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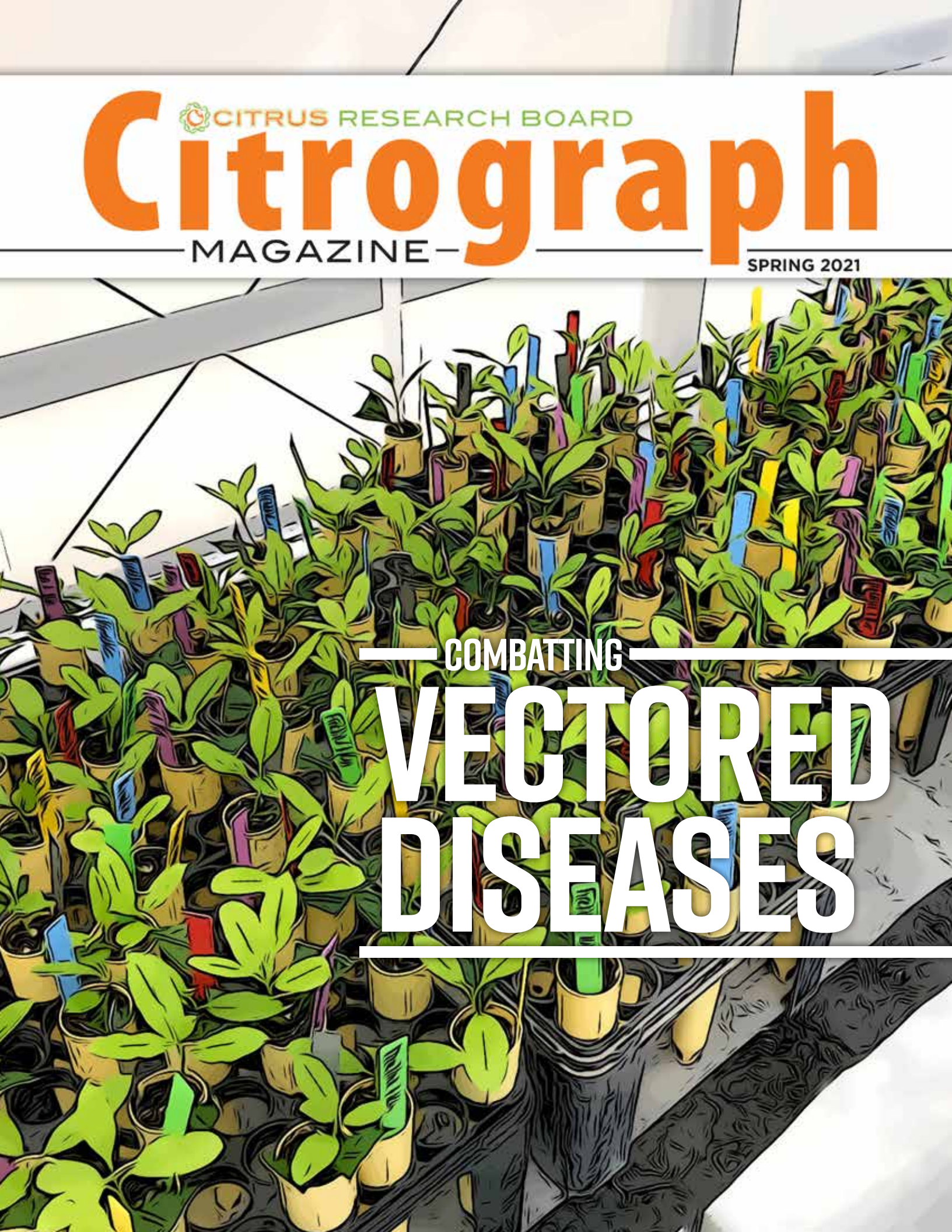
SPRING 2021



