Greg Douhan and Ben Faber

The Citrus Research Board (CRB) and the University of California Cooperative Extension (UCCE) hosted their annual citrus growers' educational seminar series in Palm Desert, Santa Paula and Exeter, California, on June 25, 26 and 27, respectively.

The combined 450 attendees were able to connect with regional farm advisors, citrus researchers and local growers and celebrate the CRB's 50th anniversary. Qualifying attendees earned continuing education units from the California Department of Pesticide Regulation and Certified Crop Advisor hours. Lunch was served at the seminars as part of the CRB's 50th anniversary celebration.

CRB Board Member Mark McBroom emceed the Palm Desert seminar, and CRB Chairman Dan Dreyer emceed the Santa Paula and Exeter seminars. Each location featured talks from:

- Melinda Klein, Ph.D., CRB chief research scientist, who presented a CRB update and a recap of the International Research Conference on Huanglongbing (HLB);
- Peggy Mauk, Ph.D., University of California, Riverside director of agricultural operations, who delivered an update on the use of canines for early HLB detection;
- Mamoudou Sétamou, Ph.D., associate professor of agronomy and resource sciences, Texas A&M University-Kingsville Citrus Center, who shared his research on optimizing area-wide management of Asian citrus psyllid (ACP) through phenology-based sprays and border control approaches;

- Riverside County Agricultural Commissioner Ruben Arroyo who spoke in Palm Desert, Andy Calderwood of the Ventura County Agricultural Commissioner Office who spoke in Santa Paula, and Fresno County Agricultural Commissioner Melissa Cregan and Tulare County Agricultural Commissioner Tom Tucker who both spoke in Exeter, all on the subject of regional pesticide laws and regulations; and
- Beth Grafton-Cardwell, Ph.D., University of California, Riverside; Neil McRoberts, Ph.D., University of California, Davis; Sara García-Figuera, University of California, Davis; and Holly Deniston-Sheets, Citrus Research Board, who provided growers with an interactive presentation on the Citrus Pest & Disease Prevention Committee (CPDPC) Voluntary Action Program. *(See results on page 22.)*

Key regional topics were covered at each location. In Palm Desert, Glenn Wright, Ph.D., of the University of Arizona, discussed lemon varieties, rootstocks and pre-harvest fruit drop trials in southern California. In Santa Paula, Mauk gave a citrus rootstock update. In Exeter, Ashraf El-kereamy, Ph.D., research extension specialist at the Lindcove Research and Extension Center, covered high density planting, root health, nutrition and ACP.

Casey Creamer, president of California Citrus Mutual (CCM) presented an organization update in Palm Desert and Exeter.

At each seminar, the local UCCE farm advisor provided attendees with a regional update on their respective citrus season.

Southern California Regional Update

Riverside and San Diego counties UCCE Farm Advisor Sonia Rios reported that July 6, 2019 was the first of three days of intense temperatures in southern California. Heat stress, Santa Ana winds and fires are just a few of the challenges citrus growers have had to work through this year. Planning for unpredictable weather can be challenging for growers. Then there is dealing with the devastating aftermath of excessive heat and how to prepare their groves if it were to occur again.

Central Coast Regional Update

Ben Faber, Ph.D., Ventura County UCCE farm advisor, reported the 2018-19 rainy season has differed from previous drought years. He stated, "It really did rain, but no more than in a 'normal' year, although the rains were well spaced out and well into the spring. This meant less irrigation was needed, and the soils got a good leaching for the first time in five years. The winter was cold, but there were no freezes along the coast. May 22, 2019 was characterized by a hailstorm that rattled the Santa Paula area, and then on June 11, there was a heat spike of 105°F in Ojai. Both of these incidents led to some fruit drop. Another complicating factor is the lemon harvests that have been disrupted by wet and foggy weather."

Weather data are available for the central coast region at the Western Regional Climate Center (*https://wrcc.dri.edu/Climate/reports.php*) where, in spite of the recent drought, rainfall patterns for this area seem to be normal. What have been unusual are the increasingly warm winters for this area.

These changing weather patterns have resulted in different pest/disease patterns, as well – more snails and brown rot, more botrytis fruit ridging and some unusual arthropods like *Lorriya formosa* mite, *Toxoptera aurantii* black citrus aphid and orange tortrix. None of them are of great concern, but are different from the "normal" infestation patterns. Some of these pests, diseases and disorders can be viewed in photographs at the University of California Integrated Pest Management citrus web site: *http://ipm.ucanr.edu/PMG/selectnewpest.citrus.html*

Another way to stay abreast of citrus problems is to tune into the UC Ask an Expert webinars offered every month. These hour-long, CEU-approved presentations cover a variety of topics including weed management, pests and diseases for citrus and other sub-tropicals *https://ucanr.edu/sites/ucexpertstalk/*.

Knowing how to irrigate in anticipation of the upcoming changing weather patterns can help citrus growers better prepare for the problems that come with changes in the weather.

Citrus Research Board (CRB) Interim President Franco Bernardi addressed seminar attendees and introduced the CRB's 50th Anniversary video in Palm Desert, California during lunch.

Ben Faber, Ph.D., (left) and Citrus Research Board President Dan Dreyer at the seminar in Santa Paula, California.

Central Valley Update

The Tulare and Fresno counties UCCE Farm Advisor Greg Douhan, Ph.D., reported that the 2018-19 citrus season in the San Joaquin Valley resulted in a good crop across the board. However, most growers complained to him of small fruit sizes, which left some growers with the decision to pick or not to pick because the value was minimal, if not a loss, for some. Douhan stated, "This was the first year I personally noticed many fields not picked and/or fruit left to drop because of this situation." Growers who produced large fruit likely did very well.

For the past couple of seasons, the Central Valley has experienced decent amounts of rainfall compared to the five years or so of drought, especially this past winter and spring where there was considerable precipitation conducive to dieback wood rotting fungi such as *Diaporthe* spp. and members of the Botryosphaeriaceae. Douhan reported that last year, he saw much more *Colletotrichum* spp. than this past year. Anecdotally, many groves with their fungal pathogens had numerous inner dead branches. These are sites where these fungi can live and sporulate, usually in the spring to infect new branches resulting in tip dieback.

The cooler, wet weather also seemed to lead to fewer issues with red scale than had been seen with hotter, drier conditions during the drought years. However, the prolonged cool wet weather this past year created issues with thrips control. Many growers reported they had to do more scouting and spraying to control these insects. The wetter weather also should have been more conducive for root pathogens (*Phytophthora* spp. and *Fusarium* spp.), but Douhan stated he did not see a noticeable difference compared to the past several years. Douhan ended on a positive note, sharing with central valley growers that all HLB-positive trees still are limited to residential areas in southern California. None have been found in commercial groves, and ACP finds in the valley have been lower than in previous years.

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Attendees at the Palm Desert, California seminar provided their answers via clicker during participation in the interactive talk on the Citrus Pest & Disease Prevention Committee's voluntary action plan.

Grower Surveys Reveal Diverse Opinions about Managing ACP and HLB

Neil McRoberts, Sara García Figuera, Holly Deniston-Sheets and Elizabeth Grafton-Cardwell

Project Summary

The Data Analysis and Tactical Operations Center (DATOC) provides on-going research-based information to the Citrus Pest and Disease Prevention Program (CPDPP) regarding all aspects of the epidemiology and management of huanglongbing (HLB). "Economic Returns to Coordinated Actions to Control HLB" is a collaborative project between the University of California, Riverside and the University of California, Davis, in which biological simulation and economic analyses are used to estimate the economics of coordinated activities, such as area-wide pesticide applications, to control the spread of HLB. For both projects, it is important to understand the actions that individual growers plan to take in response to the threat of HLB, so information about growers' opinions of the proposed voluntary response plan is of interest. The results presented here come from surveys of growers who attended the June 2019 Citrus Research Board Citrus Growers Educational Seminar Series. These survey results provide a snapshot of growers' opinions about various aspects of Asian citrus psyllid (ACP) and HLB management, particularly in relation to the recently released CPDPP "Best practices in response to huanglongbing in California citrus."

Introduction

HLB is a regulated disease in California, so confirmation of a diseased tree through an approved diagnostic method triggers a regulatory response. Regulations stipulate mandatory removal of the tree, establishment of a quarantine zone around the infection and intensive surveying in a 400-meter radius of the infected tree to determine whether additional infected trees are present. However, it is hoped that growers voluntarily will take additional action to help limit the spread of the disease, either individually or in coordination with nearby growers. Recommended best practices were developed by a committee of growers, University of California scientists and other citrus program advisers including members of the DATOC panel, and recently were published by the CPDPP (*https://citrusinsider.org/psyllid-and-disease-control/voluntary-best-practices-for-growers-response-to-huanglongbing/*).

To assess the citrus industry's willingness to adopt these practices, clicker handsets were used to gather attendee responses at the Citrus Growers Educational Seminar Series, conducted this past June in Palm Desert, Santa Paula and Exeter, California *(see page 18)*. Preliminary highlights of the survey results are presented here, while more detailed analyses are on-going.

Who Participated?

The majority of respondents were grove owners or managers; a smaller fraction were Pest Control Advisers. About one-fifth of respondents in each location chose "other" to define their involvement in the citrus industry (**Figure 1**). In Palm Desert, responses were split between small (less than five acres) and large (greater than 500 acres) producers. In Santa Paula, 50 percent of respondents farmed 25 acres or less. In Exeter, the majority of respondents farmed 500 acres or more (**Figure 2**). We asked the audience at each seminar to consider the questions in relation to the coming year.

Communication and Risk Perception

The first voluntary activity in the response plan is for growers to stay aware of the situation in their area. Most people were "likely" or "very likely" to discuss HLB with their grower and/or residential neighbors in the year ahead (**Figure 3**). Similar results (not shown) were obtained when respondents were asked how likely they would be to communicate with their Grower Liaisons.

Respondents were mostly optimistic that HLB would not be detected in their groves in the year ahead. The perceived

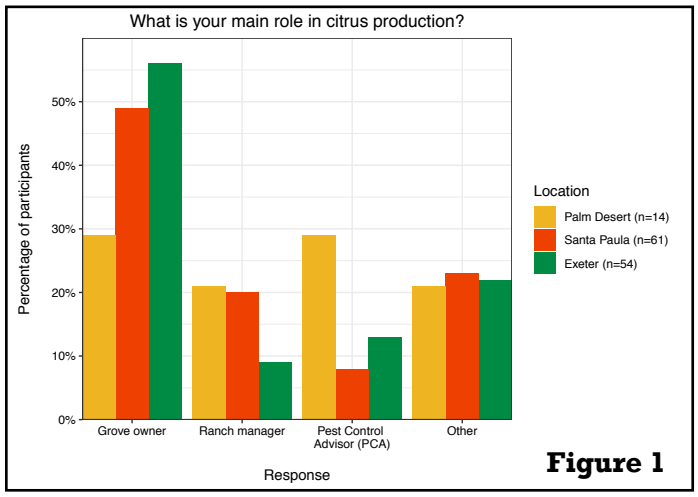

Figure 1

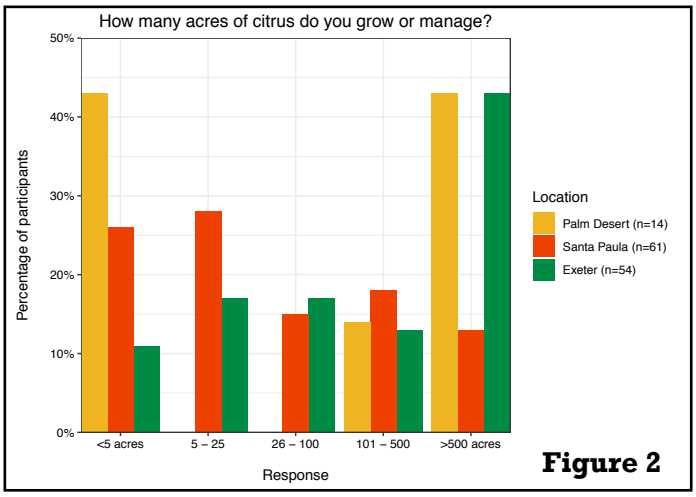

Figure 2

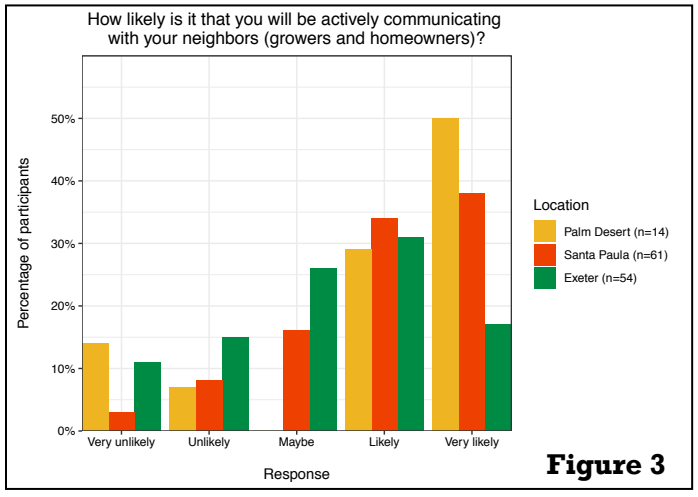

Figure 3

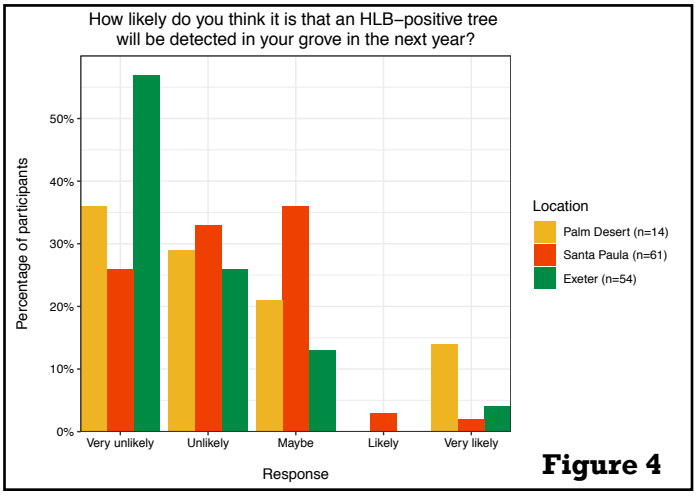

Figure 4

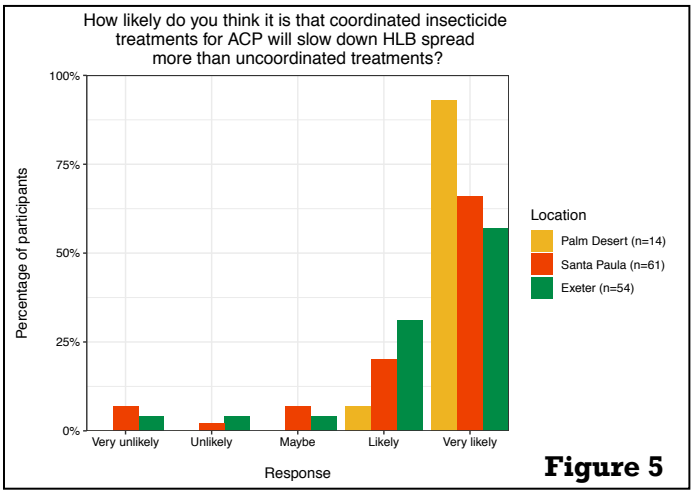

Figure 5

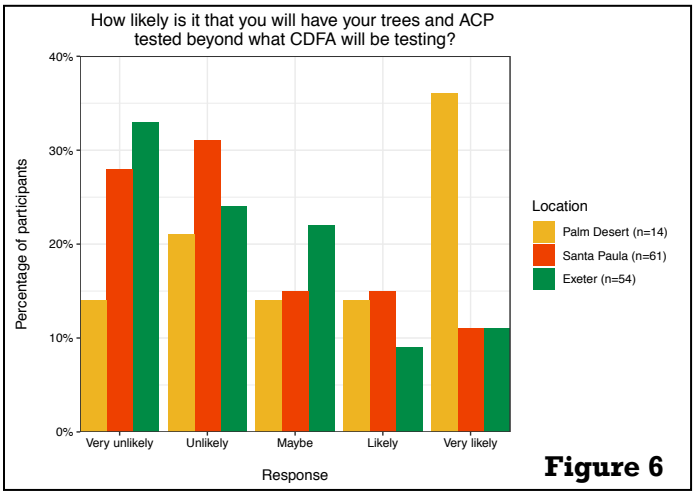

Figure 6

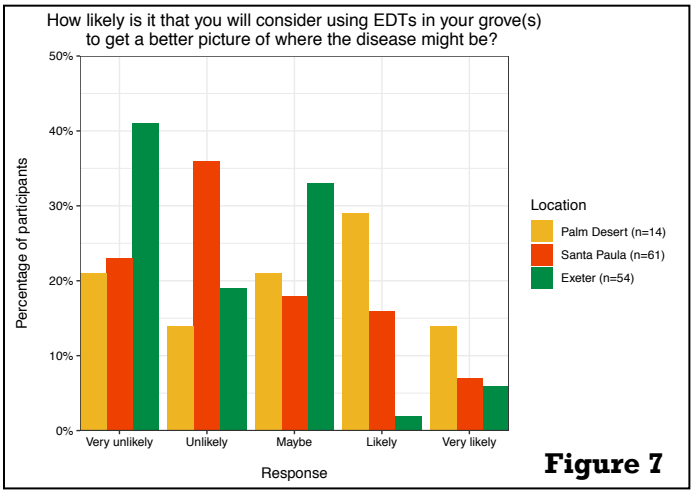

Figure 7

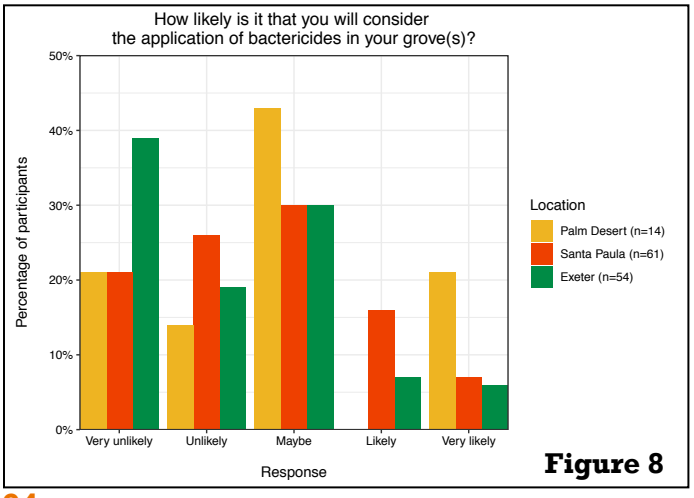

Figure 8

level of threat appeared to correspond with the distance from known HLB detections. In Palm Desert, 36 percent of respondents said that an HLB detection was "very unlikely," while the corresponding "very unlikely" numbers for Santa Paula and Exeter were 26 percent and 60 percent, respectively (**Figure 4**).

Area-wide ACP Control

The need for area-wide management to control ACP has been a consistent and regular outreach message from the CPDPP for several years. Based on responses from all three seminars, the message largely has been received (**Figure 5**). A majority of respondents at all three locations thought it "very likely" that coordinated treatments would slow down the rate of progress of HLB more than uncoordinated treatments would.

Additional Activities: Testing for HLB, Bactericide Use and EDTs

Growers were more likely to participate in recommended pesticide applications for ACP than to undertake additional actions to find disease or protect their trees. In all locations, the majority of respondents were "very unlikely" or "unlikely" to test additional ACP or tree samples for disease beyond the testing conducted by the California Department of Food and Agriculture (**Figure 6**).

Although Early Detection Technologies (EDTs) and bactericides (two products currently are approved for use on citrus in California) are not recommended in the voluntary plan, we were interested to know if growers and ranch managers are considering using them. At the three locations, just 19 percent of respondents across the three regions indicated that they were "likely" or "very likely" to use bactericides and/or EDTs, suggesting that although some growers may use them in the near future, neither is a popular option at this time (**Figures 7** and **8**). These opinions do not seem to be based on a lack of knowledge about the options; typically (in our experience of administering opinion surveys with growers), respondents to this type of survey select "maybe" when uncertainty is due to a lack of information. This tendency is not strongly reflected in the results, although it is more evident in the results for bactericides than EDTs.

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Photo A: The group of speakers who presented on day one of the conference, covering post-harvest diseases and pathogens. (Front to back, left to right): Row 1 – Chang-Lin Xiao, Ph.D., United States Department of Agriculture – Agricultural Research Service (USDA-ARS), Parlier, California; Elizabeth Baldwin, Ph.D., USDA-ARS, Fort Pierce, Florida; Sandipa Gautam, Ph.D., University of California, Riverside. Row 2 – Cheryl Lennox, Ph.D., Stellenbosch University, South Africa, and James Adaskaveg, Ph.D., University of California, Riverside. Row 3 – Joseph Smilanick, Ph.D., USDA-ARS (retired); Spencer Walse, Ph.D., USDA-ARS, Parlier, California; and Elizabeth Mitcham, Ph.D., University of California, Davis. Row 4 – Seiya Saito, Ph.D., USDA-ARS, Parlier, California; Mary Lu Arpaia, Ph.D., University of California, Riverside; and David Obenland, Ph.D., USDA-ARS, Parlier, California. Row 5 – Geert de Wever, Janssen PMP, Belgium, and James Cranney, California Citrus Quality Council. Photo B: Day two conference speakers focused on food safety. (Front to back, left to right) Row 1 – Dan Thullen, AC Foods; Ted Batkin, Batkin Ag Services; and Heidi Irrig, Syngenta. Row 2 – Michelle Danyluk, Ph.D., University of Florida; Trevor Suslow, Ph.D., Produce Marketing Association; and Mary Lu Arpaia, Ph.D., University of California, Riverside. Row 3 –Bonnie Fernandez-Fenaroli, Center for Produce Safety.

Citrus Post-harvest Pest Control Conference Focuses on Food Safety

Carolina Evangelo, Joey S. Mayorquin and Mary Lu Arpaia

The 40th Annual Citrus Post-harvest Pest Control Conference was held this past April with 70 researchers, industry personnel and service company representatives in attendance.

The meetings, which originally had been organized by Joseph Eckert, Ph.D., a renowned citrus post-harvest pathologist, began in 1978 as a University of California, Riverside extension course. Mary Lu Arpaia, Ph.D., has had organizational responsibility since 1993. Since 2001, the Citrus Research Board (CRB) has been an active participant, originally serving as one of the sponsors. The CRB's participation has grown through the years, with the Post-harvest and Communications Committees providing input on the technical program and logistics. In 2017, the CRB became the co-organizer of the meeting along with the University of California.