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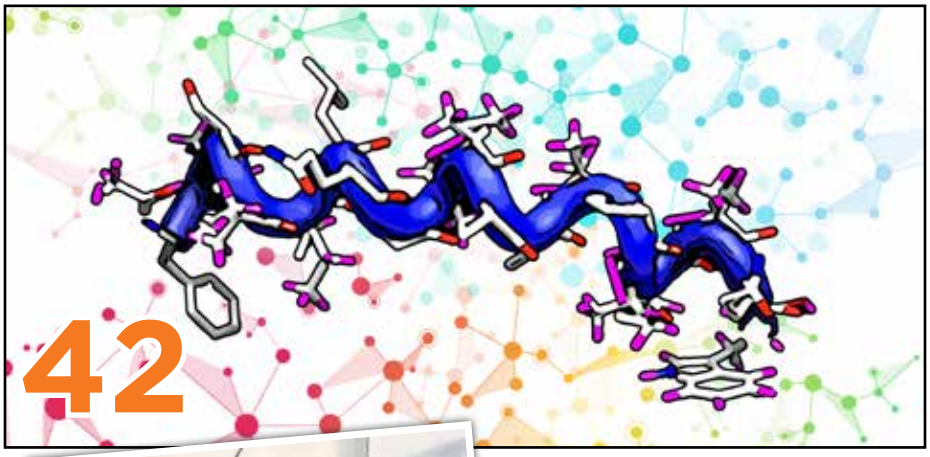

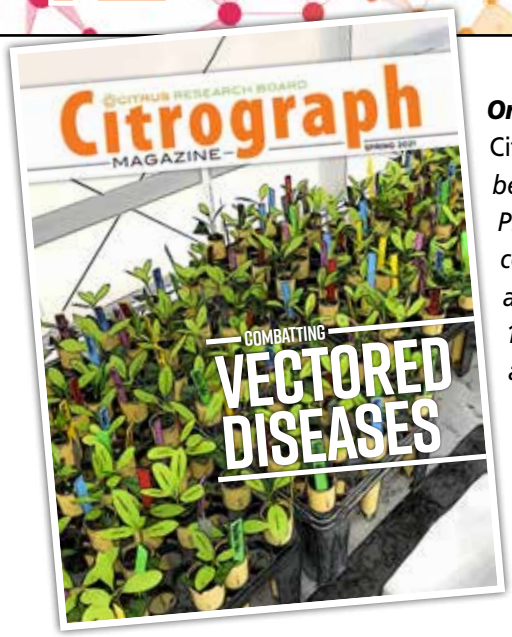

On the Cover: *The spring issue of* Citrograph *focuses on the research being conducted on vectored diseases. Pictured on the cover are 180 citrons confined in a single screen cage that are being exposed to approximately 1,000* 'Candidatus *Liberibacter asiaticus'-infectious Asian citrus psyllid adults. See page 52 to learn more about the results of this research in "Protecting Citrus from HLB" by Greg McCollum, Ph.D.*

Photo provided by Cool Carriers.

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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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MAY 11

Citrus Research Board (CRB) meeting.
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MAY 12

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

JUNE 1

2021 Citrus Research Board Webinar Series.
For more information and additional Webinar Series dates, see page 20.

AUGUST 10

Citrus Research Board (CRB) meeting.
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AUGUST 11

Citrus Pest and Disease Prevention Committee (CPDPC) meeting.
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SEPTEMBER 21

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TAMUK CITRUS CENTER DIRECTOR

JOHN V. DA GRAÇA RETIRES

Dr. da Graça in front of his backyard barbecue pit in McKinnie, Texas.

Mamoudou Sétamou

We recently said farewell to a dedicated servant/leader of the Texas A&M University-Kingsville (TAMUK) Citrus Center in Weslaco when John V da Graça, Ph.D, who served respectively as the deputy director and Director of the Citrus Center retired. John devoted 21 years of his life to leading the Citrus Center and assisting the Texas citrus industry as an active scientist and a mentor to many students.

Before joining the TAMUK Citrus Center, John spent about two decades as a scientist and professor of virology at his alma mater, the University of Natal in South Africa. During that time, he also took sabbaticals at citrus research centers in Israel, Spain and the U.S., including eight months at the TAMUK Citrus Center. Prior to his teaching career, John worked for five years at the Citrus and Subtropical Fruit Research Institute in South Africa where, among other things, he assisted in the establishment of a certified virus-free citrus budwood program.

In 1999, John joined the TAMUK Citrus Center as deputy director, becoming the Center director in 2008. During his tenure, the Citrus Center moved to a new building after decades spent in renovated old military barracks. John's vision and leadership led to the enactment of the Texas citrus budwood program, the sole source of clean budwood that supplies an average of 250,000 certified buds annually to nurseries statewide. This program ensures that Texas citrus nurseries receive top quality, true-to-type and pathogen-free budwood. "John has left a lasting impact on the south Texas citrus industry, and we will miss him", said Dean of College of Agriculture and Natural Resources at TAMUK Shad Nelson, Ph.D.

Another excellent accomplishment under John's leadership was the creation of the U.S. Department of Agriculture (USDA)-certified disease diagnostic laboratory, which has detected major exotic citrus pathogens including huanglongbing (HLB), sweet orange scab and canker in Texas.

John excelled as a leader and collaborator on pest and disease management efforts for the citrus industry. Throughout the last decade, he was at the forefront of industry efforts to curb the threat of HLB. He played an essential role in the creation of the Texas Citrus Pest and Disease Management Corporation, which focuses on developing strategies to manage invasive pests and diseases affecting the citrus industry. In collaboration with scientists at the Citrus Center, USDA-Animal and Plant Health Inspection Service, Texas AgriLife Extension and the citrus industry at large, John helped with the development and implementation of the area-wide management program for the Asian citrus psyllid (ACP), the vector of the HLB pathogen.

He also masterminded the transition of the Center from its primary research-only focus to become a research and education center that mentors students in both fundamental and applied research. From the inception of this training and mentoring program in 2000, the Center has graduated 12 Ph.D. and 104 M.S. students, thus touching the lives of many in south Texas and beyond.

Aside from his work at the TAMUK Citrus Center, John served on the Citrus Research Board (CRB) scientific review panel for HLB research in 2017 and as a CRB and Citrus Research and Development Foundation (CRDF) proposal reviewer on several occasions. At the international level, John was the chairperson of the International Organization of Citrus Virologists (IOCV) from 2004 to 2007.

Asked what he was most proud of during his tenure as Center director, John said, "working with great people for a wonderful grower community in south Texas," and also added "the enactment of the Texas budwood program, which ensures that disease-free trees are planted in Texas."

The TAMUK Citrus Center and Texas citrus industry will continue to gain the benefits of John da Graça's legacy for many years to come. Although he has retired, he will continue to serve on the USDA-National Clean Plant Network Quality Steering Committee. Following his retirement, John and his wife Sheila moved to McKinnie, Texas, to be closer to their daughters who live in Dallas. I know John has a full bucket list, including traveling across the Hill Country in Texas to "sample" wine, but I am hopeful this list will include a trip to the Rio Grande Valley during harvest season to taste the best grapefruit in the world.

All of us at the Center wish John a happy and healthy retirement.

Mamoudou Sétamou is a professor of entomology and interim center director of the Texas A&M University, Kingsville Citrus Center in Weslaco, Texas. For more information, contact **Mamoudou.Setamou@tamuk.edu**

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MEET THE VECTORED DISEASES COMMITTEE

Greg Galloway

VECTORED DISEASES COMMITTEE MEMBERS

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- **John Konda**
- Rodrigo Krugner, Ph.D.
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- Ed Russell, Ph.D.
- **Ram Uckoo, Ph.D.**
- Judy Zaninovich

The Citrus Research Board (CRB) Vectored Diseases Committee is focused on minimizing crop damage and economic losses by looking for tools, strategies and solutions to detect, eradicate, control and manage insect-vectored diseases, which are spread by insects like stubborn and huanglongbing (HLB). There are seven CRB Board members and four industry representatives who serve on the committee *(see list on previous page)*.

Research priorities are established annually, and most current and recently funded projects have focused on seeking tools for Asian citrus psyllid (ACP) management and solutions to the HLB problem. We have learned much from our friends in Florida, Texas and other citrus-growing countries. Their experiences and research will help inform and drive our research to meet the expectations of the California citrus industry. Correlating the results of other funding organizations' research should be welcomed into a best-practices update.

Some of the most recent research endeavors funded by the committee include work to improve HLB testing under California field conditions, research support for ongoing Citrus Pest and Disease Prevention Program activities and potential antimicrobial compound development (including iRNA, citrus tristeza virus, microbiome identified compounds, small peptides, etc. [*see "Antimicrobials: A Brief Introduction" on page 26*]). Several funding cycles ago, we received proposals for just a few antimicrobial projects. The following year, we received many proposals for a wide array of antimicrobial therapeutics, and the needs of this area of research – confirming the safety and specificity of these types of compounds – have resulted in a CRB-led effort to develop a national Antimicrobial Summit.