The conference's original and continuing objective is to provide information about post-harvest pest management practices related to fungicides, trade issues, quarantine requirements and food safety to California's citrus packinghouses and their technical personnel, who are employed by service companies.

Attendees represented all of the major service companies that assist the citrus industry. Technical service company personnel have a state-mandated requirement as pest

Post-harvest Conference attendees asked questions of keynote speaker Cheryl Lennox, Ph.D., of Stellenbosch University in South Africa.

control advisors (PCAs) and applicators to receive continuing education (CE) credits. This conference provides the only opportunity in the state to receive CE units for post-harvest pest management. Although there are occasional non-citrus presentations, the bulk of the program always has been focused on citrus.

This conference has greatly benefited the industry through the years since it provides a forum not only for continuing education, but also networking and information outreach in a very specialized field of endeavor. It has helped the California citrus packinghouse community stay abreast of changes in regulations, chemical registrations and management strategies to prolong the useful life of the chemicals used to control post-harvest decay and insect pests. As quarantine trade issues have become more prevalent, presentations covering fumigants and other quarantine treatment strategies have been added. In 2002, the meeting introduced speakers to address food safety, and this focus has continued to expand over the years. This year's conference, for the first time, included a citrus industry food safety forum to openly discuss food safety issues important to the industry. The forum was moderated by Ted Batkin, president of Batkin Agricultural Services and former CRB president.

Post-harvest Disease Control

Most of the presentations at this year's conference focused on various aspects of post-harvest disease management, including new biopesticides, sanitizers and fumigants. Keynote speaker Cheryl Lennox, Ph.D., from Stellenbosch University in South Africa delivered two presentations. Her participation was co-sponsored by JBT and Wonderful Citrus. Lennox's first presentation provided an overview of citrus post-harvest disease management in South Africa, and the second presentation focused on her lab's current research into the management of fungicide-resistant populations of the green mold and sour rot fungal pathogens that are major contributors to rejected consignments.

CRB-funded researcher James Adaskaveg, Ph.D., also shared two presentations. The first provided an update on new biopesticides and sanitizers to manage post-harvest diseases of lemons; and the second covered new fungicides for pre- and post-harvest treatments of citrus brown rot, which are needed due to the increased resistance of *Phytophthora* to traditional treatments for brown rot.

Elizabeth Baldwin, Ph.D., gave two talks. Her first was on the efficacy of ginger essential oil against gray mold and Mucor rot when applied as a nano-emulsion (small particles that can deliver chemicals) coating; and her second focused on the correlation of Diplodia stem-end rot with pre-harvest fruit drop in huanglongbing-affected citrus.

CRB-funded researcher Chang-Lin Xiao, Ph.D., highlighted the efficacy of natamycin, an antifungal biopesticide, in managing emerging post-harvest diseases of mandarins, which is a concern for the industry as California mandarin acreage continues to increase in response to consumer demands.

Several of this year's speakers discussed post-harvest fumigation treatments. David Obenland, Ph.D., presented preliminary data suggesting that post-harvest treatment

Citrus Research Board Chairman Dan Dreyer provided an update on post-harvest work being funded by the Board.

Cheryl Lennox, Ph.D., Ted Batkin, Mary Lu Arpaia, Ph.D., and Joseph Smilanick, Ph.D., enjoyed the conference's networking reception.

Charlene Jewell, JBT; Jim Adaskaveg, Ph.D., University of California, Riverside; and Jim Cranney, CCQC caught up at the evening reception.

with phosphine does not cause citrus fruit surface damage as does methyl bromide. Elizabeth Mitcham, Ph.D., discussed the tolerance of unwaxed fruit to ethyl formate fumigation. Both studies included sensory panels, which determined that panelists could not detect a difference between fumigated and non-fumigated fruit. Joe Smilanick, Ph.D., provided an update on several post-harvest fumigants including sulfur dioxide fumigation, which has been used routinely to prolong the storage of table grapes and could be a potential option for the citrus industry. Seiya Saito, Ph.D., discussed the use of sulfur dioxide-releasing pads and packaging to manage post-harvest diseases in blueberry.

Export Requirements

CRB-funded researchers Spencer Walse, Ph.D., and Sandipa Gautam, Ph.D., provided updates on their research into the management of post-harvest insect pests of concern for export markets. Walse reviewed the registration status of ethyl formate and noted his efforts in working with the Environmental Protection Agency to classify ethyl formate as a "biopesticide" and add a special use for citrus. Gautam discussed the use of post-harvest fumigants, including propylene oxide and phosphine in conjunction with cold treatments to increase the mortality of thrips and various mite species.

Jim Cranney, president of the California Citrus Quality Council (CCQC), talked about various regulatory issues including the registration review of imazalil following the European Union's threat to revoke imazalil, the demands that Food and Drug Administration regulations will put on California exporters, and New Zealand's declaration to reject any damaged or rotten fruit following a recent spotted wing Drosophila detection.

Geert de Wever, Ph.D., from Janssen PMP discussed ongoing work to defend the use of imazalil in citrus, as there currently are no reasonable alternatives.

Chad Cox, Fruit Growers Supply; Doug McDowell, Jet Harvest; Tarcisio Ruiz, Fruit Growers Supply; and Geert de Wever, Janssen PMP.

Tony Ecuyer, Limoneira; Alejandro Gonzalez, JIFKINS, Mexico; Miguel Espinoza, JIFKINS, Mexico; and Tony Stolz, Pace International.

Attendees at the food safety forum took notes and listened closely.

Heidi Irrig of Syngenta reviewed the process of citrus maximum residue level (MRL) management with a focus on MRLs in Asia.

Food Safety

Several presentations were given on different aspects of food safety. Michelle Danyluk, Ph.D., delivered two talks. The first focused on the importance of biofilm management and the various mechanical and chemical strategies available as biofilms allow pathogens to persist in the environment. The second covered the Food Safety Modernization Act and related activities occurring in the Florida citrus industry.

Bonnie Fernandez-Fenaroli provided an update on the current research areas being funded by the Center for Produce Safety, including funding for post-harvest preventive controls and interventions of *Listeria*.

Trevor Suslow, Ph.D., shared information on *Listeria* harborage in packinghouses and post-harvest water treatment systems.

Agriculture Capital Operations Food Safety Manager Daniel Thullen, who also is the co-founder and president of the Central Valley Food Safety Committee, discussed the history of third-party audits and highlighted the impact that hazard analysis and risk-based preventive controls may have on the industry.

The conference concluded with the Citrus Food Safety Forum, a round-table discussion for the post-harvest citrus industry to openly discuss current food safety issues and to provide an opportunity to set priorities for the food safety industry. This forum included lengthy discussions on several topics including molecular diagnostics, consistent auditing processes and dealing with customer requests. Ultimately, the group agreed there is need for a citrus food safety working group to address the transitional needs of the industry. Cranney of the CCQC agreed to organize a citrus-specific Food Safety Workshop, which was held this past July 16 in Visalia, California. Visit *www.citrusresearch.org* to read about the results of the workshop.

***Carolina Evangelo is the director of communications and co-publisher of* Citrograph *for the Citrus Research Board. Joey S. Mayorquin, Ph.D., is a research associate at the Citrus Research Board and also serves as an associate science editor of* Citrograph. *Mary Lu Arpaia, Ph.D., is a statewide extension specialist for subtropical fruit at the University of California, Riverside. For additional information, contact* Carolina@citrusresearch.org**

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Pattern of canine alerts near urban interface. Green lines depict path of survey, and red dots indicate trees with canine alerts. Note, there were no alerts on the interior grove. Trees range from 20-30 years old.

UCR and USDA-ARS Prepare for HLB

Peggy A. Mauk, Marylou Polek, Elizabeth Grafton-Cardwell, James E. Adaskaveg, Tracy L. Kahn, Georgios Vidalakis, Mikeal Roose, Wenbo Ma, Carolyn Slupsky and Tim Gottwald

Since the first Asian citrus psyllid (ACP) was found in 2011 on a backyard tree in Riverside, California, the University of California, Riverside (UCR) Agricultural Experiment Station-Citrus Research Center has been developing the best integrated approaches possible to prevent, minimize or eliminate the impact of the devastating disease huanglongbing (HLB) on UCR's Citrus Research Center and possibly for the entire California citrus industry. UCR has active research programs to solve HLB, which is associated with the bacterium *'Candidatus* Liberibacter asiaticus' (CLas). We used an integrated approach to address the threat of HLB on the UCR research station including biological control and insecticides, early detection of CLas, bactericides (also known as antibiotics), cross-protection using Citrus dwarfing viroid, breeding for resistance and building protective structures to enclose germplasm collections.

During the past three years, we have been developing a program for UCR's citrus research station to identify and treat or eliminate infected trees years before CLas is detected using quantitative polymerase chain reaction (qPCR), the molecular process that is used to detect CLas DNA in the plant. This qPCR test requires the bacterium to be in the leaf that is tested. The problem with this approach is that when only a few leaves are infected, finding a positive leaf in a tree with up to 100,000 leaves has extremely poor odds. To improve these odds, the program under development by the UCR/ARS team employs the U.S. Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) HLB-sniffing canines from Florida as part of an early detection system. The canines are trained to sit next to putative positive trees and may be focused on either the odor of the diseased tree or the bacterial pathogen itself. Canine teams can survey large blocks of citrus trees more rapidly than humans can conduct a visual examination of a tree or collect tissue samples and perform laboratory diagnostics. Our intent is to use the canines to target potentially infected trees so that resources can be utilized more efficiently. Instead of conducting a qPCR test of every single tree in a research block or orchard, the more time consuming and costly diagnostic assays would be performed only on the trees the canines sat by. For this article, we are referring to trees that the canines identified as putative positive as "canine alert" trees.

Early Detection Using Trained Canine Teams

On February 28, 2017, the canine team was brought to UCR, and 2,999 trees were surveyed by scanning the perimeters of several research blocks of various citrus types, tree by tree. Some blocks had no canine alerts, others had up to five percent alerts. Overall, canines alerted on three percent of the trees surveyed. Since that time, these alert trees have been monitored using three different early detection technologies (EDTs), including the detection of bacterial structural proteins, bacterial-secreted proteins and plant metabolites. These methods were compared to the USDA-APHIS-approved diagnostic qPCR method. The assay using bacterial structural proteins had the most agreement with the canine alert trees, followed by plant metabolites, and the secreted protein assay had the least agreement with the canine-alerted trees. However, the results of the structural protein, the plant metabolite and the secreted protein assays did not always agree with each other. After a series of three sample collections over the course of one year and qPCR testing, all samples have tested negative.

In March 2019, the canine team returned to California, and 3,903 trees were surveyed at UCR and 2,577 trees at the Lindcove Research and Extension Center (LREC). Since UCR is surrounded by an urban area where ACP populations are relatively high, and is currently included in an HLB quarantine zone, it was not surprising that among the 3,903 trees surveyed at UCR, there were eight percent canine alerts. Although UCR has a stringent proactive psyllid treatment program in place (late summer, fall, winter and pre-harvest treatments) and there are no indications of ACP breeding populations, feeding injuries still can be found in research orchards, suggesting that psyllids are present. The LREC is located in the central San Joaquin Valley where, in contrast to Riverside, psyllids have not become established, nor has HLB been found. In the canine team survey at LREC, there were no canine alerts.

Immediately after the 2019 canine survey, we collected leaves from the canine-alert trees for the fourth time. Whereas the lab team is still in the process of performing the qPCR assays, all samples tested so far had negative results for CLas. Results also are forthcoming for the other EDTs. The fifth round of sample collection will commence this fall once daytime temperatures drop below the mid 90s.

At UCR, there was a strong pattern of canine alerts adjacent to the urban–ag research station interface. Additionally, canine alerts were prominent on the west and south borders (prevailing winds are from the southwest). These patterns are very similar to those of other psyllid infestations matching prevailing winds mapped by Mamoudou Sétamou, Ph.D., of Texas A&M University, Kingsville in Texas citrus groves (Sétamou 2019).

To determine if canines alert to other pathogens or because of a previous canine alert, several situations were tested prior to the HLB citrus orchard surveys. There was no correlation between alerts and the presence of other pathogens including *Phytophthora*, viroids, *Citrus tristeza virus* or *Spiroplasma citri*. In 2019, we also addressed the question of whether a canine will alert on a tree because they sense that another canine sat previously. For this test, three canines were used. The first canine surveyed one row of trees and alerted on one tree. The alerted tree was marked via GPS so that there were no visible flags. The second canine was commanded to sit next to each tree in the same row. The third canine surveyed the same row and alerted only on the tree that the first canine identified as potentially positive for HLB. This test has been done multiple times in different locations. Our conclusion was that the detection canines were not influenced by other canine activities.

Integrating Bactericides with Other Management Practices

At UCR, researchers also are determining if bactericides can be a viable option for controlling HLB in California. There are several small-scale trials that are ongoing, and we have collaborated to survey these using the canines. Bactericides have been widely applied throughout Florida with highly variable efficacy, and researchers are focused on explaining why this is the case. One explanation could be that in plant agriculture, disease management is most successful when bactericides and fungicides (antimicrobials) are used as protective and not as curative treatments. In Florida, however, bactericides were first used in advanced disease stages of a widespread epidemic. Because the disease incidence is still very low in California, the proactive use of bactericides may prevent the disease from developing. Cost-effective and discriminatory strategies used for applying insecticides, such as treating orchard perimeters or treating the prevailing windward side of an orchard, also can be adopted for bactericides.

An Integrated HLB Management Program

At this time, southern California is at risk for a major outbreak of HLB in commercial orchards. The current available methods of reducing the impact and spread of the disease are to regularly use registered insecticides for controlling ACP, construct barriers to psyllid establishment, treat perimeters with registered bactericides, full orchard insecticide treatments, and remove infected trees as soon as they are diagnosed. Research at the UCR research station and Texas A&M indicate that the majority of ACP detections and canine alerts occur on grove perimeters and near the urban interface. Thus, the strategic use of canines and other early detection methods and the application of registered insecticides and bactericides for managing ACP and CLas

HLB-sniffing canine Bello alerting on an HLB suspect tree at the UCR Research Station in Riverside.

may help protect commercial citrus trees from HLB in the near term by preventing disease establishment and providing much needed time for development of long-term solutions such as breeding for resistance in citrus to the HLB threat.

References

Sétamou, M. 2019. ACP barrier and border treatment work [PowerPoint presentation]. 2019 Citrus Growers Educational Seminar Series.

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UNTIL THEY FIND A CURE... WE CAN PROVIDE A CONTROL

BEFORE – OCT 2006

AFTER – APR 2012

The grove above was diagnosed with HLB in 2006. Our team worked directly with the grower to develop a program for his groves to strengthen and protect his trees. It combined K-PHITE 7LP and other Plant Food Systems nutritionals, along with psyllid control. The photo on the right is the same row in the same grove six years later – healthy and productive.

Plant Food systems

KPHITE® 7LP

SYSTEMIC FUNGICIDE BACTERICIDE

Finding a cure is a long term effort at best. And when and if a cure is eventually found, its implementation may take years to actually execute. In the meantime, it does not make sense to go totally unprotected when your company or your family's livelihood is at stake. K-PHITE 7LP Systemic Fungicide/Bactericide in combination with superior nutritional programs has shown excellent control that is supported by years of positive University of Florida Research. Trees that are symptomatic have actually been returned to asymptomatic producing quality fruit on vigorously growing trees.

An early preinfection program with K-PHITE 7LP will also provide valuable results in the control of Phytopthora Gummosis, Phytopthora Root Rot, Pythium, Dry Root Rot, Brown Rot, and Foaming Canker. K-PHITE 7LP is an EPA and California registered biopesticide providing a strong and effective management tool for today's citrus grower while being sensitive and eco-friendly to the environment.

While we completely support the future possibilities of research, ***UNTIL THEY FIND A CURE... WE CAN PROVIDE A CONTROL.***

To find out more about K-PHITE 7LP and K-PHITE 7LP Programs, contact your California Plant Food Systems Representative directly: Mark Brady – Western Marketing Manager 559 731-1267.

A Hamlin sweet orange tree in decline due to severe HLB infection in a Florida citrus grove.

Current Efforts to Culture '*Candidatus* Liberibacter asiaticus' *in vitro*

Mojtaba Mohammadi, Joey S. Mayorquin and Melinda Klein

'Candidatus *Liberibacter asiaticus' (CLas) is a bacterium associated with huanglongbing (HLB), a devastating citrus disease currently threatening the California citrus industry. CLas is a 'fastidious bacterium,' meaning it is difficult to cultivate* in vitro[1] *and cannot survive outside of its citrus host or insect vector, the Asian citrus psyllid (ACP). Several research teams have been working to culture CLas on artificial media over the last decade; however, these attempts have resulted in limited success in mimicking appropriate growth conditions, including environmental conditions, both physiologically and nutritionally. While that work continues, alternate approaches to culturing CLas* in vitro *are being pursued to develop disease mitigation strategies.*

Culturing Unculturable Bacteria

Plant bacteria occupy a wide range of ecological niches in their hosts to obtain nutrients necessary for growth and survival. Some bacteria are beneficial, such as Rhizobia[2], which fix atmospheric nitrogen into ammonium in legumes. Other bacteria are pathogenic, such as *Xanthomonas axonopodis* pv. *citri* (citrus canker), which can be grown on plant tissue releasing enzymes that break down plant cell walls to form lesions leading to defoliation, fruit drop and tree decline. Two examples of pathogenic bacteria that live in vascular bundles include *Xylella fastidiosa*, a bacterium that lives in xylem vessels[3] and causes citrus variegated chlorosis, and *Spiroplasma citri*, the causal agent of stubborn disease of citrus, which can be found in phloem tissue. These bacteria are fastidious, but due to progress made with *in vitro* culturing techniques, they now can be grown on artificial media. Culturing these previously unculturable bacteria on artificial media has helped advance research (Rapicavoli et al. 2017) and deepened our knowledge of disease management.

To grow CLas on synthetic media, researchers need to mimic the physiological and nutritional conditions that exist inside the phloem cells where CLas resides (**Figure 1**). Environmental conditions such as pH (acidity/alkalinity), oxygen tension, nutrients, osmotic pressure and energy level in the phloem and the psyllid hemolymph (a fluid in insects that is analogous to mammalian blood) are taken into consideration when developing culture media to support free-living growth of CLas *in vitro* (Merfa et al. 2019).

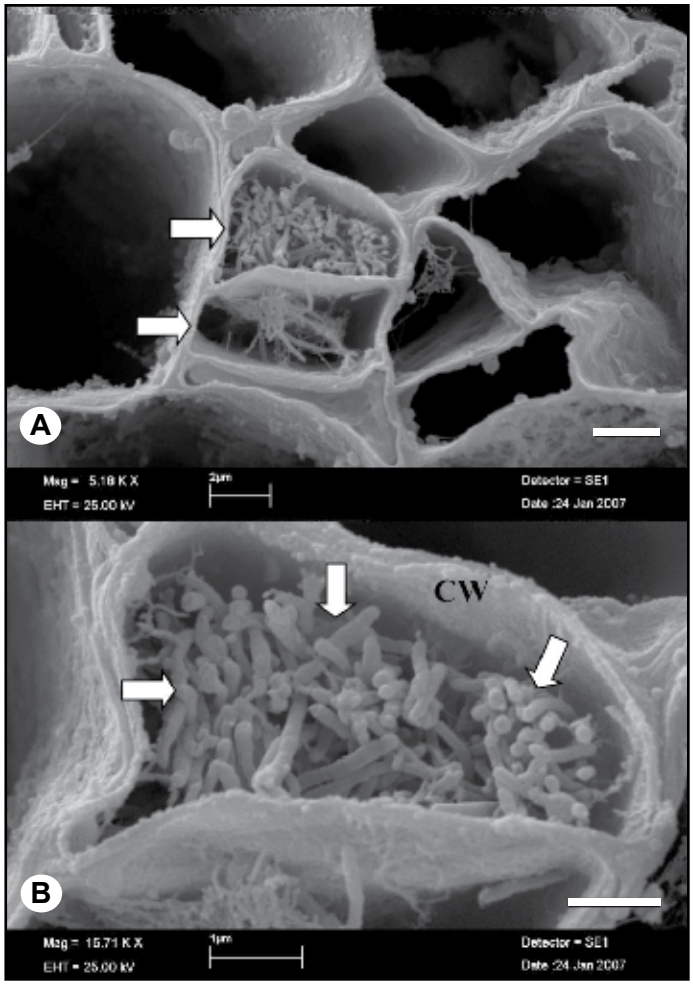

Figure 1. A) Scanning electron micrograph of phloem vessels (arrows) in cross section in a frozen-fractured leaf midvein of a periwinkle (Catharanthus roseus) experimentally infected by 'Candidatus Liberibacter americanus' using dodder. One of the vessels is filled by bacilliform cells of 'Candidatus Liberibacter americanus' (bar = 2 .µm). B) Detail of A, showing the bacterial cells (arrows) within the phloem element (bar = 1.µm).
Image credit: F.A. Tanaka

Why is Culturing CLas so Important?

Culturing CLas is a priority of the research community for several reasons. By culturing this bacterium:

1. *Scientists can confirm that CLas is the causal agent of HLB*. To date, CLas has not been "proven" to be the pathogen responsible for HLB disease symptoms. One of the criteria to establish the causative relationship between a microorganism and a disease (known as Koch's postulates), requires a pure culture of that organism. Inoculation of the isolated CLas bacterium into a healthy citrus tree should result in the development of shoot chlorosis and leaf blotchy mottle, typical of HLB symptoms (**Figure 2**).
2. *Disease-causing genes from CLas can be identified and characterized.* It is critical to learn how CLas suppresses citrus and psyllid defenses to overcome citrus host defense mechanisms (Bendix and Lewis 2018). Understanding CLas pathogenicity (disease causing) genes and CLas genes involved in transmission by psyllid can focus strategies to manage and mitigate HLB in California such as developing molecular blockers to disrupt small molecules (proteins, RNAs) involved in CLas acquisition and transmission and to treat HLB.
3. *The efficacy of antimicrobial compounds on CLas can rapidly be screened.* Having a pure culture can significantly streamline the process of screening antimicrobial compounds (*i.e.*, bactericides, proteins) against CLas. Researchers currently use biofilms (CLas in association with other bacteria), related bacterial species like *Liberibacter crescens* and plant-based assays to conduct such studies (see **"Current alternate approaches in CLas research"** below), but these assays are not as straightforward and efficient as a direct assay would be.

Challenges in Culturing CLas with Synthetic Media

Researchers have been making efforts to culture CLas for years. There are a number of potential theories about the "pickiness" of this bacterium and why it hasn't yet been grown outside of plant and insect hosts including:

1. complex nutritional requirements,
2. reliance on other microorganisms for necessary nutrients and energy,
3. genes essential for free-living growth may have been lost and
4. activation of virus-like genes within CLas when the bacterium is transferred to a synthetic medium resulting in death (Bendix and Lewis 2018; Merfa et al. 2019).

Previous attempts to isolate CLas in pure form have met with limited success. These included co-culturing CLas with another bacterium (Davis et al. 2008) or phloem microbiota (Fujiwara et al. 2018) and growing CLas in growth media in the presence of citrus vein extract (Sechler et al. 2009) or citrus juice (Parker et al. 2014). Biofilm formation in juice cultures indicated that CLas is dependent on other microorganisms for obtaining additional nutrients, chemical signals, energy or some other environmental modifications to grow efficiently *in vitro* (Parker et al. 2014).

Current Alternate Approaches in CLas Research

While research is ongoing to culture CLas outside of plant and insect hosts, several alternative methods are being evaluated for testing antimicrobials and characterizing CLas pathogenicity genes.

Biofilms. Published reports indicate that CLas is dependent on other microorganisms in the phloem to obtain necessary nutrients and energy for survival and growth (summarized in Merfa et al. 2019). Scientists have been able to produce a CLas biofilm *in vitro*, consisting of a mixture of CLas and other bacteria (Gang et al. 2019). CLas biofilm has been extracted from ACP and used to test for antimicrobial efficacy indirectly (Krystel et al. 2019).

Hairy root culture. Hairy root cultures are a type of plant tissue culture in which citrus roots are grown in liquid media, transformed with *Agrobacterium rhizogenes* to induce continuous root growth and then inoculated with CLas-containing mixtures from plants or insects to mimic infected plant material. This *in vitro* assay system allows for high throughput screening of possible antimicrobial compounds for effectiveness in reducing CLas growth, but also to avoid potential adverse effects – like plant toxicity – before testing in the greenhouse or field (Mandadi et al. 2019).

Single leaf assay. Single citrus leaf assay is another approach to study the efficacy of antimicrobial compounds against CLas. In this case, petioles of detached leaves from CLas infected plants are kept in a solution containing various concentrations of antimicrobial compounds. CLas titer is then measured at different time points using qPCR and compared to titer before treatment (Robert Shatters, personal communication).

Bioinformatics. Researchers are also working to create a computational model of CLas to identify potential new and effective HLB management tools and strategies. This approach has been used to characterize gene expression and metabolic activity in other bacterial microorganisms (Lerman et al. 2012) and potentially can identify critical metabolites or compounds needed for CLas growth *in vitro*.

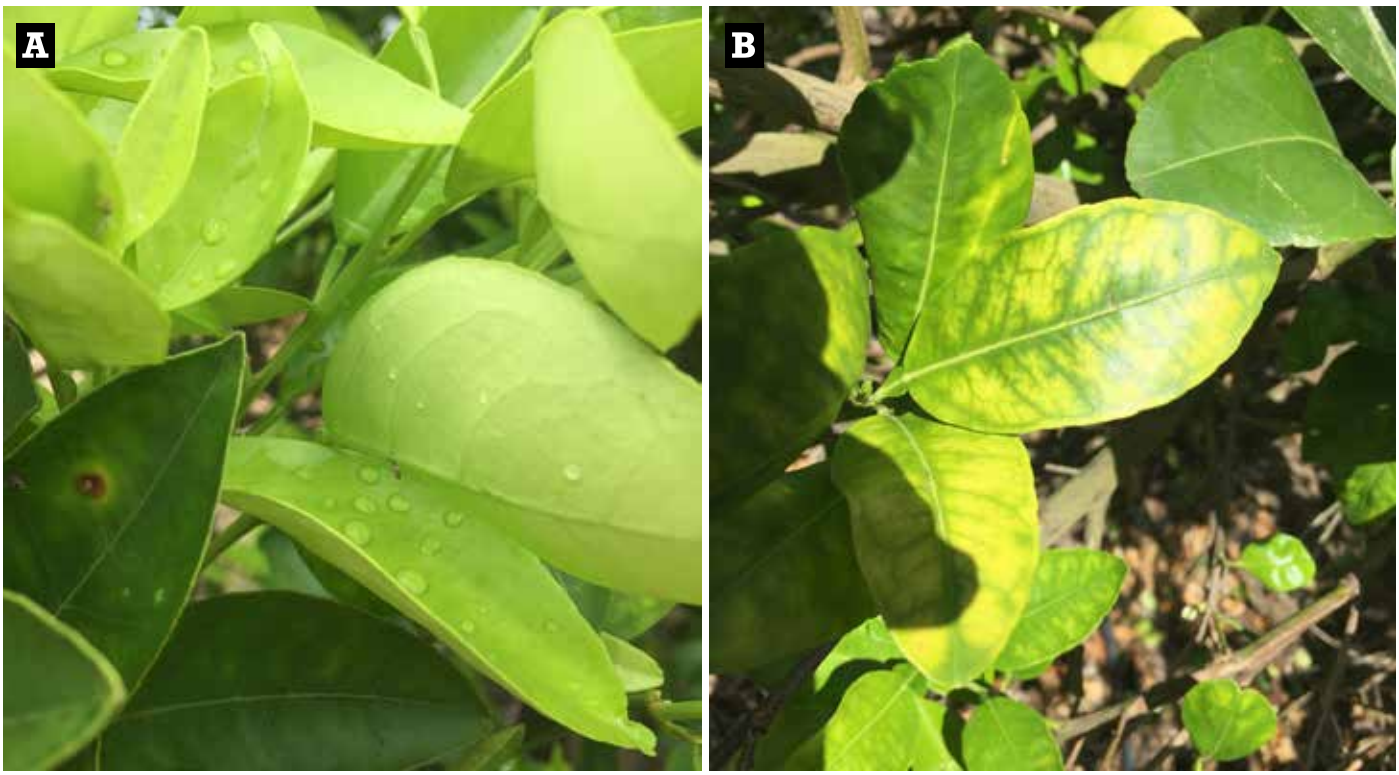

Figure 2. HLB symptoms: A) shoot chlorosis and B) leaf blotchy mottle in Hamlin sweet orange grown on Swingle citrumelo rootstock in Florida.

Related bacterial species. Work currently is underway to complement related non-pathogenic bacteria with CLas genes to identify those factors involved in virulence (aggressiveness) and pathogenicity, as well as a rapid method to screen antimicrobial compounds (Duan et al. 2019). In some cases, related bacterial strains such as *Liberibacter crescens* are being used directly as a surrogate bacterium to test antimicrobials (Naranjo et al. 2019). *Liberibacter crescens* is a non-pathogenic bacterium isolated from mountain papaya that does not live in vascular tissue (Fagen et al. 2014).

Conclusion

In the absence of a successful culturing protocol for CLas, research continues to seek ways to slow the spread and limit the effects of HLB. Once CLas can be cultured outside of its citrus and psyllid hosts, researchers expect to identify weak links in the infection process leading to the development of chemical, biological and genetic control methods to contain HLB in the field. In the meantime, novel ideas and innovative approaches are being developed to overcome the challenges faced by researchers and the citrus industry in the wake of this disease.

References

Bendix, C. and Lewis, J.D. 2018. The enemy within: phloem-limited pathogens. *Molecular Plant Pathology* 19(1):238-254.

Davis, M.J. et al. 2008. Co-cultivation of *'Candidatus* Liberibacter asiaticus' with actinobacteria from citrus with huanglongbing. *Plant Disease* 92:1547-1550.

Duan, Y. et al. 2019. Identification, assessment and delivery of antimicrobial compounds for the management of citrus HLB. *Abstract (IRCHLB-P8-95) in Proceedings of IRCHLB IV*, Riverside, California.

Fagen, J.R. et al. 2014. *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the genus *Liberibacter. International Journal of Systematic and Evolutionary Microbiology* 64:2461-2466.

Fujiwara, K.; Iwanami, T.; Fujikawa, T. 2018. Alterations of '*Candidatus* Liberibacter asiaticus'-associated microbiota decrease survival of *Ca*. L. asiaticus in *in vitro* assays. *Frontiers in Microbiology* 9:1-12.

Gang, D. et al. 2019. Culturing *'Candidatus* Liberibacter asiaticus,' the associated agent of citrus greening disease (huanglongbing, HLB). *Abstract (IRCHLB-O2b-01) in Proceedings of IRCHLB IV*, Riverside, California.

Krystel, J. et al. 2019. An *in vitro* protocol for rapidly assessing the effects of antimicrobial compounds on *'Candidatus* Liberibacter asiaticus.' *Abstract (IRCHLB-P8-89) in Proceedings of IRCHLB IV*, Riverside, California.

Lerman, J.A. et al. 2012. *In silico* method for modelling metabolism and gene product expression at genome scale. *Nature Communications* 3:929.

Mandadi, K. et al. 2019. Hairy root matrix used to culture CLas to rapidly screen antimicrobials for improved HLB management. *https://ucanr.edu/sites/scienceforcitrushealth/Research_Snapshots/Mandadi/*

Merfa, M.V. et al. 2019. Progress and obstacles in culturing *'Candidatus* Liberibacter asiaticus,' the bacterium associated with huanglongbing. *Phytopathology*: *https://doi.org/10.1094/PHYTO-02-19-0051-RVW.*

Naranjo, J. et al. 2019. *In vitro* antimicrobial activity and mode of action of Zinckicide™ against *Liberibacter crescens*, a surrogate of '*Candidatus* Liberibacter asiaticus.' *Abstract (IRCHLB-P8-96) in Proceedings of IRCHLB IV*, Riverside, California.

Parker, J. et al. 2014. Viability of *'Candidatus* Liberibacter asiaticus' prolonged by addition of citrus juice to culture medium. *Phytopathology* 104:15-26.

Rapicavoli, J. et al. 2017. *Xylella fastidiosa*: an examination of a re-emerging plant pathogen. *Molecular Plant Pathology* 19:786-800.

Sechler, A. et al. 2009. Cultivation of *'Candidatus* Liberibacter asiaticus,' *'Ca*. L. africanus' and 'Ca. L. americanus' associated with huanglongbing. *Phytopathology* 99:480-486.

Tanaka, F.A. et al. 2007. Detection of the *"Candidatus* Liberibacter americanus" in phloem vessels of experimentally infected *Cataranthus roseus* by scanning electron microscopy. *Fitopathologia Brasileira* 32(6):519.

Glossary

[1]*in vitro:* A term meaning 'in a laboratory vessel (tube, culture dish, etc.)' and outside the natural setting of a living organism.

[2]Rhizobia: A family of bacteria that convert nitrogen gas from the atmosphere into ammonium nitrogen within root nodules for legumes.

[3]Xylem vessels: Vascular tissue used by plants to move water and dissolved nutrients from the roots to leaves.

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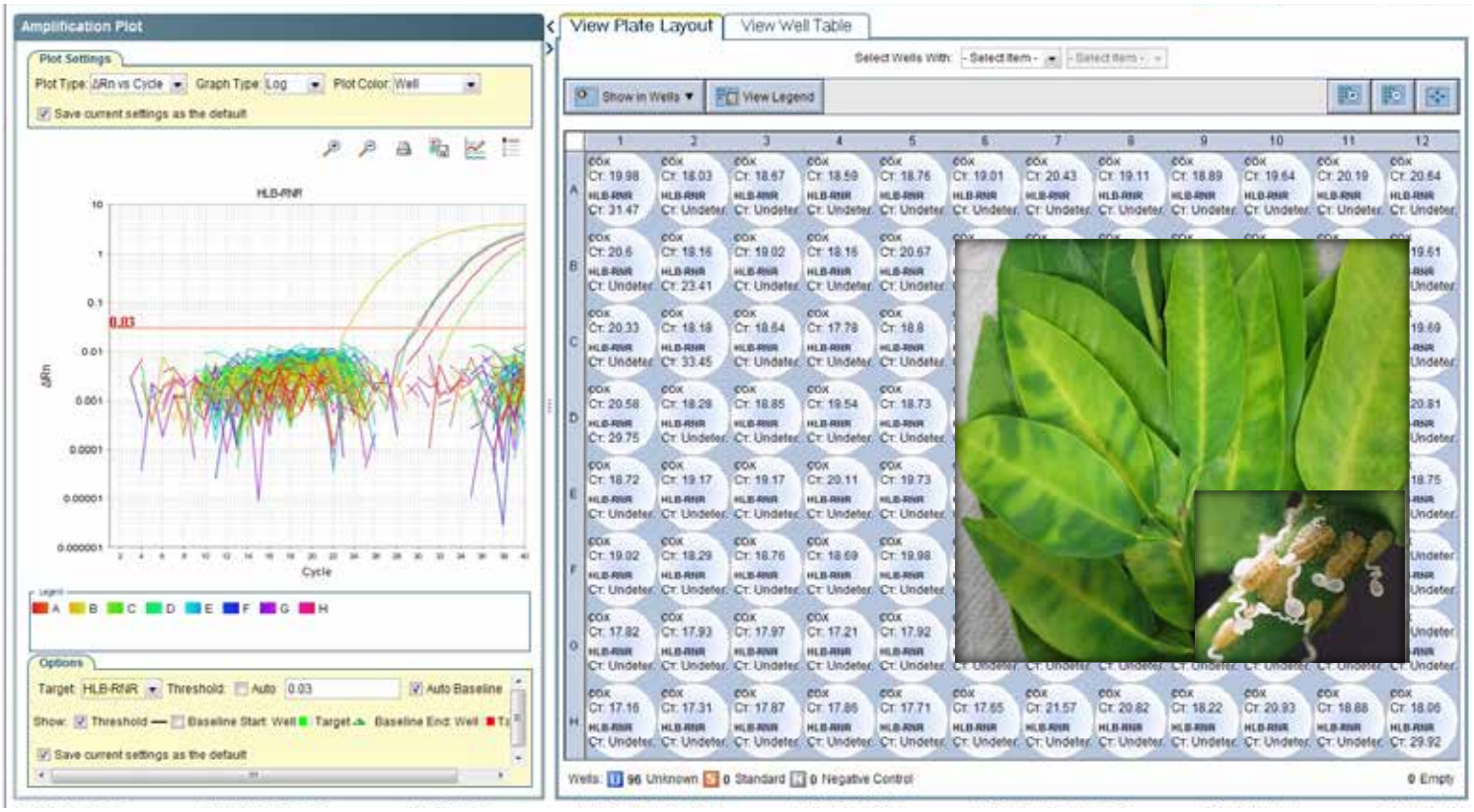

qPCR analysis of DNA extracted from Asian citrus psyllid nymphs collected from citrus leaf flush tissue. **(Image credit: Lucita Kumagai)**

An Update from the Early Detection Technologies Task Force

Robert Atkins and Ed Civerolo

Summary

An ad hoc huanglongbing (HLB) Early Detection Technologies (EDT) Task Force (referred to hereafter as the "Task Force") was established by the Citrus Research Board (CRB) in 2014. The original objective of the Task Force was to assess the potential effectiveness of several techniques to detect 'Candidatus Liberibacter asiaticus' (CLas)-infected or HLB-affected trees before HLB symptoms developed and/or CLas could be detected by quantitative polymerase chain reaction (qPCR)[1]. Several studies were conducted in the field and greenhouses in Texas, California and Florida from 2014 to 2018. Direct detection of CLas by qPCR or by a commercial immunoassay test were, overall, the most promising HLB EDTs in these studies.

Introduction

HLB was first found in Los Angeles County, California, in 2012 (Kumagai et al. 2013), and detection of HLB-affected trees continues to increase in southern California. Currently, HLB is known to occur only in residential areas in the southern part of the state and has not been detected in commercial orchards as of June 2019. Limiting the spread of CLas (the bacterium associated with HLB) is critical for protecting commercial citrus production from HLB.

A challenge posed by CLas/HLB is that once a tree is infected, there is no known effective treatment or cure, and resistant plants are not yet available. Another challenge is that the

disease is difficult to detect until the symptoms become obvious or the bacterium has spread sufficiently enough within an infected tree and is present at high enough levels to be detected. Unfortunately, infected plants are sources of CLas inoculum soon after they are infected. So, roguing of infected, asymptomatic trees can be an effective HLB management strategy, especially in areas in which the disease is not endemic or widespread (Gottwald et al. 2007; Xie et al. 2016).

In 2014, the CRB established the Task Force to assess the potential of several plant disease diagnostic technologies to detect CLas-infected trees before development of HLB symptoms or before CLas can be detected by qPCR. HLB symptoms may be difficult to distinguish from other abiotic and biotic stresses.

Prior to the formation of the Task Force, there had been surprisingly little research into early detection of the disease. Because of several policy decisions, HLB was found throughout Florida soon after the initial detection. HLB symptoms originally were described for citrus in Asia more than a century ago, and the disease was so entrenched that there was no need for early detection. Tree roguing was only done when trees produced bitter fruit or dropped so much of the crop as to be useless. PCR provided a tool for identifying trees before that stage of infection, but California's fresh-marketed fruit created a need to find HLB earlier.

Studies to evaluate potential HLB EDTs were conducted in 2014-2018 in Texas, California and Florida. Analyses of the comparative performance results of five of the most promising were presented to joint meetings of the Task Force, the CRB Vectored Diseases Research Committee and the Research Development and Implementation Committee in February and May 2019 by Neil McRoberts, Ph.D. and Brianna McGuire.

The main challenge for direct detection methods is to collect samples of infected tissue from diseased trees. CLas is not uniformly distributed in asymptomatic trees, at least not in the early stages of disease development (Bové 2006; Gottwald 2010; Li et al. 2009; Park et al. 2018). However, if sampling limitations can be overcome, these tests can enhance early detection of HLB-diseased trees as they conclusively identify the presence of CLas.

The main challenge for indirect detection methods is to identify the symptoms or physiological changes of infected trees that are specific to CLas infection, not other responses to biotic or abiotic stressors. Therefore, if citrus host responses specific to CLas infection can be identified, the diagnosis of HLB can be reliably confirmed without having to rely on trying to find CLas-infected tissue (e.g., leaves) to sample for testing.

Based on presentations made to the Task Force in February and May 2019, 12 HLB EDTs were evaluated based on the results of eight studies in California, Florida and Texas conducted between 2014 and 2018. The results of five of these HLB EDTs were analyzed in the McRoberts laboratory at University of California, Davis (*see California-based EDT Experiment* on page 44 and *Florida-based Experiment Demonstrates Earliness and Accuracy of EDTs* on page 50). The most promising direct methods to detect CLas in these studies were tested for the presence of bacterial DNA by qPCR (Li et al. 2006) or outer membrane proteins of the CLas bacterium (Ding et al. 2016; Rider et al. 2003; **Figure 2**) or proteins released from the CLas bacterium (Franco et al. 2017; Pagliaccia et al. 2017). Indirect methods to diagnose HLB, based on host responses to CLas infection or HLB disease, were tested for changes in the chemical composition of the plants (metabolome) (Chin et al. 2014; Chin et al. 2015; Slisz et al. 2012) and changes in the microorganisms on the leaf surface (microbiome) (Leveau and Rolshausen 2016).

Detection Technologies

Early detection technologies (or EDTs) can broadly be classified as either direct or indirect bacteria detection methods. Methods to directly detect CLas include PCR, which measures the amount of bacterial DNA present, or immunoassays, which measure the amount of bacterial-specific proteins present. Indirect detectors identify plant responses to CLas infection rather than the bacterium itself . These include metabolite changes, changes in the microbiota (microbiomes)[2] on leaf surfaces, volatile organic compounds (VOC's, **Figure 1**) emitted from leaves (including odors detected by canines), and changes in light reflected from leaves (spectral imaging).

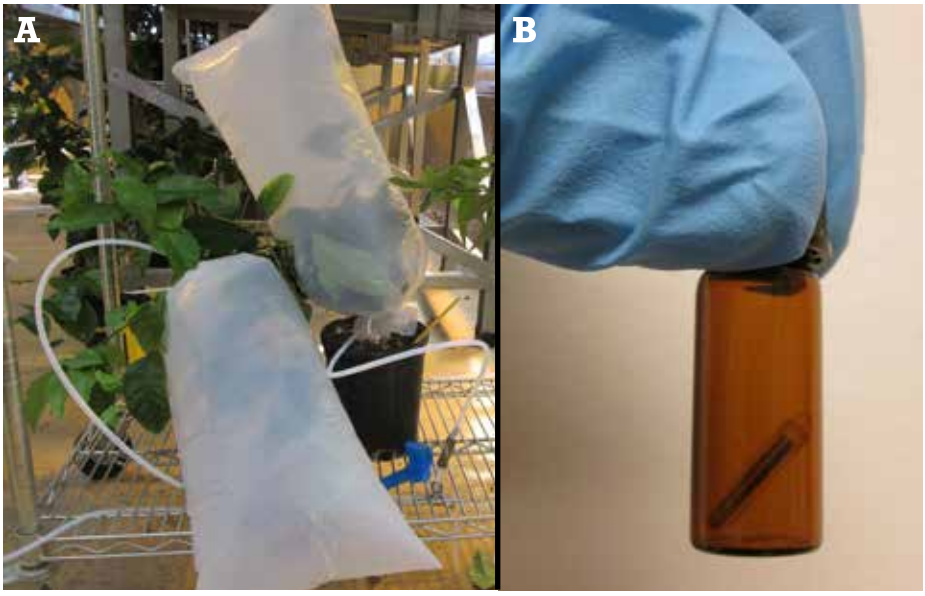

Figure 1. (A) Sampling of volatile organic compounds in a greenhouse environment requires the use of bags. (B) The Twisters used to sample the volatile organic compounds are placed in a sterile vial until the compounds are analyzed.