Our goals for the current year are commercialization and deployment of high-throughput early detection technologies for HLB-affected trees, management of existing groves during an HLB epidemic, development of tools to prevent *'Candidatus* Liberibacter asiaticus' (CLas) transmission or suppression of HLB disease and research on additional exotic or endemic disease that pose a threat to the California industry. Twelve continuing and six new projects, with support this fiscal year in the amount of $1,395,637, are currently underway.

Continuing projects are centered on areas such as mapping risk assessments across the state and model development in support of ongoing Citrus Pest and Disease Prevention Program activities, potential antimicrobial compound and delivery system development, and improved disease diagnostics.

New projects are looking at the potential role of canines for ACP detection, the role of biofilms in bacterial survival and reducing disease spread at the insect level.

Three separate projects that have had CRB funding are proceeding toward potential commercialization. Canine activities have moved forward at full speed with both ACP and HLB detection research being conducted in California. Support of a jointly funded project with the Citrus Research and Development Foundation, Coca Cola, PepsiCo and Bayer has allowed for the continuation of a project that has more recently received significant National Institute of Food and Agriculture (NIFA) funding with the expectation of potential HLB control.

We on the Vectored Diseases Committee understand and appreciate the important responsibility that we have to the industry. The decisions we make about which research projects our committee should fund are vital to determining whether California citrus growers can continue to survive and thrive amid the many challenges that are presented by vectored diseases.

***Greg Galloway is the chief operating officer for Sierra Crest Agriculture and also serves as chair of the Citrus Research Board Vectored Diseases Committee. For more information, contact* galloway.gc@gmail.com**

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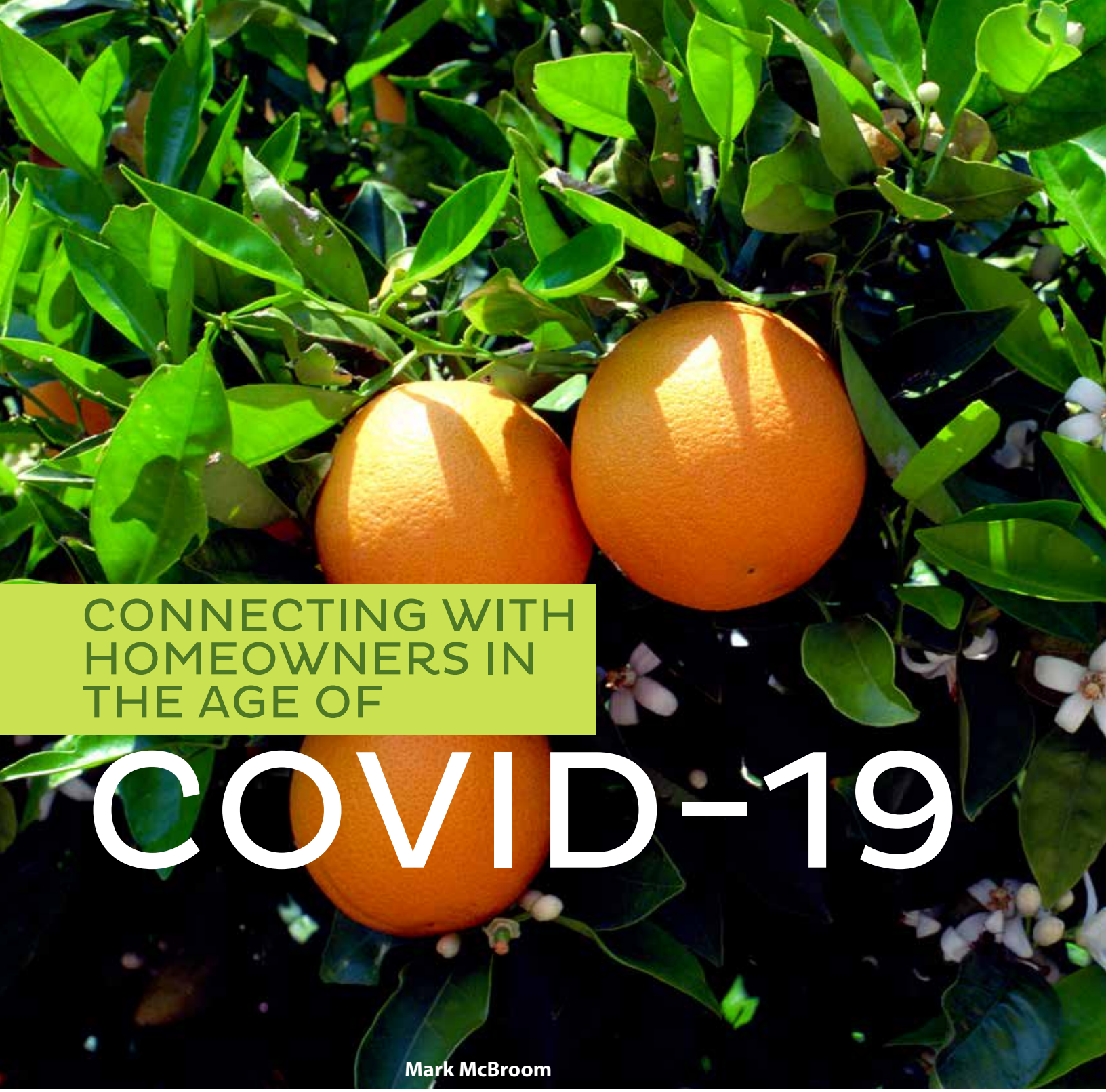