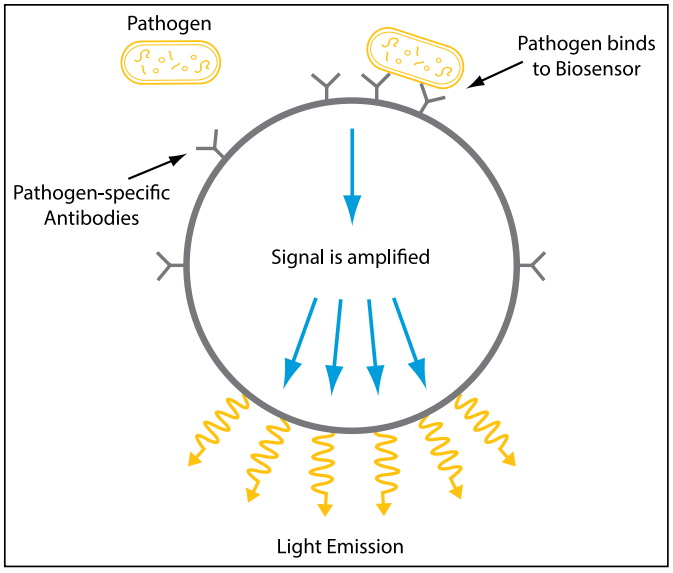

Figure 2. CANARY (Cellular Analysis and Notification of Antigen Risks and Yields) is a cell-based biosensor technology licensed by PathSensors, Inc. and is an example of an immunoassay, which delivers extremely rapid and sensitive detection of pathogens (such as CLas) as compared to other immunoassays. If pathogens are present, they bind to target-specific cell surface antibodies on the biosensor, initiating an intracellular signaling cascade that results in the release of calcium ions. Aequorin, a calcium-responsive luminescent protein, senses this calcium flux, which results in the release of light. PathSensors' instruments measure the light output and report if the target is present in the sample. **(image credit: PathSensors, Inc.)**

Results

Generally, qPCR using the 16S gene primers (Li et al. 2006) and the immunoassay using a monoclonal antibody[3] against a CLas cell surface protein (Rider et al. 2003) had the highest overall performance ratings based on the analyses above. Metabolomic profiling (Chin et al. 2014; Chin et al. 2015), leaf surface microbiome profiling (Leveau and Rolshausen. 2016) and serological detection of CLas outer membrane using a polyclonal antiserum[4] (Ding et al. 2016) or CLas-secreted proteins also may be useful for HLB diagnosis. Use of more than one HLB EDT, as noted by Neil McRoberts, Ph.D., and in accordance with international guidelines for plant pest diagnostic protocols (IPPC 2016), may be the best strategy for increasing the reliability of detecting asymptomatic CLas-infected trees.

Diagnostic Improvements

The Task Force asked if sampling the flush where nymphs were found could point to CLas-infected tissue rather than the then-approved work instructions of sampling HLB symptomatic tissue. Greg McCollum, Ph.D., showed that it is possible to detect CLas in ACP nymphs where the infected adults were found feeding. The California Department of Food and Agriculture (CDFA) is now developing the field procedures and tissue selection process to use when flush and nymphs are found. Additionally, the CDFA now has determined that identification of an infected nymph indicates that the tree has been exposed to CLas and will be removed as a public nuisance.

Over the course of the EDT Task Force, improvements also have been made in qPCR – the most widely used technique for HLB diagnosis. A new set of primers, referred to as the RNR primers, were developed (Zheng et al. 2016) and validated by the U.S. Department of Agriculture-Animal and Plant Health Inspection Service and the California Department of Food and Agriculture (CDFA) for CLas detection. Use of the RNR primers in qPCR assays increased sensitivity of CLas detection three-fold (pers. comm., Lucita Kumagai, CDFA). Furthermore, use of the RNR primers in qPCR increased specificity and reliability, significantly reducing cross-amplification of the target DNA sequence of non-HLB bacteria by about 60 percent (Zheng et al. 2016).

References

Bové, J.M. 2006. Huanglongbing: a destructive, newly emerging, century-old disease of citrus. *Plant Pathology* 88(1):7-37.

Chin, E.; Lobo, R.; da Graça, J.; et al. 2015. Early detection of HLB with metabolomics. *Citrograph* 6(2):32-34.

Chin, E; Mishchuk, D; Bruce, J.; et al. 2014. An interdisciplinary approach to combat HLB: research in UC Davis' Contained Research Facility. *Citrograph* 5(1):28-34.

Ding, F.; Yongping, D.; Yuan, Q.; et al. 2016. Serological detection of '*Candidatus* Liberibacter asiaticus' in citrus, and identification by GeLC-MS/MS of a chaperone protein responding to cellular pathogens. *Scientific Reports* (6):29272 *https://www.nature.com/articles/srep29272*

Franco, J.; Thapa, S.; Pagliaccia, D.; et al. 2017. The citrus response to '*Candidatus* Liberibacter asiaticus' infection. *Citrograph* 8(2):32-36.

Gottwald, T.R. 2010. Current epidemiological understanding of citrus huanglongbing. *Annual Review of Phytopathology* 48:119-139.

Gottwald, T.R.; da Graça, J.V. and Bassanezi, R.B. 2007. Citrus huanglongbing: the pathogen and its impact. *Plant Health Progress* 8(1):31. *Online doi:10.1094/PHP-2007-0906-01RV*.

International Plant Protection Convention (IPPC). 2016. International Standard for Phytosanitary Measures 27-Diagnostic protocols for regulated pests. *International Plant Protection Convention*, Rome, FAO. 11 pages.

Kumagai, L.B.; Levesque, C.S.; Bloomquist, C.L; et al. 2013. First report of '*Candidatus* Liberibacter asiaticus' associated with citrus huanglongbing in California. *Plant Disease* 97(2):283.

Leveau, J. and Rolshausen, P. 2016. A Microbiota-based approach to citrus tree health. *Citrograph* 7(2):60-63.

Li, W.; Hartung, J.S. and Levy, L. 2006. Quantitative real-time PCR for detection and identification of '*Candidatus* Liberibacter' species associated with citrus huanglongbing. *Journal of Microbiological Methods* 66(1):104-115.

Li, W.; Levy, L. and Hartung, J.S. 2009. Quantitative distribution of '*Candidatus Liberibacter* asiaticus' in citrus plants with citrus huanglongbing. *Phytopathology* 99(2):139-144.

Pagliaccia, D.; Shi, J.; Pang, Z.; et al. 2017. A pathogen secreted protein as a detection marker for citrus huanglongbing. *Frontiers in Microbiology* (8):2041. *https://doi.org/10.3389/fmicb.2017.02041*

Park, J-W.; Louzada, E.S.; Braswell, W.E.; et al. 2018. A new diagnostic real-time PCR method for huanglongbing detection in citrus root tissue. *Journal of General Plant Pathology* 84(5):359-367.

Rider, T.H.; Petrovick, M.S.; Nargi, F.E.; et al. 2003. A B cell-based sensor for rapid identification of pathogens. *Science* 301(5630):213-215.

Slisz, A.M.; Breksa, A.P.; Mishchuk, D.O.; et al. 2012. Metabolomic analysis of citrus infection by '*Candidatus* Liberibacter' reveals insight into pathogenicity. *Journal of Proteome Research* 11(8):4223-4230.

Xie, F.; Gao, S.; Yu, D.; et al. 2016. A citrus huanglongbing model with varying pulse rouging and general incidence. *Journal of Advances in Applied Mathematics* 1:222-233.

Zheng, Z.; Xu, M.; Bao, M.; et al. 2016. Unusual five copies and dual forms of *nrdB* in '*Candidatus* Liberibacter asiaticus": Biological implications and PCR detection amplification. Scientific Reports (6):39020. *https://doi.org/10.1038/srep39020*

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Glossary

[1]Quantitative PCR (qPCR): A molecular method to amplify and make multiple copies of a specific DNA segment, which is quantitatively monitored in real time by measuring fluorescence.

[2]Microbiota (Microbiomes): All the microorganisms, including viruses, bacteria and fungi, in a specific environment or habitat (e.g., leaf surfaces here).

[3]Monoclonal antibody: A type of protein that binds or adheres to only one site on a substance, such as a bacterial protein. The high specificity of a monoclonal antibody to bind or adhere to a single site on the target (e.g., CLas surface protein here) reduces the probability of cross-reactivity with other bacteria.

[4]Polyclonal antiserum: A mixture of antibodies taken from blood serum that bind to multiple locations of a substance (such as a bacteria protein). By adhering to multiple locations, the detection capabilities of the antibodies increase.

California-based EDT Experiment

Baseline Data Collection and Quarantine Zone Testing

Brianna McGuire and Neil McRoberts

Project Summary

The purpose of this study was to examine whether a group of potential early detection technologies (EDTs) for pre-symptomatic diagnosis of huanglongbing (HLB) were specific to HLB or could be triggered by other citrus diseases. The study was aligned with a larger HLB Multi-Agency Coordination (MAC) Group-funded project, designated California-1, that was aimed at establishing baseline data for healthy citrus in California based on a stratified random sample of 1,000 trees from commercial orchards across the state. The Citrus Research Board (CRB)-supported research project described here was composed of two studies that augmented the larger baseline project; this larger project was designated CA-1 because it was the first large-scale EDT study carried out in California.

*The first study was CA-1b Quarantine Zone (QZ), which sought to examine the accuracy of the EDTs using samples from the HLB QZ previously tested by the California Department of Food and Agriculture (CDFA) in Sacramento. CA-1b Non-QZ (NQZ) was designed to test whether the EDTs would produce diagnostic errors in trees infected with pathogens other than '*Candidatus *Liberibacter asiaticus.' The results showed that five out of six EDTs improved their process following collection and analysis of the baseline measurements. EDTs had a wide range of accuracy in classifying samples from the quarantine zone, and the most accurate tests from that study were a commercial antibodies test and a quantitative polymerase chain reaction (qPCR) performed by the McCollum lab.*

Introduction

Once the first case of HLB was detected in California in 2012, stakeholders understood that early detection would become a part of their strategy to stop the spread of the disease. A field experiment carried out in Texas identified promising early detection methods – tests that indicate the presence of HLB before signs or symptoms of the disease can be seen. However, since many promising methods are influenced by environmental conditions, the Texas experiment highlighted the need for field studies based in California. In addition, the leading methods from Texas relied on looking for changes in infected trees compared with a known baseline profile for healthy trees. Because the profiles of healthy trees show some variation from one tree to the next, it is important to know what range is typical so that healthy trees can be distinguished from trees with HLB. Data for that baseline in California were lacking, and no systematic effort had been made previously to collect data from healthy California trees. These factors provided the justification for the CA-1 study, which was funded by the U.S. Department of Agriculture-Animal and Plant Health Inspection Service (APHIS) HLB MAC Group, with additional support from the CRB.

CA-1 was conducted from March 2017 through January 2018 to collect leaf samples from 1,001 healthy citrus trees located in California commercial citrus production areas. The purpose of the CA-1 experiment was to provide baseline data from a variety of cultivars and locations more appropriate to California commercial citriculture (**Figures 1**, **2** and **3**).

CA-1 compared six different EDTs :

1. metabolome profiling – the relative abundance of a set of plant metabolites is measured,
2. phytobiome profiling – the relative abundance of different types of microorganisms on the leaf surface is measured,
3. qPCR (experimental) – the abundance of pathogen DNA is measured,
4. ELISA – an antibody assay to detect pathogen secreted proteins,
5. CANARY – A commercial antibody-based assay to detect CLas membrane proteins and
6. volatile profiling – the relative abundance of volatile compounds is measured.

In addition to testing samples for CLas/HLB, the Vidalakis lab at the University of California, Riverside (UCR) tested all samples for viruses, viroids and spiroplasmas (VVS).

Eighty leaf samples were collected from the CA-1bNQZ study in December 2017 and January 2018 from trees within the Central Valley that were known to be infected with two other non-HLB diseases, citrus tristeza and citrus stubborn disease (CSD), to assess the capacity of the EDTs to filter out non-HLB disease signatures. A total of 289 samples, some HLB-positive, were collected for the CA-1bQZ study in 2017 and 2018 to re-assess the accuracy of EDTs in California.

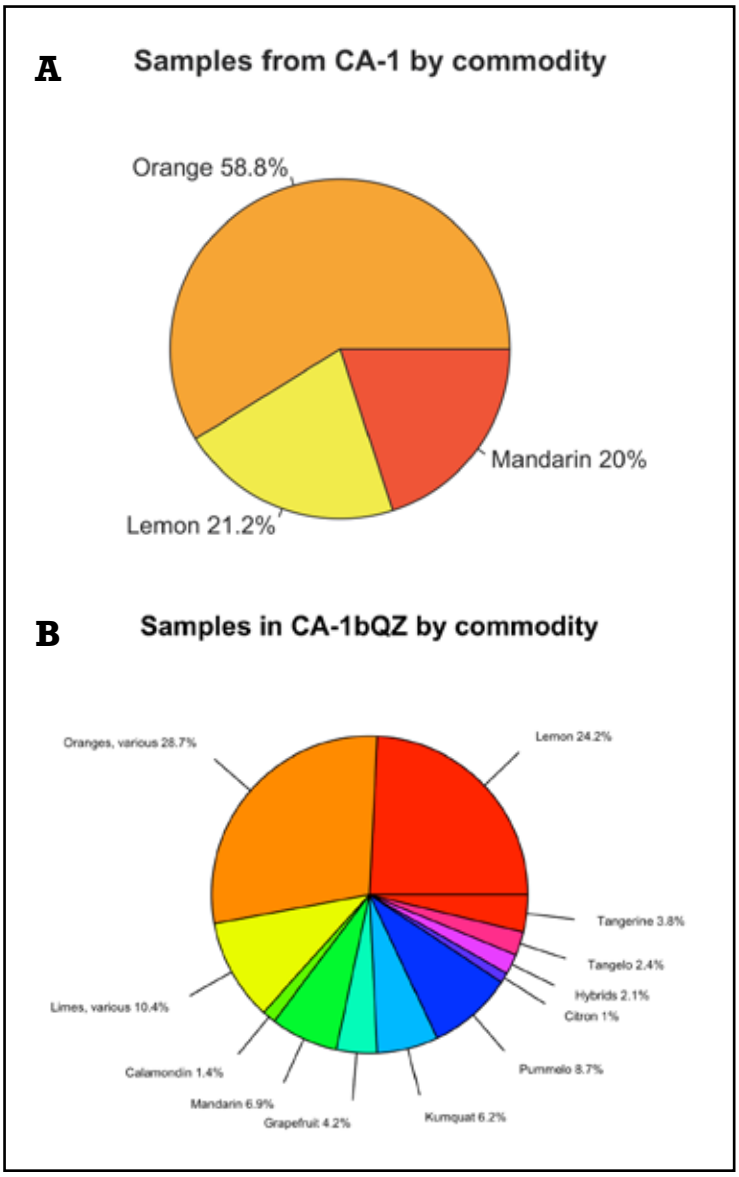

Figure 1. Distribution of samples collected by commodity for (a) CA-1 and (b) CA-1bQZ studies. Orange was the only commodity collected for the CA-1bNQZ study.

The McRoberts lab (University of California, Davis) was the primary coordinating lab responsible for grower recruitment for the project and mailing all CA-1 and CA-1bNQZ leaf samples to the Vidalakis lab at UCR. Field collectors were accompanied by CDFA staff, who also collected a regulatory sample for reference purposes from every tree in CA-1 and CA-1bNQZ.

The McRoberts lab also oversaw the analyses and reporting of results to the CRB EDT Task Force and APHIS for the HLB MAC. The Vidalakis lab processed all leaf samples in CA-1 and CA-1bNQZ, sent the samples to EDT labs, retained extra tissue for reference and tested this tissue using their VVS panel.

The CDFA collected CA-1bQZ samples from the quarantine zone. The tissue for these samples was treated similarly to those processed by the Vidalakis lab, but processing was handled at the CDFA lab in Sacramento.

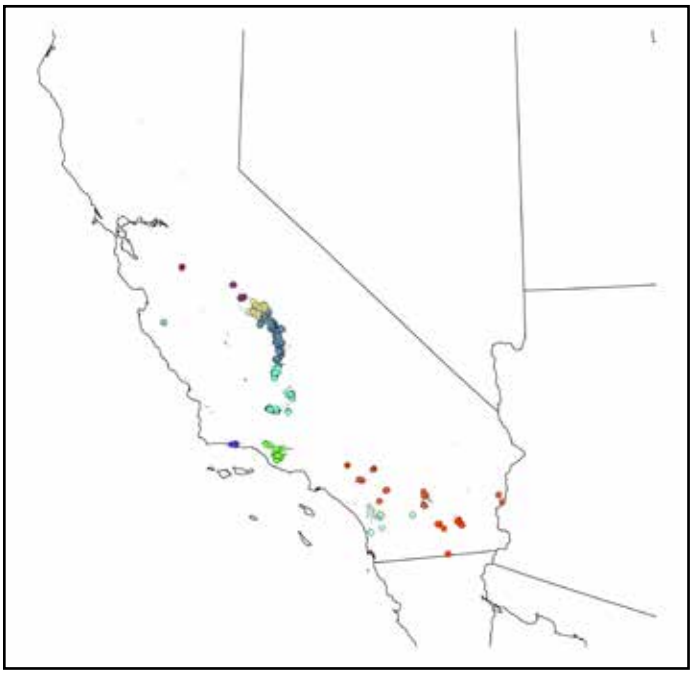

Figure 2. Sample locations across each county in California. Samples were taken proportionally from each county based on county citrus acreage. Different colors correspond to different counties.

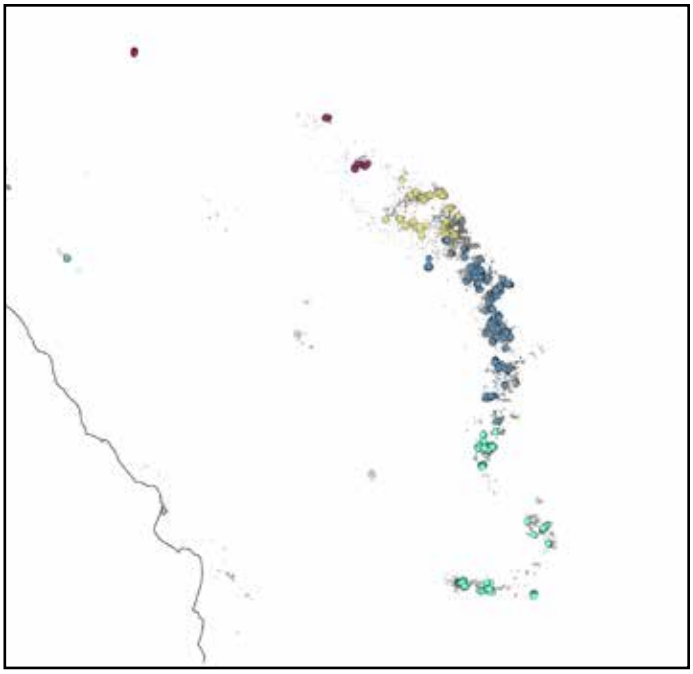

Figure 3. A close up from the CA-1 samples taken from the Central Valley.

Results

Performance of EDT Labs in Experiments

Section A: CA-1

Overall, 1,001 samples were collected, of which 996 were eventually tested by all labs. Labs showed 92 percent agreement over the classification of CA-1 samples as CLas-negative. There were some initial disagreements in the classification of 81 out of 996 samples, but upon re-testing by the CDFA, none of the samples were classified as CLas-positive by regulatory-approved qPCR. Of the 1,001 samples tested successfully in the VVS panel, 312 samples tested positive for at least one virus. Of the 81 samples that were classified by an EDT lab as suspect, only 28 percent of them tested positive for a VVS. Thus, it does not appear that classification of a sample as suspect was the result of cross-detection of the pathogens in the VVS panel.

Because all CA-1 samples are from areas without known HLB-affected trees, the value of the CA-1 study is due to improvements in accuracy or processing volume. Many labs involved reported that the sheer volume of samples helped determine ways to increase efficiency, improved their EDT model, reduced variability, increased the confidence of the EDT and identified questionable HLB markers to avoid.

Section B: CA-1bNQZ

Labs involved in this study showed 79 percent agreement regarding the classification of CA-1bNQZ samples as CLas-negative. Sixteen samples of the 75 samples received and tested successfully by all labs showed disagreement in their classification. When these trees were resampled and retested using qPCR, all 16 were found to be negative for CLas.
Of the 79 samples tested successfully by the Vidalakis lab in their VVS panel, 65 tested positive for at least one VVS. Of this total number, 60 were positive for one VVS and five were positive for two VVSs.

Because the metabolomics and phytobiome methods can associate a particular profile with a certain disease, it is possible for them to go beyond simply determining if a sample is HLB-positive or not; given enough data they can potentially associate a particular profile with each of the other diseases. These two techniques were able to identify which samples from CA-1bNQZ were infected with *Citrus tristeza virus* (CTV) or *Spiroplasma citri*. Diagnostic plots provided by the two labs show the diagnostic differentiation among different diseases that they were able to produce. The phytobiome method showed 100 percent sensitivity and specificity, correctly identifying all CTV and *S. citri* samples. The phytobiome performance is detailed in **Figures 4** and **5**. The metabolome method showed 100 percent sensitivity and specificity for all samples infected with CTV, and 97.5 percent sensitivity and specificity for all samples infected with *S. citri*, identifying one sample that was truly positive for *S. citri* as positive for CTV. The metabolome performance is detailed in **Figure 6**.

Section C: CA-1bQZ

Because CA-1bQZ samples were collected in two different years with changing quarantine zone boundaries, each set was analyzed separately.

Table 1. Detection technology summary results for diagnostic determinations provided by EDT labs for samples collected in the HLB quarantine area.

Detection Technology	Number of positives	Number of negatives	Number of inconclusive/ suspicious
Metabolome	32	33	24
ELISA	13	76	0
Phytobiome	9	80	0
CANARY	3	86	0
qPCR (experimental)	2	87	0
qPCR (regulatory)	1	87	1

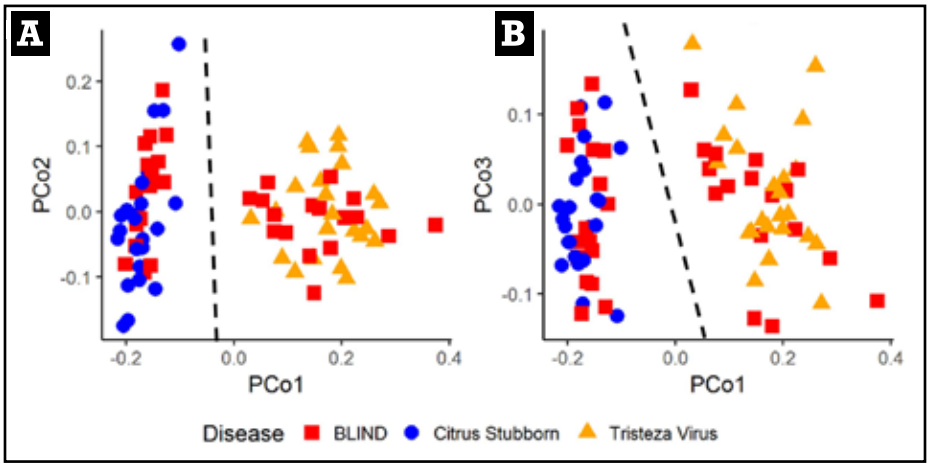

Figure 4. Phytobiome Principal Coordinates Analysis (PCoA)[1] plots of fungal communities based on Bray-Curtis distance matrices[2].

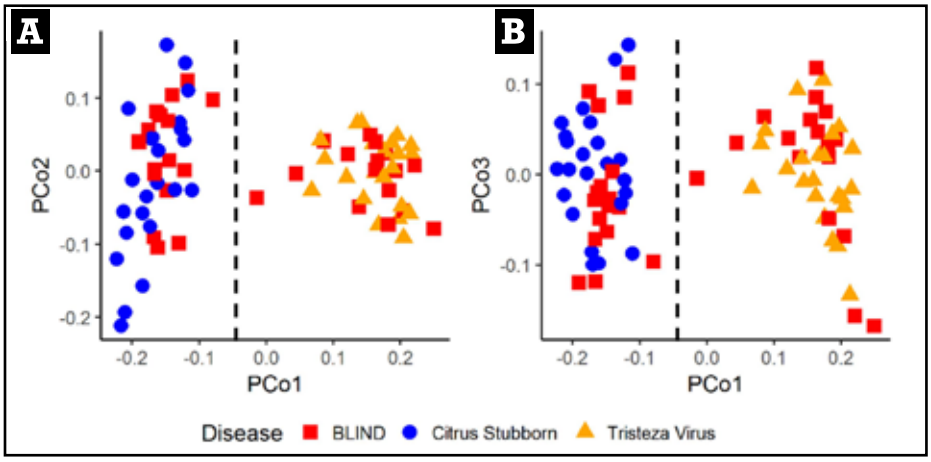

Figure 5. Phytobiome Principal Coordinates Analysis (PCoA)[1] plots of fungal communities based on Jaccard distance matrices[3].

Of the 89 samples collected in 2017, 27 percent were agreed upon by all labs to be CLas-negative; 65 samples received at least one positive or suspicious determination by an EDT (**Table 1**).

Diagnostic accuracy in this case is defined by how well each test does in replicating the results from CDFA's qPCR test, which is the accepted diagnostic method, described here as the "gold standard" though it is known to have features that could be improved.

Receiver Operating Characteristic (ROC) curves visualize the accuracy of a test. The ROC curves of each EDT are given below. In application to disease diagnostics, the graph plots the proportion of actually diseased trees that are diagnosed as diseased on the vertical axis against the proportion of actually healthy trees that are diagnosed as diseased on the horizontal axis. A highly accurate test's line looks like a right angle with its corner in the upper left corner of the graph. Tests that are closer to the gray diagonal line going across the center of the plot are about as effective as flipping a coin. Experimental qPCR and CANARY technology (which has the same score and is under the qPCR line) have the ROC curve most similar to that of a highly effective test (**Figure 7**).

Of the 200 samples collected in 2018, 34.5 percent were agreed upon by all labs to be CLas-negative. Overall, 131 samples total received at least one positive or suspicious determination by an EDT (**Table 2**).

Each test's proximity to the "true value" is measured by its degree of similarity to the CDFA's test, which is assumed to be the gold standard.

ROC curves show that experimental qPCR has the ROC curve most similar to that of a highly effective test (**Figure 8**).

Latent Class Analysis

All previous analyses of the CA-1b dataset used the CDFA qPCR results as

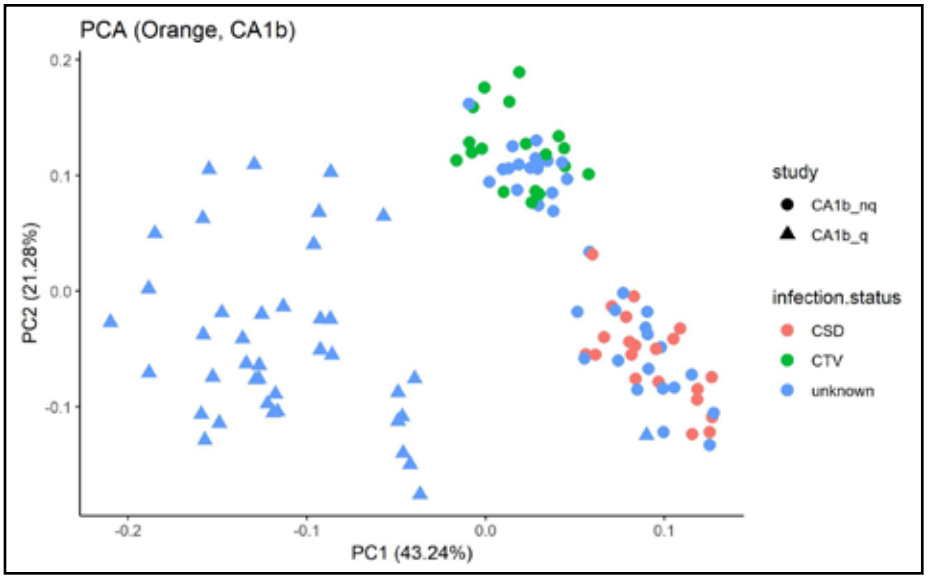

Figure 6. Principal Component Analysis (PCA)[4] of the leaf metabolome of known and unknown samples from the CA1b study. Circles denote samples from the non-quarantine zone, and triangles denote samples from the quarantine zone. Those with CSD are colored red, those with CTV green and those of unknown status are blue.

the gold standard to evaluate EDT performance. However, if the goal of an EDT is to identify disease earlier than qPCR, it may be possible that some EDTs may be accurate in signaling on more samples than tested positive by regulatory qPCR results. Using qPCR as the benchmark in this circumstance might unfairly penalize the EDTs by comparing them with a test that requires selection and testing of infected tree material.

What options are available other than using qPCR as a gold standard? Latent class analysis is a method for estimating the diagnostic accuracy of a test when there is no true gold standard available. In the present case, we assume that the effectiveness of a given EDT should not necessarily be measured based on how similar it is to the regulatory qPCR data.

Table 2. Detection technology classifications of all data.

Detection Technology	Number of positives	Number of negatives	Number of suspicious
Metabolome	80	120	0
ELISA	71	129	0
CANARY	11	183	6
qPCR (experimental)	18	182	0
qPCR (regulatory)	22	178	0

Output from latent class analysis of the first 89 samples in CA-1bQZ found that 87 percent of samples were considered to be CLas-negative, 2.25 percent of the samples were considered to be CLas-positive and 8.8 percent of the samples were considered to be CLas suspect. This analytical method will be important in interpreting results of future studies involving a range of HLB diagnostics, including the canine detection teams, and we will report more fully on this work in the future.

Conclusions

- EDTs agreed on 92 percent of all trees tested in CA-1, classifying them as CLas-negative. Follow-up testing by the CDFA lab with qPCR confirmed that all tested trees from all commercial orchards in the survey were CLas-negative.
- CA-1 study samples improved throughput and processes for five of the six EDTs.
- EDTs agreed on 79 percent of all trees tested in CA-1bNQZ, classifying them as CLas-negative; follow-up testing by the CDFA lab with qPCR confirmed that all tested trees from CA-1bNQZ were CLas-negative.
- The phytobiome- and metabolome-based HLB profiling HLB EDTs correctly identified the diseases present in CA-1bNQZ (tristeza, citrus stubborn disease) in almost 100 percent of cases.
- The CDFA regulatory qPCR test revealed that 97.75 percent of the first 89 samples in CA-1bQZ were CLas-negative, and 2.25 percent were CLas-positive.
- When comparing EDTs with the CDFA regulatory qPCR results, the best matches for the first set of 89 samples in CA-1bQZ were for experimental qPCR and antibodies that detect CLas (CANARY technology).
- The CDFA lab qPCR test revealed that 89 percent of the second 200 samples in CA-1bQZ were CLas-negative, and 11 percent were CLas-positive.
- The best match with the CDFA results for the second set of 200 samples in CA-1bQZ was experimental qPCR.

The CA-1 experiments contribute to broader understanding of baseline California citrus health, as well as current EDT performance, throughput and limitations.

CRB Research Project #5300-181

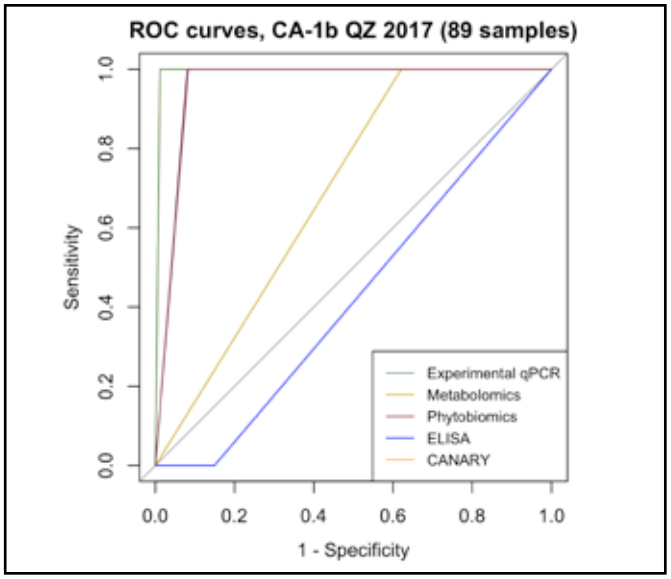

Figure 7. ROC curves for the first 89 samples collected in CA-1b quarantine zone in 2017. The ROC curve for qPCR describes experimental qPCR, which is conducted in a USDA-ARS lab, and is different from regulatory qPCR conducted by CDFA. The curve for the CANARY test is not visible because it is beneath the qPCR curve.

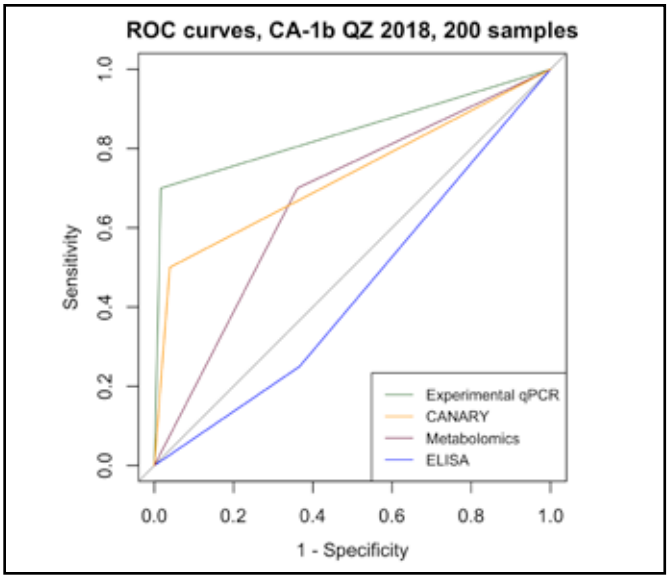

Figure 8. ROC curves for 200 samples collected in CA-1b quarantine zone in 2018. The ROC curve for qPCR describes experimental qPCR, which is conducted in a USDA-ARS lab, and is different from regulatory qPCR conducted by CDFA.

Glossary

[1]Principal Coordinates Analysis (PCoA): A statistical procedure used to visualize the level of similarity or dissimilarity between data.

[2]Bray-Curtis distance matrices: A measure of dissimilarity between two sets of data.

[3]Jaccard distance matrices: A measure of similarity between two sets of data.

[4]Principal Component Analysis (PCA): A statistical procedure used to simplify potentially correlated, complex data while retaining trends and patterns.

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Florida-based Experiment Demonstrates Earliness and Accuracy of EDTs

Brianna McGuire and Neil McRoberts

Project Summary

The Florida-based (FL)-1 experiment aimed to determine how soon several early detection technologies (EDTs), could detect huanglongbing (HLB) after pot-grown trees were exposed to feeding by infected psyllids.

The FL-1 study was planned by researchers from the University of California, Davis (UC Davis) and the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) in Ft. Pierce, Florida. This study comprised three greenhouse experiments, each using 30 potted trees, some of which were exposed to 'Candidatus Liberibacter asiaticus' (CLas)-positive psyllids. Each set of trees was sampled four times during the two-to-five-month period after psyllid exposure. These repeated measurements monitored changes within the plant month-by-month to get an idea of the earliest month an EDT could detect CLas after exposure. Three EDTs were tested: quantitative polymerase chain reaction (qPCR), a metabolomics-based test and a phytobiome-based test.

Data were analyzed by the McRoberts lab at UC Davis. In this study, it was found that qPCR was the earliest to correctly identify CLas-positive trees 71 percent of the time; 90 percent of those trees were identified within two months of exposure to CLas-positive psyllids. There was no evidence to suggest that other EDTs were routinely earlier, more sensitive or specific in detecting infections than qPCR.

Research on improving the sampling methodology of qPCR to capitalize on its high accuracy, or on improving indirect EDT diagnostic reliability is suggested. These improvements could lead to earlier CLas detection and HLB diagnosis than is currently possible. Removal of HLB-affected trees is important to mitigate the spread of HLB.

Introduction

qPCR is the current standard technique to confirm HLB. Though qPCR is able to detect very low levels of CLas, the presumptive causal agent of HLB, it only can detect CLas in infected tissue. It is not always obvious which tissues are infected before visual symptoms appear. Sometimes, qPCR may suggest a tree as uninfected even when there is infected tissue present on the tree if the sample does not include infected tissue. Early detection matters because a tree can be infected with CLas for a long time without showing any visible symptoms. EDTs are technologies intended to detect the presence of CLas/HLB as soon as possible after infection to prevent symptomless, infected, trees from spreading disease.

Table 1. Comparative early detection technology (EDT) performance based on Youden's J score.

EDT	Experiment 1	Experiment 2	Experiment 3
qPCR	0.6	0.93	0.82
Metabolomics	0	0.2	0.5
Phytobiome	-0.16	-0.16	NA

The FL-1 study was part of a series of studies that evaluated EDTs for CLas or HLB. The experiment specifically examined EDT accuracy and earliness. Experiments prior to the FL-1 study that measured the effectiveness of EDTs primarily measured only EDT accuracy at a single time point after a long or unknown interval following infection. Diagnostic test accuracy is reported using "sensitivity" and "specificity." Sensitivity refers to how well a test can identify an infected tree, and specificity refers to how well a test can identify a healthy tree.

Indirect EDTs measure systemic changes in the tree and do not require CLas-positive tissue to diagnose disease as does qPCR. Examples of indirect EDTs used in this study are measures of changes in the tree metabolism (the metabolome) or changes in the microbial community on the leaf surface (the phytobiome). The direct EDT used in this study (qPCR) measured the presence of CLas.

The FL-1 study was proposed in 2017 to measure the same set of trees exposed to CLas-positive psyllids four times, starting eight weeks after exposure. Repeatedly measuring the same trees could give laboratories precise estimates of how early their EDTs could detect CLas or HLB. Since this experiment required the timing of exposure to be carefully controlled and also for trees to be completely protected from infection before and after the exposure period, it could not be done under field conditions. Consequently, the experiments used 18 month-old, potted trees (Valencia scion on Swingle rootstock) produced in a standardized manner at the USDA-ARS greenhouse in Ft. Pierce.

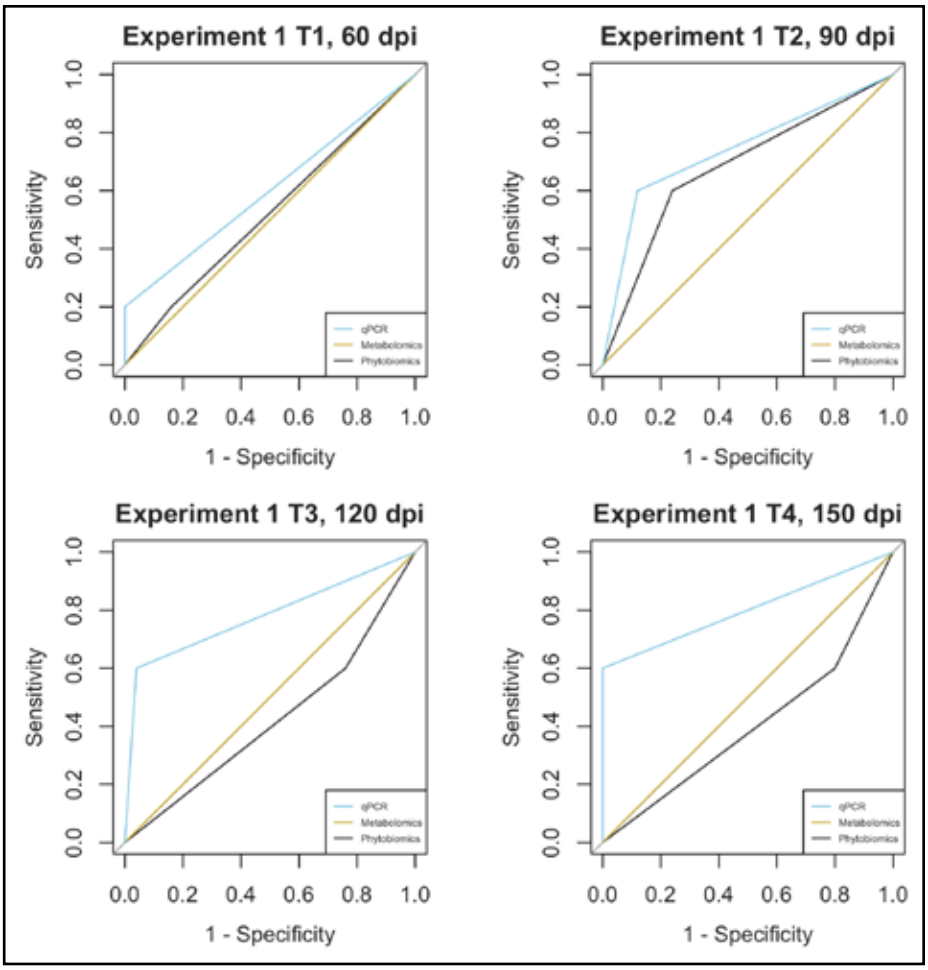

Figure 1: Performance of EDTs over time in Experiment 1.

The proposal was funded by the Citrus Research Board (CRB) in 2017 and involved three labs from UC Davis: Neil McRoberts, Ph.D. (project director and data analysis), Carolyn Slupsky, Ph.D. (metabolomic analysis), Johan Leveau, Ph.D. (phytobiome characterization) and Greg McCollum, Ph.D., at the USDA-ARS in Ft. Pierce (tree production, infection, sample collection and distribution, qPCR).

The study included three replicated experiments. In each experiment, 24

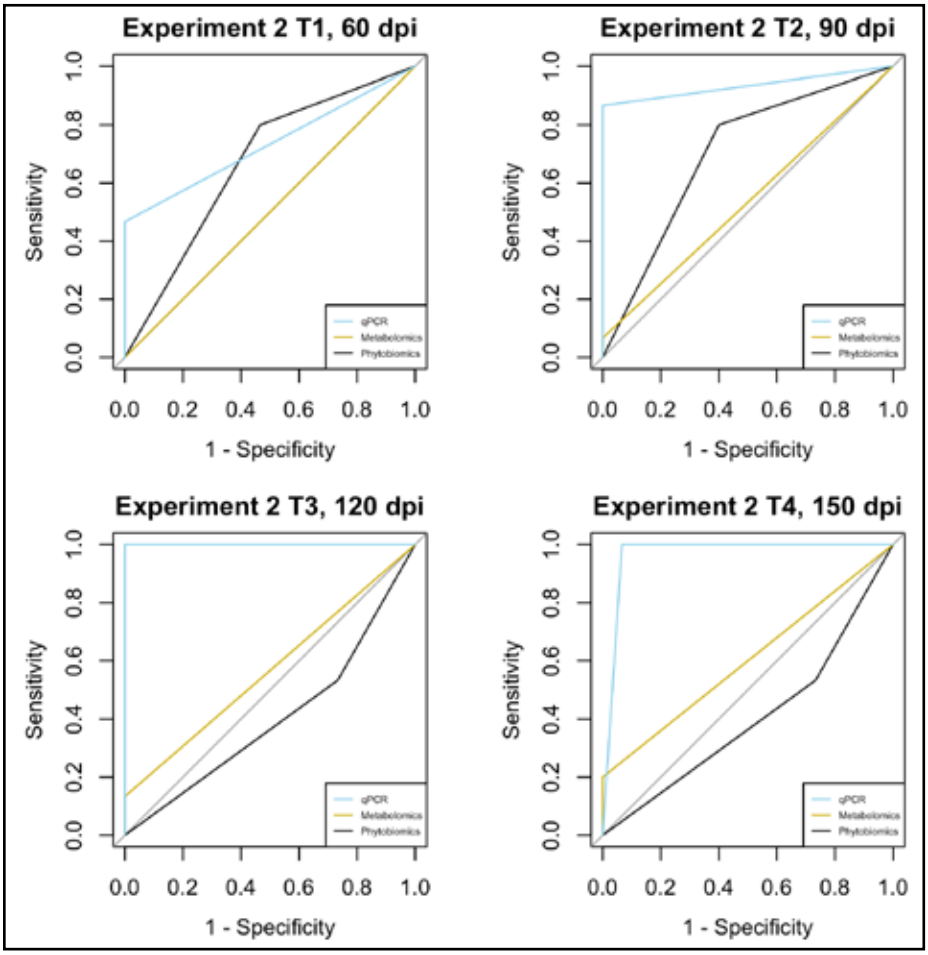

Figure 2: Performance of EDTs over time in Experiment 2.