CONNECTING WITH HOMEOWNERS IN THE AGE OF COVID-19

Mark McBroom

During the past year, the Citrus Pest & Disease Prevention Program (CPDPP) has remained nimble in adapting to the myriad challenges presented by the COVID-19 pandemic, all while continuing to apply steady pressure against the progression of the Asian citrus psyllid (ACP) and huanglongbing (HLB). From increased safety protocols for staff to conducting virtual public meetings, the fight continues to ensure that citrus can be a staple in California homes and groves for years to come.

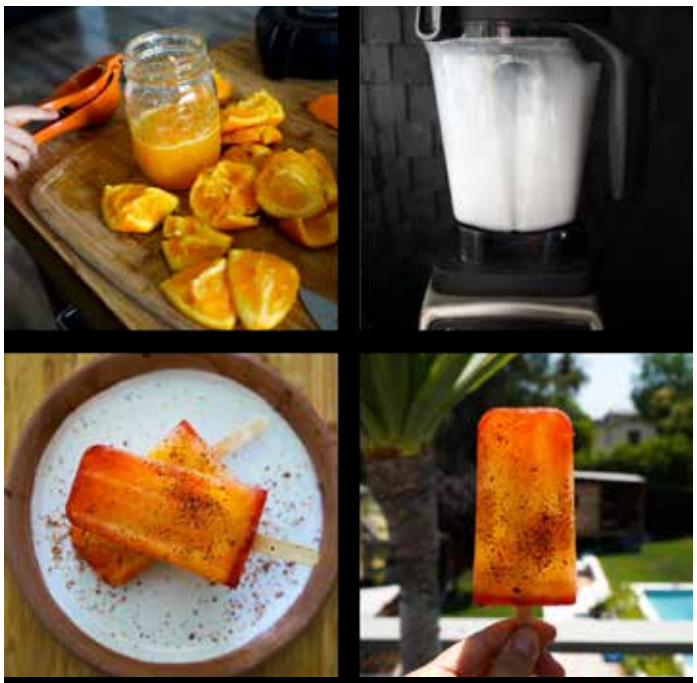
Furthermore, as California's residents have shifted their daily routines and news consumption habits, the CPDPP's outreach program has pivoted quickly to ensure the program can meet homeowners where they are and through sources they trust.

Engaging Local News

As local media outlets, reporters and journalists scrambled to cover critical news while adhering to social distancing and other health department guidelines, many traditional outreach tactics were abandoned. Television stations were no longer accepting in-studio guests; and traditional media events, where camera operators and reporters stood shoulder-to-shoulder to obtain remarks and video footage, were quickly dismissed. To continue to build residents' awareness of ACP and HLB, the program created virtual media opportunities that capitalized on major detection milestones or other relevant angles – such as the releases of *Tamarixia radiata* to suppress ACP populations – to reach the community via local broadcast, print and digital news sources.

For example, instead of hosting an in-person media event to discuss the releases of *Tamarixia* throughout California, the program developed a ready-for-press digital media kit, which included pre-recorded video footage, interviews, images and details about releases in key media markets. Additionally, virtual interview opportunities were offered with state biocontrol experts, to secure placements with CBS Los Angeles, ABC Los Angeles, NBC San Diego and the *Bakersfield Californian*.

Leveraging pre-recorded and virtual content allowed the program to secure coverage throughout the state.



Influencer Bricia Lopez, a restaurateur in Los Angeles, developed a traditional paletas recipe featuring citrus fruit while communicating our program's key messages.

The Digital Shift

As Californians abruptly shifted their lives online due to stay-at-home orders, their daily activities – shopping, school, work and entertainment – were primarily happening in the digital sphere. While the program has traditionally seen success

Ensuring homeowners participate in best practices while harvesting their backyard citrus fruit is a top priority for the CPDPP.

connecting with homeowners face-to-face at community events across the state, digital connections quickly became a primary conduit to maintain urgency among homeowners, particularly among multicultural audiences.

A paid media plan was developed to reach critical multicultural audiences that focused on each community's interests. The plan included partnering with California-based, Hispanic and bilingual influencers who helped communicate the program's key messages while delivering the information via fun and engaging social media content. Garnering an estimated seven million impressions, influencer partnerships allowed us to successfully reach homeowners in a culturally relevant and safe way.

In addition, the program developed in-language social media content in Chinese, Vietnamese and Korean to connect with audiences via social media platforms and encourage them to act. The video campaign delivered more than 950,000 impressions at a 1.63 percent engagement rate, which is five times higher than the industry benchmark of 0.26 percent.

Relevant Content for Concerned Residents

Understanding that COVID-19 impacts were top of mind for residents, the program created content to appeal to residents' concerns, while also encouraging desired behaviors. At the beginning of California's stay-at-home order in spring 2020, the program sought opportunities to promote at-home gardening as a safe way to get outside, relieve stress and reinforce inspection behaviors homeowners should take. By creating engaging content – including social media graphics, videos, pre-formatted articles and informational fliers – homeowners were encouraged to find solace in their gardening activities, visit University of California's website for helpful gardening tips and safely donate any harvested fruit to local food banks in their area.

Zooming into Public Meetings

In addition to leveraging traditional and social media trends to support the program's outreach, the Citrus Pest & Disease Prevention Division (CPDPD) also was tasked with moving the critically important public meetings to a COVID-safe format. To support that effort, the program developed a web page dedicated to providing localized information for virtual public meetings being held by the CPDPD. Residents were invited to attend these virtual public meetings via a personalized mail with meeting log-in information and links to helpful websites to inform them about the important actions that need to be taken to protect the area's surrounding citrus trees.

Looking Forward in 2021

California's citrus is part of our livelihood, and the widescale effort to preserve backyard and commercial citrus in California has reached a critical point. While the future of COVID-19's impact remains uncertain, CPDPP's outreach team will continue to innovate and explore various strategies to ensure we're reaching homeowners in culturally relevant ways that garner their support in the fight against ACP and HLB.

Mark McBroom is the vice chair for the Citrus Pest & Disease Prevention Committee and outreach subcommittee chairman. He also serves as secretary-treasurer of the Citrus Research Board. For more information, contact **desertcitrus@aol.com**