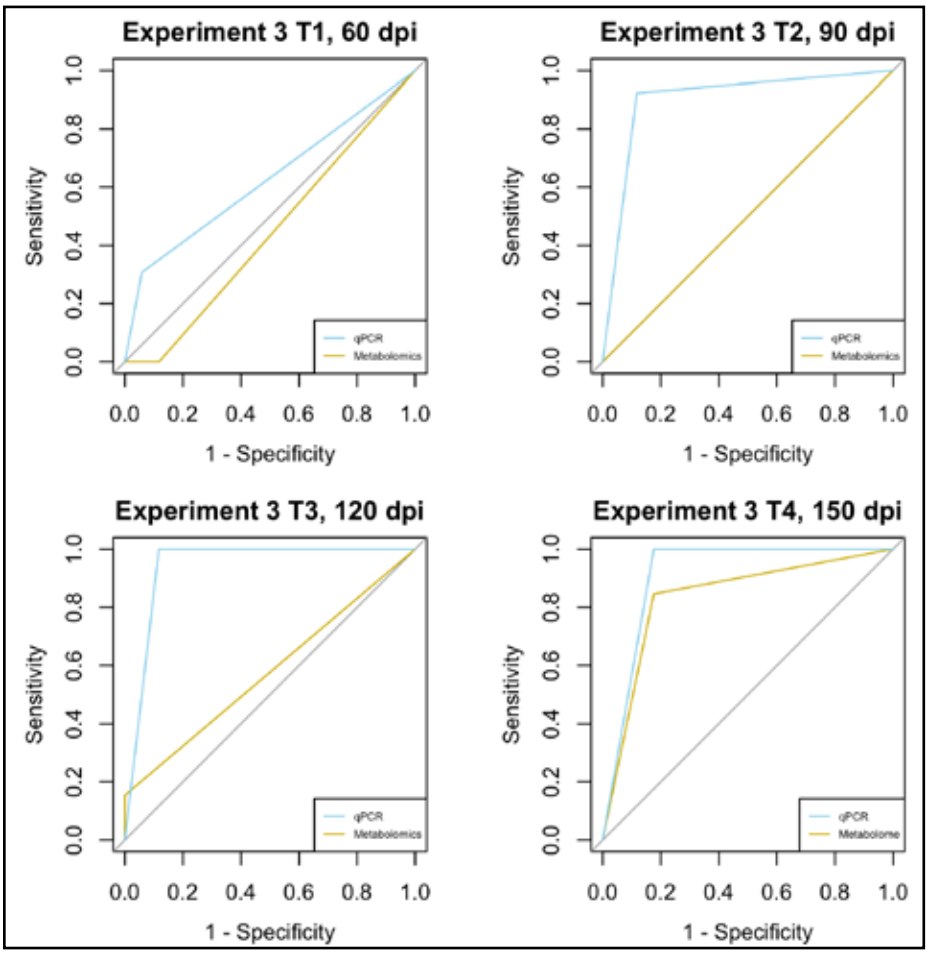

Figure 3: Performance of EDTs over time in Experiment 3.

Valencia trees grafted onto Swingle rootstock were exposed to psyllids that had previously fed on CLas-positive tissue, while six additional trees were left unexposed for that same time period. Leaf material was then sampled from all 30 trees four times between March and August 2018 for analyses by potential EDTs.

Any experiment measuring the performance of diagnostic tests requires knowing the "true status" of each sample to properly evaluate the diagnostic tests. In this experiment, the standard used to determine the true status of each sample was visual rating. Every tree was assessed by two visual raters, who gave a "HLB-positive" or "HLB-negative" rating of HLB symptom development (e.g. blotchy mottle, stunting, leaf malformation). This was done at three time points. Visual ratings of experiment 1 occurred at three, five and six months post-psyllid feeding (also called post-inoculation, or p.i.).

Visual ratings of experiment 2 occurred at four, six and eight months p.i., and visual ratings of experiment 3 occurred at three, four and five months p.i. If the raters disagreed on a tree's symptom presence, the HLB status of the tree was rated as inconclusive. In this report, a "true positive" was visually rated as HLB-diseased by both raters by the final time point; inconclusives were therefore considered negative.

Results

Comparative early detection technology (EDT) performance is shown in **Table 1**. A higher Youden's J score indicates a better performing test; Youden's J score is an overall measure of accuracy and is equal to Sensitivity+Specificity-1. The highest Youden's J score for each experiment is highlighted in green. Experiment 3 was not processed by the phytobiome lab due to sample damage from transit, and receives an "NA" designation.

The results presented in **Figures 1-3** show the ROC curves for each EDT in the different experiments. ROC curves, or receiver operator characteristic curves,

visualize diagnostic test accuracy. They plot sensitivity (i.e. the proportion of truly infected trees detected by the EDT) on the vertical axis against 1- specificity (i.e. the proportion of truly healthy trees, wrongly diagnosed as infected) on the horizontal axis; each graph summarizes one experiment. The closer the ROC curve is to looking like a right angle with its corner in the upper left-hand corner of the graph, the better the EDT's performance is. Each graph summarizes one experiment, with each timepoint given in four panels. If the EDT became more accurate over the course of the experiment, the line corresponding to that EDT will appear closer to the upper left-hand corner of the graph by Timepoint 4.

Results from **Figures 1-3** show the most accurate test in each experiment, but not the earliest test. **Figure 4** shows which test was earliest at correctly identifying true positive samples.

In experiment 1, both qPCR and the phytobiome test identified the majority of positives by timepoint 2, 90 days post-psyllid feeding (90 dpi). In experiment 2, qPCR identified 100 percent of positives at 60 dpi, and the phytobiome test identified more than half of the positives at 60 dpi, while the metabolomic test identified only 15 percent of positives by 150 dpi. Experiment 3 shows similar performance: qPCR can identify the majority of positives by 90 dpi, while it takes longer for the metabolome to correctly identify true positives. In experiments 1 and 2, the phytobiome profile was earliest 29 percent of the time and qPCR was earliest 71 percent of the time. In experiment 3, qPCR was earliest 100 percent of the time.

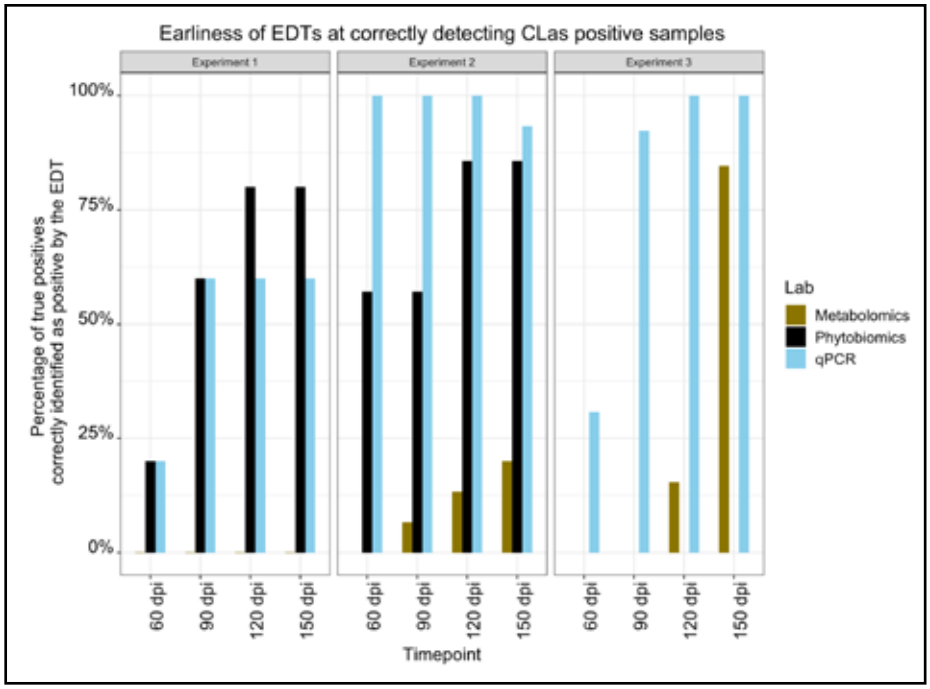

Figure 4: Earliness of EDTs at correctly detecting true CLas positive samples.

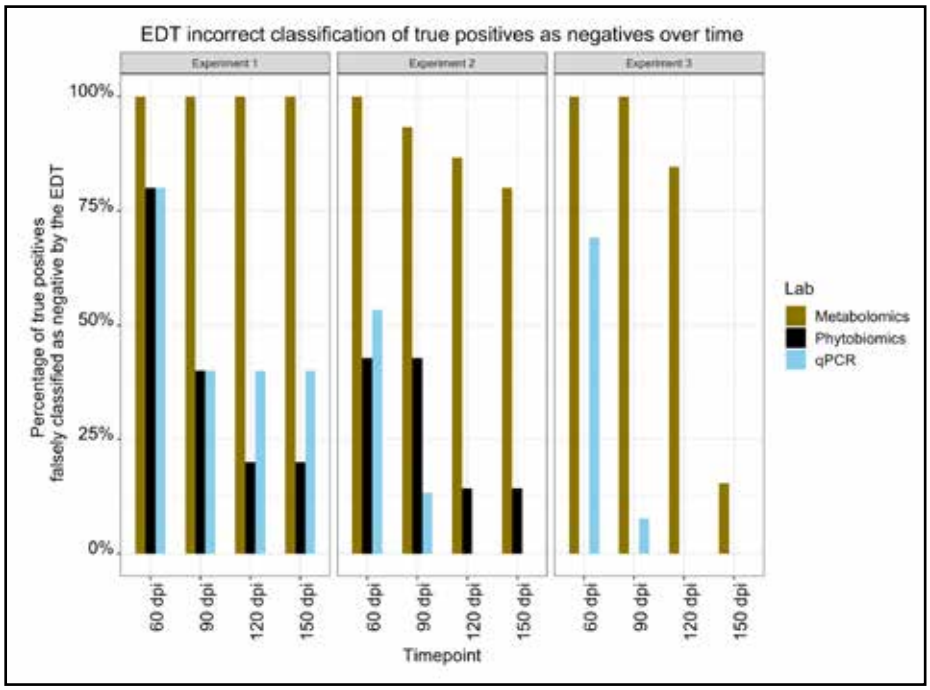

Figure 5: EDT classifications of true positives as negatives.

It is important not only to consider which EDTs were earliest at being right, but which types of errors each EDT made. **Figure 5** provides context to **Figure 4**, by showing how early and how often the EDTs identified false negatives, or true positives that the EDTs mistakenly call negative, which is a concerning error in the context of disease dynamics.

These plots show that the metabolomics test most often classified positives as negatives, while the phytobiome and the qPCR test do so at similar but lower rates.

The final type of error to consider is the rate of false positives, or true negatives that are mistakenly identified as positives by the EDT. A high false positive rate does not necessarily pose the same disease dynamics risk that a high false negative rate, but may suggest excessive removal, which would exact a high economic cost.

In these experiments, qPCR classified three percent of all samples as false positives, the metabolomic profile classified one percent of samples as false positives, and the phytobiome profile classified 38 percent of samples as false

positives. Therefore, the metabolomic test had the highest false negative rate and the phytobiome test had the highest false positive rate.

Conclusions

- **Which approach was earliest?** qPCR detected positives earliest 71 percent of the time in experiments 1 and 2, and 100 percent of the time in experiment 3. The phytobiome analyses detected positives earliest 29 percent of the time in the two experiments in which it was used, experiments 1 and 2. The metabolome EDT was not the earliest to detect positive samples in any of these experiments.
- **How early were they?** Ninety percent of all correctly identified positives detected by qPCR were detected 60 days after exposure to psyllids ended. The phytobiome analyses also detected 40 percent of its correctly identified positives and the metabolome analyses detected six percent of its correctly identified positives 60 days after psyllid exposure ended.
- **What's the major conclusion?** Under the conditions used in the experiments, qPCR was able to detect the HLB pathogen earlier than the other methods and with the highest level of accuracy.

The FL-1 study did not resolve the challenge facing citrus production to detect HLB early enough to have an impact on the rate of disease spread. However, the study does make clear that the directions for further research are either to improve the sampling problems associated with using qPCR on mature trees or continue to refine the indirect detection methods available.

CRB Research Project #5300-178

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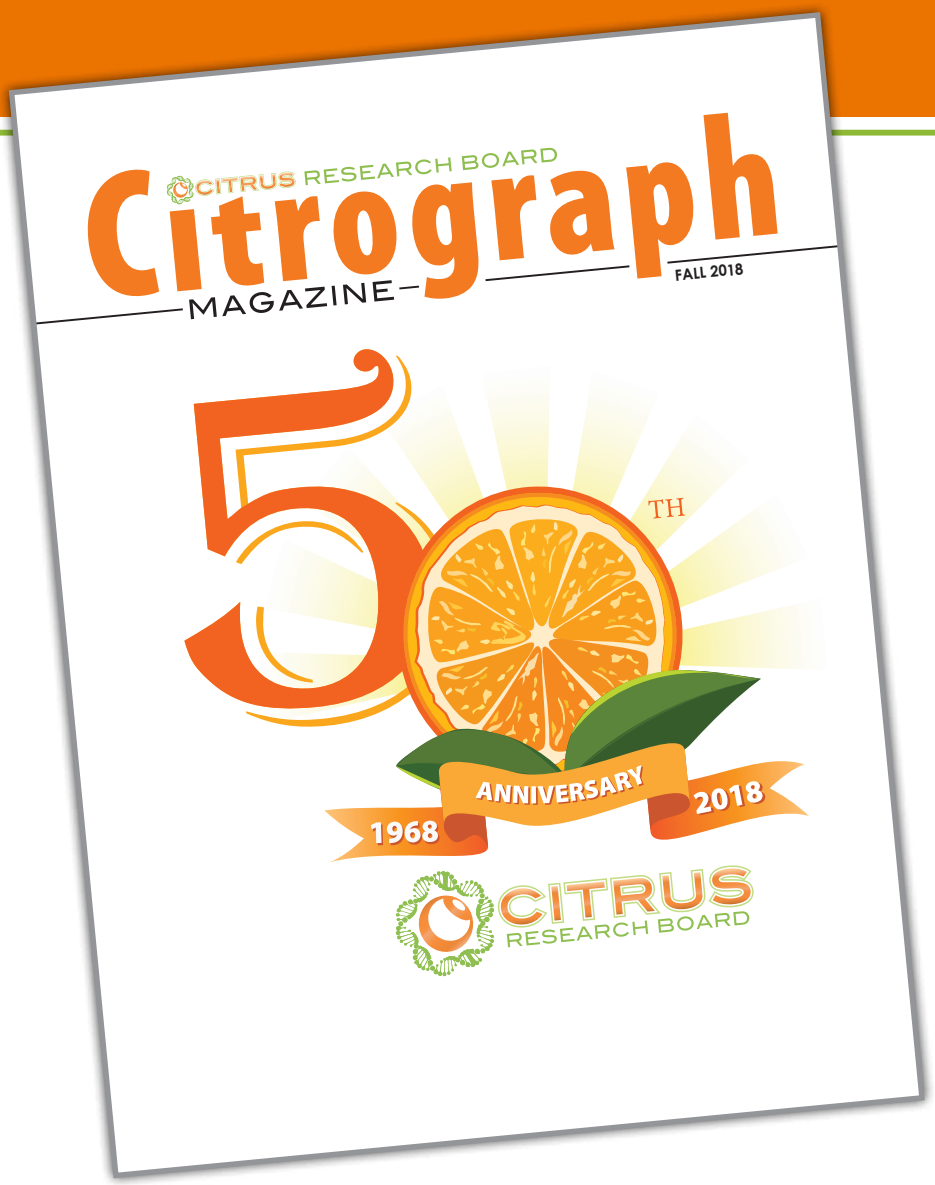

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Example of computer code used to analyze Next Generation Sequencing (NGS) data.

Next Generation Sequencing as a Routine Diagnostic Tool

Tyler Dang, Sohrab Bodaghi, Irene Lavagi, Fatima Osman and Georgios Vidalakis

Project Summary

Next generation sequencing (NGS)[1] is a powerful technology that combines molecular biology and computer science and has a wide range of applications. One application that could help the citrus industry is the detection and identification of citrus pathogens in a given sample. The goal of this project is to evaluate NGS as a tool for routine diagnostics in the citrus varieties introduction process at the Citrus Clonal Protection Program (CCPP). In this study, we determine whether NGS can perform as well or better than current diagnostic assays with infected citrus material from the CCPP disease bank (positive controls), the CCPP foundation block (negative controls) and "real life" variety introductions (unknowns). Based on initial results, NGS has high sensitivity and reproducibility for citrus pathogen detection compared to currently used methods. However, NGS protocols and sample preparation require further optimization to avoid cross contamination and improve specificity.

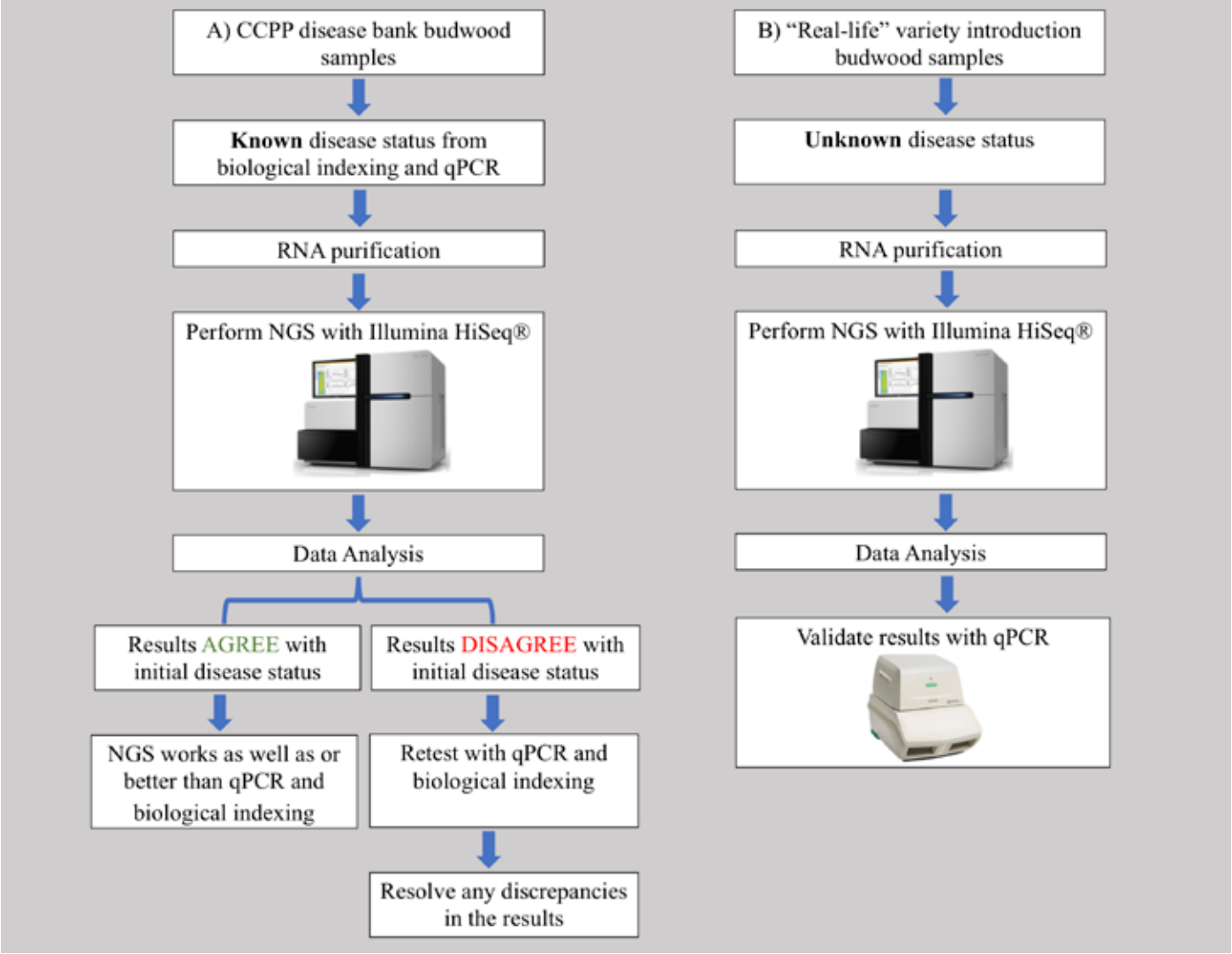

Figure 1. A graphical representation of the NGS workflow. Depending where the citrus budwood samples originate, the tissues undergo different validation procedures to show that NGS can perform as well as or better than current diagnostic assays.

Background

The citrus industry is under constant threat by various pathogens and diseases that can spread quickly through clonal propagation using infected plant materials or mechanically through contaminated tools. As a result, it is imperative to develop new diagnostic tools to further streamline the CCPP introduction process for the prompt release of pathogen-tested citrus propagative materials. NGS generally is accepted in the international scientific and regulatory communities as a tool that will revolutionize plant pathogen diagnostics and become the widely used standard, similar to biological indexing in the 1950s-60s, enzyme-linked immunosorbent assay (ELISA)[2] in the 1970s-80s, polymerase chain reaction (PCR)[3] in the 1990s-2000s and quantitative polymerase chain reaction (qPCR)[4] presently. NGS is a powerful tool capable of simultaneous detection and identification of multiple pathogens in a plant sample without the need to perform the aforementioned multiple tests.

To incorporate NGS as a diagnostic tool for the CCPP, we need to prove that it works equally well or better than the currently approved and used diagnostic assays such as biological indexing and qPCR. If successful, we anticipate developing a CCPP variety indexing (VI) protocol for citrus variety introductions, including a single initial NGS test that can progressively phase out more than 20 individual laboratory tests (PCR, qPCR, gel electrophoresis, etc.) currently required for the detection of all known graft-transmissible citrus pathogens.

Table 1. The Ribo-Zero™ rRNA depletion treatment increased the sensitvity of NGS to detect additional pathogens.

Sample ID	Ribo-Zero™ Treatment[a]	NGS Detected	qPCR Detected
3323-337	Yes	CTV, Viroids, CLas	CTV, Viroids, CLas
	No	CTV	

CTV= Citrus tristeza virus, *CLas* = Candidatus *Liberibacter asiaticus.*
[a]*Ribo-Zero™ Treatment was only used on NGS samples, not on samples for qPCR.*

Table 2. Comparison of Next Generation Sequencing (NGS) and current diagnostic assays.

#	Sample ID	Pathogens Detected	
		NGS	Current Assays[a]
	A. CCPP Disease Bank-Positive Controls	**Agreement**	
1	SY558 (Genotype: T30+VT+T36)	CTV	
2	T519 (Genotype: T30+VT)	CTV	
3	3300-4	CTV, CPsV, CLBV, Viroids	
4	C189	*Spiroplasma citri*	
		Partial Agreement	
5	SY568 (T30+VT)	CTV, CPsV	CTV
6	K-1	CLBV, CTV	CLBV
7	3207-8	Viroids, CTV	Viroids
	B. "Real-life" Variety Introductions-Unknowns	**Agreement**	
8	IPPN 122	CTLV, CTV, Viroids	
9	3323-337	CTV, Viroids, CLas	
10	724-Pakistan	Viroids	
11	Parent Navel	CVEV, Viroids	
	C. CCPP Foundation Block-Negative Controls	**Agreement**	
12	Washington Navel (VI 376)		
13	Page Mandarin (VI 58)		
14	W. Murcott (VI 462)		
15	Limoneira 8A (VI 380)		
16	Mexican Lime (VI 419)	None	None
17	S1 Citron (VI 357)		
18	Melogold Grapefruit (VI 323)		
19	Chandler Pummelo (VI 11)		
20	Nagami Kumquat (VI 276)		

[a]*Current Assays: quantitative polymerase chain reaction (qPCR), reverse transcription quantitative polymerase chain reaction (RT-qPCR), and biological indexing.*
CTV: Citrus tristeza virus.
CPsV: Citrus psorosis virus.
CLBV: Citrus leaf blotch virus.
CVEV: Citrus vein enation virus.
CLas: Candidatus *Liberibacter asiaticus*.

The NGS validation workflow (**Figure 1**) begins with either (A) citrus budwood samples collected from the CCPP disease bank with known disease status from previous biological indexing and qPCR results, or (B) "real-life" variety introduction samples from any citrus growing region in the world with unknown disease status or a series of known healthy samples from the CCPP foundation block. High-quality RNA[5] is purified from all citrus budwood tissues and is subsequently sequenced with the Illumina HiSeq® platform[6]. The HiSeq will generate millions of data points (sequences) that required bioinformatics analyses[7] with supercomputers, located at the University of California, Riverside High-Performance Computing Center, to put the data (sequences) together and analyze them. If the results from CCPP disease bank samples (A) agreed with the initial disease status (green in **Figure 1**), then NGS worked as well or better than current detection methods. However, if the results disagreed with the initial status (red in **Figure 1**), the samples would be retested by qPCR and biological indexing to resolve any discrepancies. qPCR was performed to validate the NGS results from the "real-life" variety introduction samples (B).

Based on our initial findings, NGS is an extremely sensitive method. The sensitivity was aided with the Ribo-Zero® rRNA removal kit, which depletes host plant ribosomal RNA to prevent it from oversaturating the reads generated by the Illumina HiSeq. The addition of this step allowed for the identification of more citrus pathogen sequences with

NGS results matching PCR results (**Table 1**). The same sample that was not treated with Ribo-Zero missed other pathogens that were present, such as citrus viroids and '*Candidatus* Liberibacter asiaticus' (CLas) (**Table 1**). To test the specificity of NGS, we analyzed various samples from (A) the CCPP disease bank (positive controls), (B) "real-life" variety introductions (unknowns) and (C) the CCPP foundation block (healthy controls) (**Table 2**). Overall, the results from NGS matched the current diagnostic assays (86 percent, **Table 2**) indicating that NGS can specifically detect the targeted pathogens. There were some instances (14 percent, **Table 2**) in which NGS and current diagnostic assays detected the same pathogens; however, NGS indicated the presence of additional pathogens (highlighted in red, **Table 2**).

Conclusion

NGS is a powerful tool, and it shows promise as a routine diagnostic tool for the CCPP and other citrus programs responsible for the introduction, maintenance and distribution of pathogen-tested citrus propagative material. Streamlining laboratory diagnostics with NGS will increase laboratory efficiency and reduce costs in the long term due to reduced hands-on time for running multiple assays to test for known graft transmissable citrus pathogens. As a result, NGS could potentially phase out more than 20 individual tests for the introduction and distribution of new and established citrus varieties. This will benefit citrus growers by providing faster access to pathogen-tested budwood for establishment of new citrus groves. This will be particularly critical with the constant threat posed by the introduction of exotic citrus pathogens, such as CLas, and the process by which the CCPP introduces and releases critical materials for HLB research and production of HLB-resistant and -tolerant citrus varieties.

CRB Research Project #5300-179

Glossary

[1]Next generation sequencing (NGS): Technology that allows for the sequencing of many DNA strands all at once in contrast to sequencing individual DNA strands. This technology allow for quicker turn around and cheaper prices for genomics studies.

[2]Enzyme-linked immunosorbent assay (ELISA): A serological detection method that involves an antigen (virus or bacteria) – antibody reaction. An enzyme attached to the antibody is activated when the antigen is present, causing a color change in the reaction mixture.

[3]Polymerase chain reaction (PCR): An enzyme-based biochemical reaction that, through repeated temperature stages, exponentially increases specific DNA sequences for diagnostic or other uses.

[4]Quantitative polymerase chain reaction (qPCR): Similar to PCR but the amounts of DNA produced are measured over the course of the reaction allowing for a calculation of the original amount of DNA present within a tested sample.

[5]RNA: Ribonucleic acid, a nucleic acid present in all living cells. RNAs are transcribed from the DNA genome and translated into proteins. In addition, some viruses store their genetic information in RNA, rather than DNA.

[6]Illumina HiSeq®: A commonly used system developed by Illumina for NGS. The system allows for high-throughput sequencing of large-scale genomes suitable for various applications.

[7]Bioinformatics analyses: A multi-disciplinary field of computer science and molecular biology that utilizes a special high-power computer and software to analyze the millions of sequence data points generated by NGS.

References

Al Rwahnih, M.; Daubert, S.; Golino, D.; et al. 2015. Comparison of next-generation sequencing versus biological indexing for the optimal detection of viral pathogens in grapevine. *Phytopathology* 105(6):758-763.

Jones, S.; Baizan-Edge, A.; MacFarlane, S.; et al. 2017. Viral diagnostics in plants using next generation sequencing: computational analysis in practice. *Frontiers in Plant Science* 8:1770.

Rott, M.; Xiang, Y.; Boyes, I.; et al. 2017. Application of next generation sequencing for diagnostic testing of tree fruit viruses and viroids. *Plant Disease* 101(8):1489-1499.

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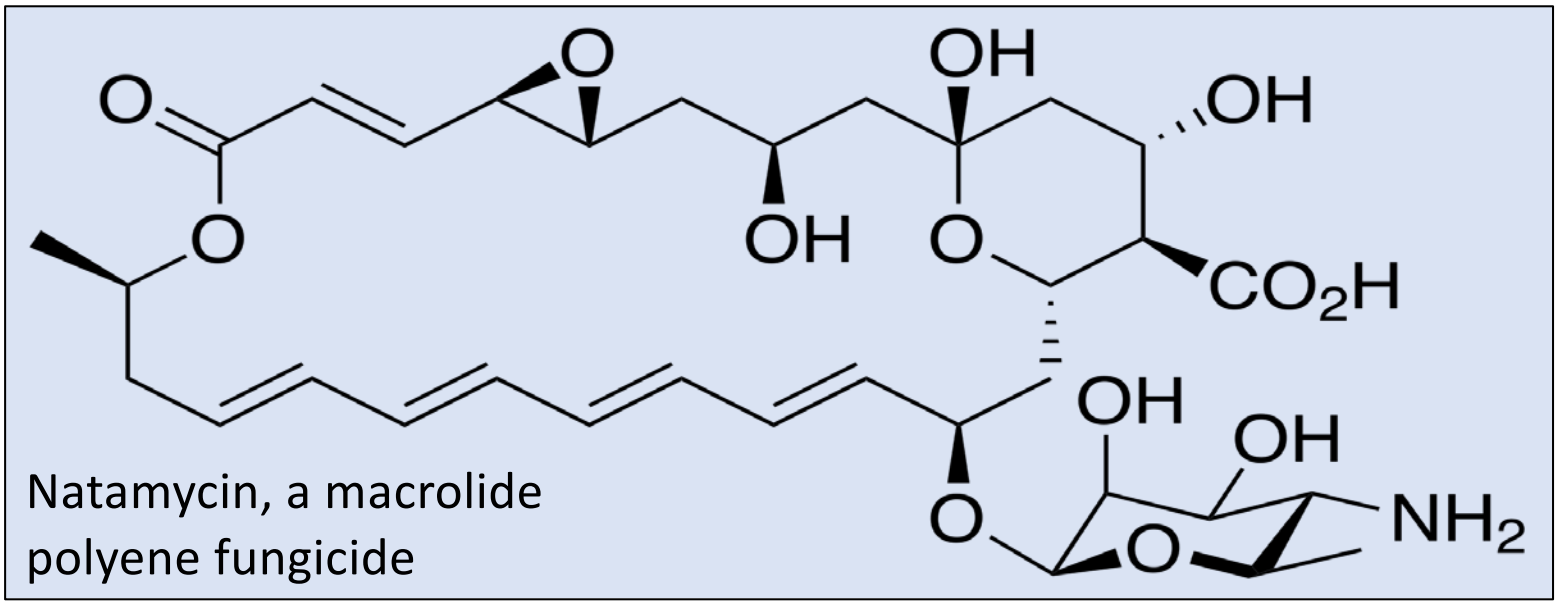

Figure 1. Chemical structure of natamycin.

Positioning Natamycin as a Post-harvest Fungicide for Citrus

James E. Adaskaveg, Helga Förster and Daniel Chen

Project Summary

Natamycin (BioSpectra®) is a new post-harvest fungicide classified as a "biopesticide[1]" with a unique mode of action[2] that can be used to manage sour rot, green and blue molds and other citrus decays. To improve its performance and overcome its lack of sporulation control, natamycin is best used in mixtures with propiconazole (Mentor®) for effective sour rot management and in mixtures with fludioxonil (Graduate®), fludioxonil with azoxystrobin (Graduate A+®), imazalil with thiabendazole (TBZ), or imazalil with pyrimethanil (Philabuster®) for highly effective Penicillium decay control on citrus. A key advantage of natamycin is its ability to reduce total populations of targeted pathogens while minimizing the risk of selecting resistant sub-populations.

The 2017 registration of natamycin (BioSpectra) is the newest post-harvest fungicide and another biofungicide (after potassium phosphite and sodium carbonate/bicarbonate) available for citrus in the United States. As with any newly registered post-harvest fungicide, there currently is limited commercial use; therefore, demonstration trials are ongoing in California packinghouses to determine where the fungicide best fits into the current portfolio of citrus decay control treatments. Similar to other new fungicides' introduction, some of the reasons for limited commercial use include the need for established maximum residue limits (MRLs) in export countries, the added cost of the product

Fungicide	Formulation	Concentration (µg/ml or ppm)
Control	---	---
Natamycin	SC	1000
Propiconazole	WP	250
Propiconazole + Natamycin	WP + SC	250 + 500

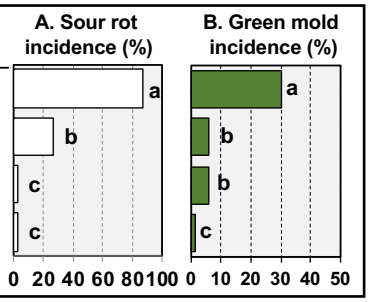

Figure. 2. Efficacy of heated (48°C) flooder applications of natamycin and propiconazole on a commercial packing line for managing sour rot (A) and green mold (B) of 'Eureka' lemon fruit caused by* Geotrichum citri-aurantii *and* Penicillium digitatum*, respectively. Sour rot and green mold were evaluated after four weeks at 12°C. Horizontal bars with the same letter are not significantly different (P > 0.05) following an analysis of variance.

we achieved our goal of providing to the citrus industry two differing modes of action effective against sour rot, in addition to other decays of citrus.

The California citrus industry is well aware of international MRLs for pre- and post-harvest pesticides since a large segment of the winter and spring citrus crops are shipped to international markets. For widespread use of any post-harvest fungicide, international MRLs and domestic tolerances must be established. This avoids the risk of contamination of fruit for export with fungicides not approved in international markets and the cost of cleaning and/or changing out packing line brushes and rollers. Thus, usage of natamycin to date is low because there are no established international MRLs, and the risk of contamination is high for fruit destined to international markets when treated on the same packing line as domestic fruit.

compared with other available fungicides, and the uncertain potential benefits to the individual packinghouse or the whole citrus industry when using the new fungicide. The purpose of this article is to discuss the characteristics of natamycin and to identify where it might fit best as a post-harvest treatment for citrus.

In the past, sodium ortho-phenylphenate (SOPP) was used to obtain moderate sour rot management. However, the potential human toxicity, phytotoxicity and disposal costs associated with this compound have dramatically limited SOPP use in California. Sanitizing agents are ineffective against wound infections, whereas alkaline treatments (e.g., sodium carbonate and bicarbonate) are only moderately effective for a few weeks of storage or until the alkaline pH reverts to an acidic pH that is conducive to fungal growth. Thus, there remains a need to develop residual fungicides with different modes of action for improved sour rot management.

Propiconazole (Mentor) was the first highly effective residual fungicide registered in the United States for controlling sour rot, and it is similar in performance to imazalil for Penicillium decay control (Adaskaveg and Förster 2013). Natamycin was pursued for registration because it has broad-spectrum activity against filamentous fungi[3]; and to avoid developing resistance to targeted pathogens, at least two fungicides with different modes of action need to be registered. Natamycin is effective against Penicillium, Alternaria and Mucor decays and, most importantly, sour rot of citrus (including grapefruit, lemons, mandarins and oranges) caused by *Geotrichum citri-aurantii* (Adaskaveg and Förster 2015; unpublished), and it has a different mode of action from other fungicides registered on citrus. Propiconazole blocks the production of ergosterol – a cell membrane component specific to fungi – while natamycin binds to ergosterol, leading to the breakdown of fungal membranes (FRAC 2019) (**Figure 1**). Natamycin has been used in the dairy and dried meat food industries with U.S. Food and Drug Administration approval as a preservative against molds. In 2017, the U.S. Environmental Protection Agency approved natamycin as a biopesticide for post-harvest treatment of citrus and other crops and gave it tolerance[4] exemption status. Therefore,

As a biopesticide, natamycin currently is exempt from residue tolerances in the United States, but not with CODEX[5] or other countries. Therefore, these MRLs need to be established. To date, the MRL and food additive tolerance (FAT) for natamycin is being pursued in Japan, but other markets are expected to follow. The biopesticide classification with the exemption residue status in the United States is both a blessing and a curse. From one point of view, having safer post-harvest treatments to ensure wholesome produce is a goal for all commodities, regulatory agencies and consumers. However, establishing a residue tolerance in the United States is a driving force for CODEX and other countries to establish an MRL.

Rates between 500 and 1,000 milligrams per liter or parts per million (ppm) of natamycin are effective for controlling sour rot and Penicillium decays (unpublished). With the current formulation of natamycin, the cost to obtain the high-labeled use rate is comparative or slightly more than the cost of propiconazole at its labeled use rates (unpublished). One way to make both of these products more affordable and possibly more effective is to use mixtures. The efficacy of natamycin used by itself or in mixture with propiconazole on a commercial packing line using 'Eureka' lemon is shown in **Figure 2**. The performance of natamycin was improved when mixed with propiconazole resulting in a less than two percent incidence for sour rot and green mold as compared to approximately 90 percent sour rot and 30 percent green mold in the untreated controls (**Figure 2**). With the addition of natamycin in a mixture, the efficacy of propiconazole was improved for green mold, but not for sour rot. Still having two different modes of action is important in developing fungicide resistance management strategies.

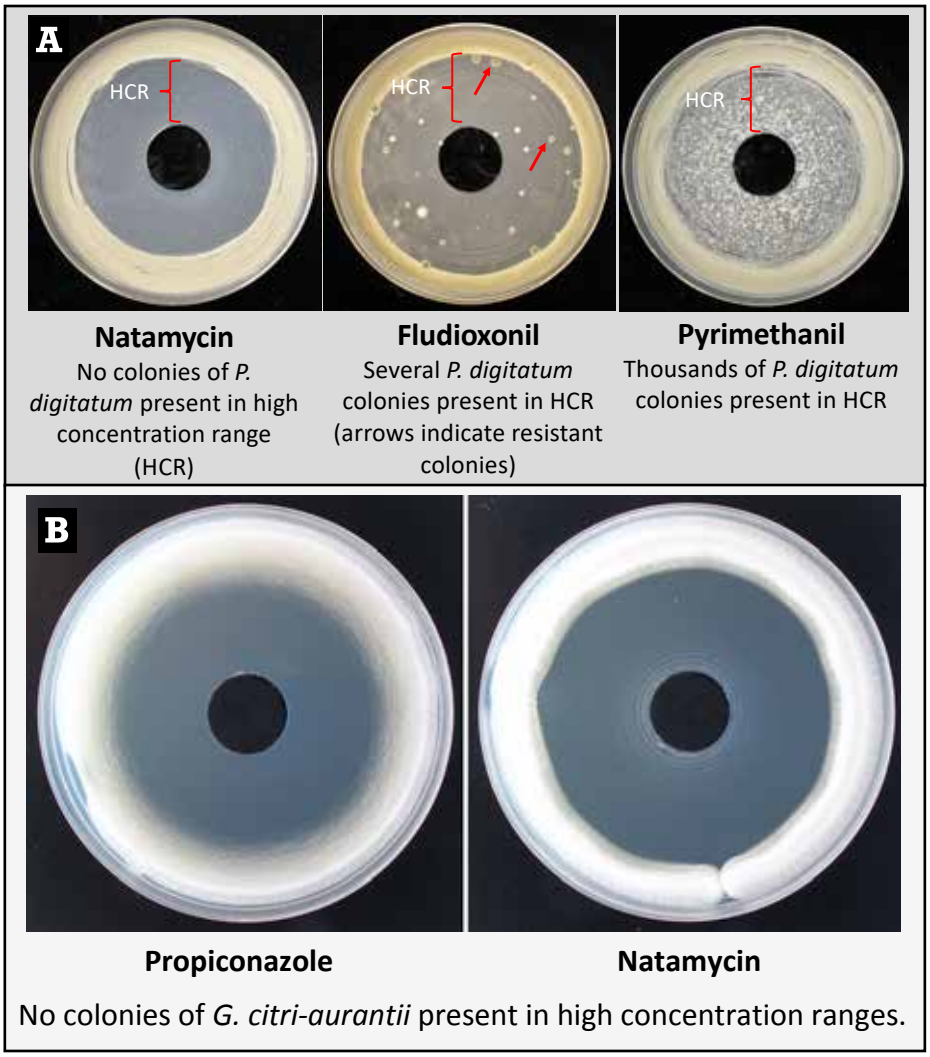

Figure 3. (A) Mass conidial platings of several isolates of* Penicillium digitatum *onto agar plates amended with natamycin, fludioxonil or pyrimethanil. (B) Mass conidial platings of* Geotrichum citri-aurantii *onto agar plates amended with propiconazole or natamycin. The spiral dilution establishes low concentrations at the outer perimeter of the plate and high concentrations toward the center of the plate. The interaction zone of growth and no growth is at the effective concentration to inhibit 95 percent of fungal growth, known as the EC95 concentration. More colonies in the center region of high fungicide concentrations indicate a higher potential risk for selection of resistant isolates. Based on the plates shown, natamycin, fludioxonil, propiconazole and pyrimethanil have zero to extremely low, low, low and high potential for development of fungal resistance, respectively.

We have demonstrated that natamycin has an extremely low to zero potential for resistance development in *Penicillium* and *Geotrichum* species. Using the spiral gradient dilution assay, mass platings of conidia (spores) from multiple isolates of *P. digitatum* were exposed to natamycin, fludioxonil, or pyrimethanil. In **Figure 3A**, low concentrations of each fungicide are present at the outer perimeter of each plate and, subsequently, a continuous dense ring of fungal growth developed. Toward the center of the plate, high concentrations of each fungicide are present. With natamycin, no colonies formed in this area, whereas some colonies developed with fludioxonil, and hundreds of colonies developed with pyrimethanil (**Figure 3A**). This indicates the potential for development of strains resistant to each of these two fungicides.

Thus, based on our evaluations, the development of resistance in *P. digitatum* to natamycin, fludioxonil, and pyrimethanil is zero to extremely low, low, and high, respectively. The resistance development potential in *G. citri-aurantii* to natamycin and propiconazole is zero to extremely low and low, respectively (**Figure 3B**).

Using natamycin in a mixture with propiconazole would improve the efficacy of natamycin and reduce the potential for resistance development in sub-populations of *P. digitatum* and *G. citri-aurantii* resistant to propiconazole to extremely low levels. Flooder applications allow for much lower rates of fungicides, while providing higher decay control. This is because high-volume applications result in optimum coverage. This type of application allows for heating of the fungicide solutions, which generally improves fungicide efficacy and efficiency of the fungicide solution by allowing re-use, thus also making the treatment very affordable. One weakness of natamycin is its incompatibility with several sanitizers, especially those that are oxidizers. However, we identified organic acidic sanitizers including citric acid and lactic acid that are compatible with natamycin and most of the other registered fungicides. These sanitizing acids and mixtures of these acids with surfactants or hydrogen peroxide are available as commercial products for use in citrus packinghouses.

In our experimental and commercial packing line trials, flooder applications of natamycin and propiconazole were highly effective in controlling sour rot and Penicillium decays, and we achieved up to 5-log reductions of surrogate bacterial contaminants (Adaskaveg et al. 2017). If there is an alkaline soda ash treatment prior to flooder applications of natamycin sanitized with organic acids, the fruit must be washed extremely well prior to the flooder treatment to prevent cross-contamination of the acidified fungicide treatments.

Unlike imazalil and fludioxonil, natamycin does not prevent fungal sporulation[6]. Decay that develops after a fungicide treatment is potentially equivalent to survivors, and reproducing "survivors" allows for additional selection leading to resistance development. This is how fungal populations change or adapt to new environments. Thus,

having sporulation control is a very important characteristic of a post-harvest fungicide in resistance management.

Instead of flooder or drench applications, another potential post-harvest application strategy for natamycin is to use it sequentially as an aqueous treatment prior to the storage wax (i.e., fruit coating) application or use it directly in the storage wax. We have demonstrated that both strategies are effective against green mold (sour rot data are pending). Natamycin used in a mixture with pack waxes, however, was significantly and dramatically less effective and commercially not acceptable (Adaskaveg and Förster 2015). One solution to this problem would be to use an aqueous treatment of natamycin and subsequently apply the pack wax. However, this stepwise application will require additional space and equipment on the packing line. Still, because natamycin does not inhibit sporulation, it should be mixed with other fungicides. For lemons going into storage, natamycin should be mixed with propiconazole for sour rot control and/or with fludioxonil (Graduate) or fludioxonil/azoxystrobin (Graduate A+) for Penicillium decay management. When packing oranges and lemons for final markets, natamycin should be applied aqueously, followed by a pack wax treatment with imazalil and TBZ or with fludioxonil and TBZ for decay and sporulation control.

Overall, natamycin is a fungicide for post-harvest management of sour rot and green and blue molds, as well as other citrus decays. It is best used in mixtures with propiconazole for sour rot control and in mixtures with fludioxonil, fludioxonil/azoxystrobin, imazalil and TBZ or imazalil/pyrimethanil (Philabuster) for Penicillium decay control. Additionally, application methods can be optimized by choosing compatible sanitizers (e.g., organic acids) for flooder applications, using natamycin in water-soluble storage fruit coatings or applying it as an aqueous treatment prior to a storage or pack wax treatment. The key advantage of natamycin is its near-zero potential in selecting resistant sub-populations of fungal pathogens. In addressing fungicide resistance in plant agriculture, a general principle is to use a broad-spectrum, multi-site material to reduce the total pathogen population before using a single-site mode of action fungicide. All post-harvest fungicides registered on citrus in the United States have single-site modes of action. Because multi-site residual fungicides are not available for post-harvest use on citrus, natamycin with its near zero resistance potential and broad-spectrum activity can fulfill this role of lowering the total pathogen population. Much smaller pathogen populations will then be exposed to the subsequent or concurrent use of other fungicides with single-site modes of action. Development of resistance then is much less likely to occur.

CRB Research Project #5400-103

References

Adaskaveg, J.; Chen, D.; Nguyen, K.; et al. 2017. New food safety rules challenge recycling post-harvest fungicide. *Citrograph* 8(4):48-50.

Adaskaveg, J. and Förster, H. 2013. New post-harvest treatments for improved management of green mold, sour rot, and brown rot of citrus. *Citrograph* (4)3:52-58.

Adaskaveg, J.E. and Förster, H. 2015. Biofungicides: New products with exempt-from-tolerance registration status for citrus disease management in the U.S. *Citrograph* (6)4:66-71.

Fungicide Resistance Action Committee, 2019. FRAC Code List: Fungicides sorted by mode of action (including FRAC Code numbering). Online document available at *http://www.frac.info*. CropLife International, Brussels, Belgium.

Glossary

[1]Biofungicide (biopesticide): Biochemical pesticides are naturally occurring substances that control pests by non-toxic or less-toxic mechanisms than conventional pesticides.

[2]Mode of Action (MOA): The specific cellular process that is inhibited by a specific group of chemical compounds.

[3]Filamentous fungi: Organisms composed of a network of tube-like cells (hyphae). This classification of fungi includes mushrooms and common packinghouse molds/pathogens.

[4]Exempt from Tolerance: Phrase used by the U.S. Environmental Protection Agency for materials that are considered safe for the environment and consumers and that are not required to have a tolerance or maximum residue limit (MRL) in the United States.

[5]CODEX: A collection of international food standards and pesticide limits developed to protect the health of consumers and promote fair practices in food trade.

[6]Fungal sporulation: Formation of spores or propagation structures for dissemination or survival of fungi.

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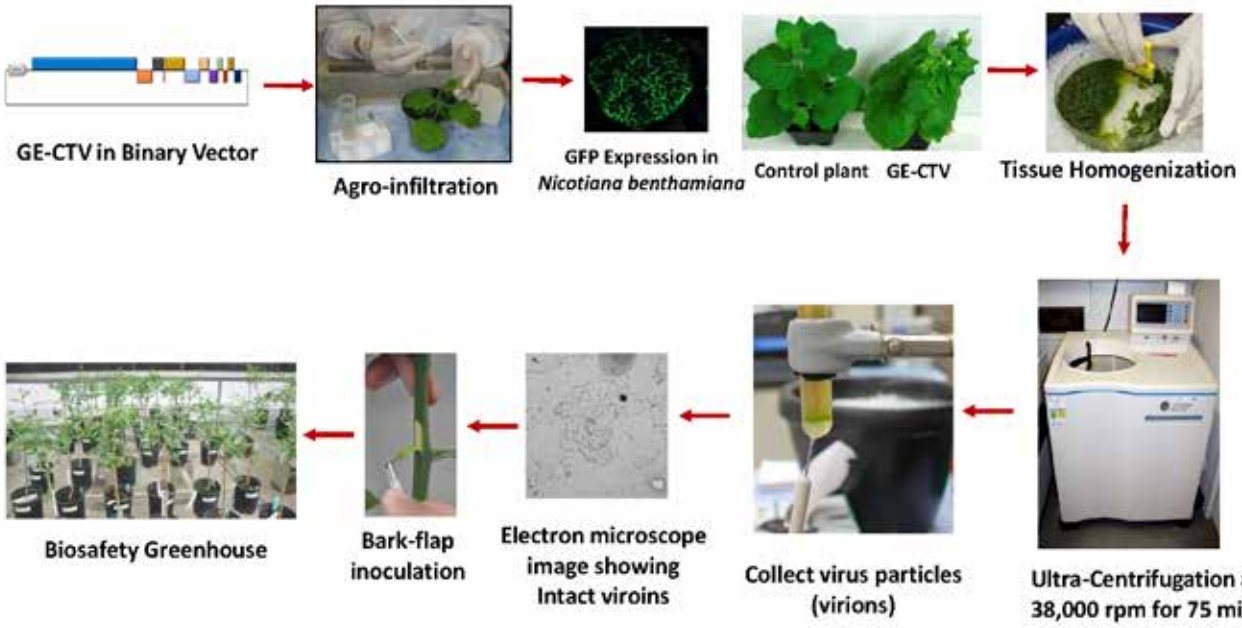

***General procedure developed to infect citrus with genetically-engineered (GE)* CTV.**

Genetically-engineered *Citrus Tristeza Virus*

Establishing and Examining its Interaction with Wild-type CTV

Raymond Yokomi, Vijayanandraj Selvaraj, Yogita Maheshwari and Subhas Hajeri

Project Summary

Genetically-engineered Citrus tristeza virus *vectors (GE-CTV) have been developed in Florida to carry and express genes to help control the huanglongbing (HLB) pathogen and its psyllid vector in existing citrus trees without the use of transgenic citrus. Such a system was developed using a T36 genotype of GE-CTV (T36-GE[1]) obtained from Florida. Several clones of T36-GE with different marker genes were established in Alemow (*Citrus macrophylla*) and produced a high virus titer with no CTV disease symptoms except for fluorescence and photobleaching[2] induced by the marker genes. T36-GE was aphid-transmissible at a very low level (0.86 percent) and readily co-infected with California wild-type CTV strains T30, S1 and VT. However, T36 prevented co-infection of T36-GE due to cross-protection[3], and, interestingly, RB-115 reduced co-infection of T36-GE. These data suggested that T36-based GE-CTV expressing genes to mitigate HLB may show limited benefit if the T36 or RB strains of CTV are already present in a citrus tree. This research benefits growers by understanding factors needed to improve efficacy of GE-CTV vectors and having an in-state system to test GE-CTV clones with different gene inserts designed to control HLB and ACP.*

Introduction

Pioneering research in Florida showed that CTV can be engineered to carry and express foreign genes to combat '*Candidatus* Liberibacter asiaticus' (CLas), which is associated with HLB and its vector, *Diaphorina citri*, the Asian citrus psyllid (ACP) (Dawson and Folimonova 2013; Dawson et al. 2015; Hajeri et al. 2014). In fact, a T36-GE expressing defense proteins from spinach to help manage HLB has been in confined Florida field trials since 2010 (USDA-APHIS 2018a) with a permit now pending for the wider release of this strain in Florida (USDA-APHIS 2018b).

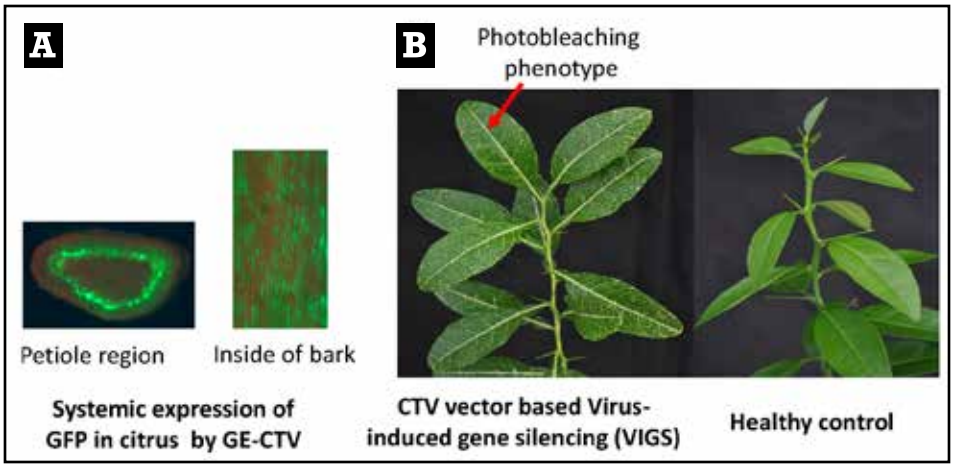

Figure 1. Expression of T36-GE marker genes in Alemow: (A) GFP fluorescence in phloem under UV illumination, (B) photobleaching in veins and bark.

CTV infects citrus and related species (Dawson et al. 2013). Just as there are different citrus cultivars with different traits and characteristics, there are strains or genotypes of CTV that are characterized by the symptoms produced in specific citrus cultivars or by their genetic sequence. Hence, CTV symptoms vary depending on the CTV strain and the citrus scions and rootstocks involved. Many CTV isolates[4] are mild or asymptomatic in commercial citrus cultivars grown on CTV-tolerant or -resistant rootstocks. CTV strains are considered virulent if they induce stunting and severe stem-pitting, regardless of rootstock (Garnsey et al. 2005) and should not be used as a GE-CTV vector because of these associated symptoms.

Mechanical Transmission of T36-GE Clones into Citrus

The objective of this research was to establish a system to measure the biological activity of GE-CTV in citrus. This system uses infectious cDNA of the GE-CTV to systemically infect citrus. Once the virions are introduced, *in planta* activity is measured, including GE-CTV titer, aphid transmissibility, symptom expression and response to co-infection with different California fully sequenced genomes of California wild-type CTV strains: RB, T30, T36, S1 and VT. Four cDNA clones of the T36-GE were obtained from the laboratory of Bill Dawson, Ph.D., Citrus Research and Education Center in Lake Alfred, Florida (**Table 1**).

T36-GE cDNA clones were transferred to tobacco via an *Agrobacterium*-based method, allowed to replicate in tobacco plants for three to four weeks and CTV virions (infectious virus particles) were collected from tobacco leaves by ultracentrifugation, then inoculated into Alemow plants (Gowda et al. 2005). Four to eight weeks after inoculation, the Alemow plants were monitored for systemic infection and stability by serology, PCR and visual expression of the markers (**Table 1**). All 28 Alemow seedlings mechanically inoculated became infected. Expression of T36-GE clone 527 (T36-GE-527) is shown in Alemow plants that were inoculated through GFP fluorescence in petiole and bark phloem (**Figure 1A**) and photobleaching in vein and stem phloem tissue (**Figure 1B**). This expression pattern demonstrates the localization and activity of these marker proteins in the phloem, which are critical steps if GE-CTV is expected to express genes to mitigate CLas activity at the site of infection – also in the phloem. This tobacco-*Agrobacterium* procedure is needed each time a new GE-CTV is constructed and was developed to evaluate current and future GE-CTV strains (Peng et al. 2018).

Table 1. List of GE-T36 clones established in citrus in Parlier, California.

Clone number	Inserted marker	Test for marker presence	Reference
500	GFP (green fluorescent protein)	Fluorescence in tissue where marker gene is present when viewed under UV light	Hajeri, Gowda and Dawson (unpublished)
525	tPDS (truncated Phytoene desaturase)	Photobleaching in citrus	Hajeri et al. (2014)
527	tPDS & GFP	Photobleaching and fluorescence in tissue where marker gene is present when viewed under UV light	Hajeri, El-Mohtar, Gowda and Dawson (unpublished)
588	GFP	Fluorescence in tissue where marker gene is present when viewed under UV light	El-Mohtar and Dawson (2014)

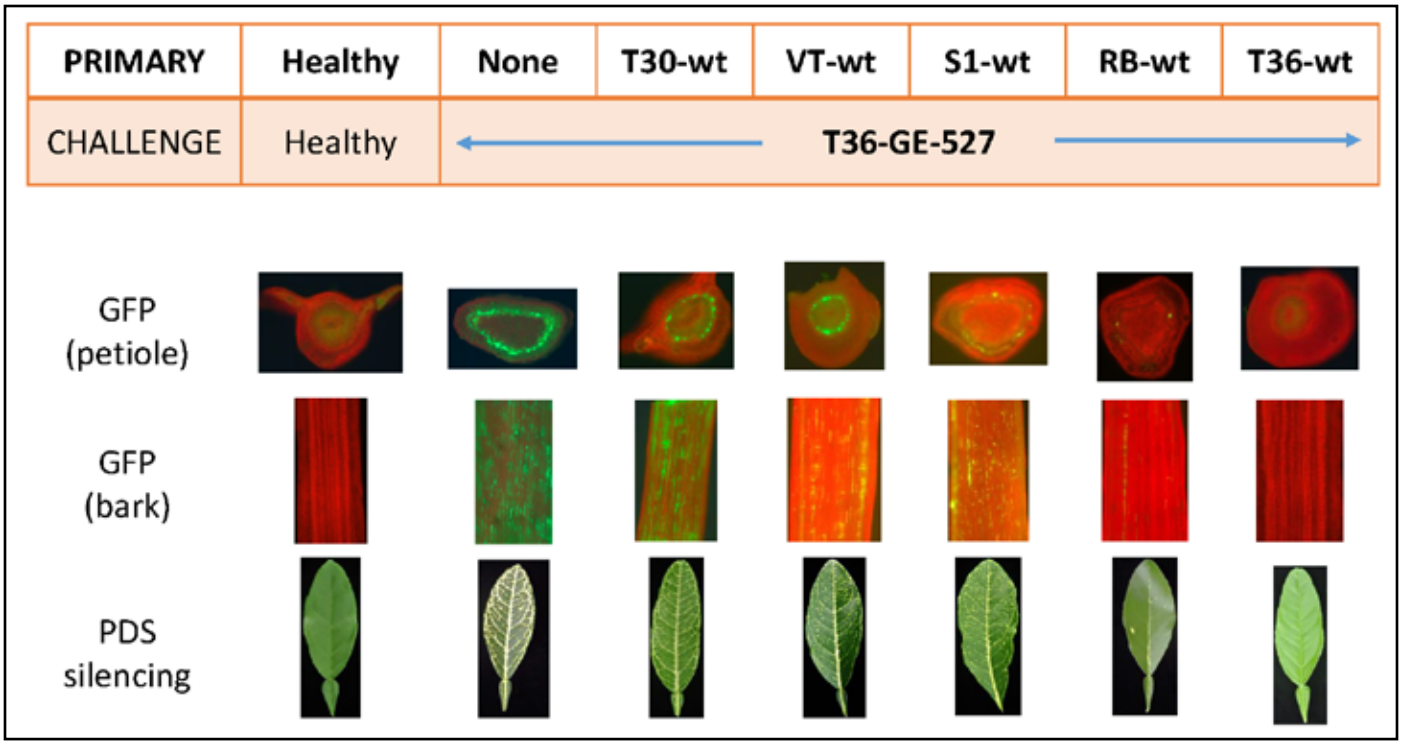

Figure 2. Challenge inoculation experiment. Alemow seedlings were initially inoculated with wild type (wt) California CTV strains to establish primary infection and subsequently graft-challenge-inoculated by T36-GE-527. Each treatment had four replications and data were collected 29 weeks post-inoculation.

Aphid Transmission of GE-CTV

Since aphid transmissibility differs with CTV strain, aphid species and citrus host, GE-CTV should optimally be non-transmissible to minimize risk of field spread, which is important for regulatory approval of field release. Therefore, aphid transmissibility was tested for all four GE-CTV clones using the cotton aphid, *Aphis gossypii*, the principle vector of CTV in California (Yokomi and DeBorde 2005), and results indicated a very low (0.86 percent) level of transmission. California is fortunate since the brown citrus aphid, a more efficient CTV vector, does not occur in state.

Interactions of Califoria Wild-type CTV Strains and T36-GE

CTV can be applied directly to trees in the grove or to trees propagated in citrus nurseries. In either case, a critical consideration for field deployment of GE-CTV is cross-protection, which is defined as a pre-existing CTV infection that prevents establishment of a new infection of the same genomic strain, but not from a different genotype strain (Folimonova et al. 2010). The overall incidence of CTV in central California is extremely low; and in central California, T30 is the predominant CTV strain with a small proportion of VT and S1 strains also present. RB and T36 strains can be found, but are rare (Hajeri and Yokomi 2019; Yokomi and DeBorde 2005).

Since CTV is endemic in California, an experiment was conducted to simulate how pre-infection of different wild type (wt) California CTV strains (T30, VT, S1, RB-115 and T36) may affect infection and expression of T36-GE-527. Wild type California CTV strains T30, VT, S1, RB-115 and T36 were first inoculated and established in Alemow seedlings in the greenhouse. After systemic infection by the wild type strains was confirmed, plants were challenge-inoculated with T36-GE-527. GFP expression and photobleaching were monitored in new flush tissue for two years. The California wild type T36 strain prevented infection by T36-GE-527 by cross protection; but T36-GE-527 readily co-infected Alemow preinfected with T30, VT and S1 wild type CTV strains. These results were expected because these strains are not closely enough related to the T36 strain to develop cross-protection (Folimonova et al. 2010). However, RB-115 significantly limited co-infection of the T36-GE clone 527 in Alemow (**Figure 2**). This observed interaction between RB-115 and T36-GE is likely due to competition, not cross-protection, since these strains have different genotypes; this also suggests that RB strains may limit the effectiveness of a T36-based GE-CTV to mitigate HLB.

Where GE-CTV is applied to nursery trees, co-infection of wild-type CTV may affect GE-CTV in new plantings. This was examined by a reciprocal experiment where Alemow was first infected with T36-GE-527 and then challenge inoculated with California strains. After one year, RB-115 still reduced the viral expresson of T36-GE-527. Therefore, there is a need to develop other GE-CTV genotype vectors to overcome problems associated with cross protection and competition.

Since susceptability and titer of CTV infection vary with citrus cultivars, the next step is to test levels of GE-CTV titer and

expression in commercial citrus cultivars in California. A U.S. Department of Agriculture, Animal Plant Health Inspection Service-Plant Protection and Quarantine/California Department of Food and Agriculture permit has been obtained to evaluate the infection and expression of Florida GE-CTV in orange, grapefruit, lemon and mandarin cultivars in a quarantine protective structure in Parlier, California.

This research benefits growers by understanding factors needed to improve efficacy of GE-CTV vectors and having an in-state system to test GE-CTV clones with different gene inserts designed to manage HLB and ACP. Additionally, data from studies like this one are needed to obtain regulatory approval for field testing in California.

CRB Research Project #5300-174

References

Dawson, W.O.; Bar-Joseph, M.; Garnsey, S.M.; et al. 2015. *Citrus tristeza virus*: making an ally from an enemy. *Annual Review of Phytopathology* 53:137–155.

Dawson, W.O. and Folimonova, S.Y. 2013. Virus-based transient expression vectors for woody crops: a new frontier for vector design and use. *Annual Review of Phytopathology* 51:321–337.

Dawson, W.O.; Garnsey S.M.; Tatineni, S.; et al. Citrus tristeza virus-host interactions. *Frontiers of Microbiology* 4:88.

El-Mohtar, C. and Dawson, W.O. 2014. Exploring the limits of vector construction based on *Citrus tristeza virus*. *Virology* 448:274-283.

Folimonova, S.Y.; Robertson, C.J.; Shilts, T.; et al. 2010. Infection with strains of *Citrus tristeza virus* does not exclude superinfection by other strains of the virus. *Journal of Virology* 84(3):1314–1325.

Garnsey, S.M.; Civerolo, E.; Gumpf, D.J.; et al. 2005. Biological characterization of an international collection of *Citrus tristeza virus* (CTV) isolates. p. 75–93. In: Hilf, M.E.; Duran-Vila; N., Rocha-Pena, M.A. (eds.), *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*. Riverside, California.

Gowda, S.; Satyanarayana, T.; Robertson, C.J.; et al. 2005. Infection of citrus plants with virions generated in *Nicotiana benthamiana* plants agroinfiltrated with a binary vector based *Citrus tristeza virus*. p. 23–33. In: Hilf, M.E.; Duran-Vila, N.; Rocha-Peña, M.A. (eds.). *Proceedings of the 16th Conference of the International Organization of Citrus Virologists*, Riverside, California.

Hajeri, S.; Killiny, N.; El-Mohtar, C.; et al. 2014. Citrus tristeza virus-based RNAi in citrus plants induces gene silencing in *Diaphorina citri*, a phloem-sap sucking insect vector of citrus greening disease (Huanglongbing). *Journal of Biotechnology* 176:42-49.

Hajeri S. and Yokomi, R. 2019. Preinfection of citrus by RB strains of *Citrus tristeza virus* (CTV) negatively affected expression of a modified T36 CTV vector. In: XXI *Conference of the International Organization of Citrus Virologists*. IOCV-05-04. Riverside, CA.

Peng, J.; Chen, A; Ng, J. 2018. Fighting HLB with a *Citrus tristeza virus*-based vector. *Citrograph* 9(3):66-70.

USDA-APHIS. 2018a. Southern Gardens Citrus Nursery, LLC Permit 17-044-101r to Release Genetically Engineered *Citrus tristeza virus*–Preliminary Pest Risk Assessment. USDA-APHIS-BRS. *https://www.aphis.usda.gov/brs/aphisdocs/17_044101r_CTV_ppra.pdf*

USDA-APHIS. 2018b. Southern Gardens Citrus Nursery, LLC Permit to Release Genetically Engineered *Citrus tristeza virus* –Draft Environmental Impact Statement. USDA-APHIS-BRS. *https://www.aphis.usda.gov/brs/aphisdocs/17_044101r_CTV_dEIS.pdf*

Yokomi, R.K. and DeBorde, R.L. 2005. Incidence, transmissibility, and genotype analysis of *Citrus tristeza virus* (CTV) isolates from CTV eradicative and non-eradicative districts in central California. *Plant Disease* 89(8):859-866.

Glossary

[1]T36-GE: Genetically-engineered CTV strain T36 from Florida.

[2]Photobleaching: Loss of green color in plants is due to sunlight damage caused by RNA silencing of the proctective phytoene desaturase gene.

[3]Cross protection: Protection against a severe CTV infection by pre-infection by a related, but milder strain.

[4]CTV isolate: A laboratory or greenhouse propagation of a field-collected strain of CTV by graft transmission to a host plant before any biological characterization is performed. This is considered a capture of the CTV population in the infected tree, as well as CTV variants, if present.

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