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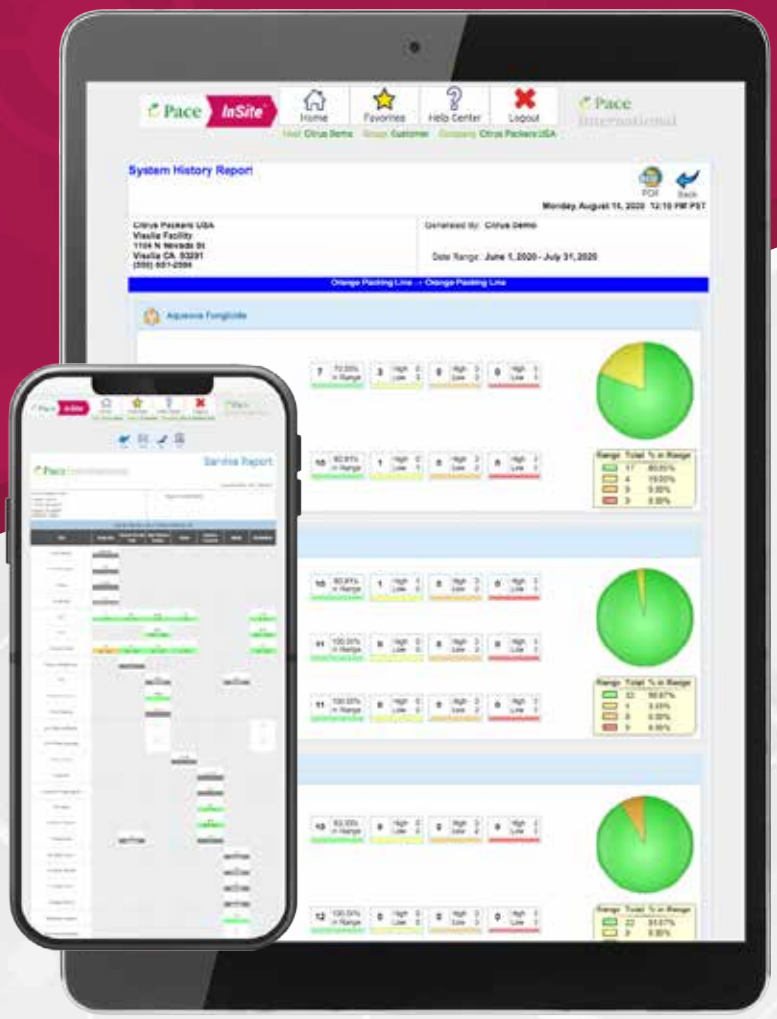

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CITRUS RESEARCH BOARD WEBINAR SERIES

Tamara Tollison and Ivy Leventhal

It soon will be time to sign up for the 2021 Citrus Research Board (CRB) Webinar Series, which will be held in coordination with the University of California Agriculture and Natural Resources Statewide Integrated Pest Management Program (UC IPM). The four one-hour free sessions, which will cover multiple topics, are scheduled for:

• JUNE 1 • JUNE 8 • JUNE 15 • JUNE 22

Normally, this would be the time of year when the CRB conducts its annual Citrus Growers Educational Seminar Series in person. However, due to the on-going COVID-19 situation, the series has been restructured into a webinar platform to ensure everyone's safety. This will be the second series of webinars conducted as a partnership between the CRB and UC IPM.

Continuing education units will be available through the California Department of Pesticide Regulation, pending their approval.

More information will be released shortly via the CRB's website, *www.citrusresearch.org*. Please watch for a list of topics and presenters, additional details and sign-up information.

***Tamara Tollison is the communications specialist at the Citrus Research Board and also serves as an editorial assistant to* Citrograph. *Ivy Leventhal is the managing editor of* Citrograph. *For more information, contact* tamara@citrusresearch.org**

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This issue of Citrograph takes a closer look at vectored diseases. We recently asked the following questions of two California citrus industry members...

WHAT HAVE WE LEARNED ABOUT VECTORED DISEASES IN THE LAST FIVE YEARS?

HOW HAS THIS KNOWLEDGE BENEFITED CALIFORNIA CITRUS GROWERS?

ANDREW BROWN
AB Farming Company
A&A Ag Services
Orange Cove, California

The last five years have reinforced the need for the use of clean nursery stock for planting and propagation, as well as the importance of vector control, specifically Asian citrus psyllid (ACP) control to prevent the spread of huanglongbing (HLB) throughout the growing districts. Throughout those years, much effort and time have been directed toward the development of Early Detection Technologies for use in finding HLB-infected trees in orchards prior to the visual symptoms becoming apparent. While there are still some promising leads and possibly some other approaches that have not been researched yet for finding the disease before it can gain a foothold in our orchards, our industry must remain focused on the proven methods of vector control.

On another note, the Citrus Research Board (CRB) has been collaborating with the Citrus Research and Development Foundation (CRDF), large food industry stakeholders and Bayer to help fund fast-tracked research into multiple methods of HLB prevention or control in citrus. This exciting project is leveraging citrus industry support to engage commercial scale research using the vast resources of companies that are experienced in successfully bringing crop protection products to market. The hope is that if a promising tool is developed, it can be placed into the hands of growers for use in protecting their orchards and thus our domestic citrus supply very quickly.

KEVIN OLSEN
Kevin Olsen Agri-Business Consultant
Clovis, California

In the last five years, we have expanded our knowledge of vectored diseases. Specifically applied to HLB, we have made significant progress on basic and applied research. Because of this, we've been able to lessen the fear factor in dealing with the problem moving forward. Though it remains a significant threat to the economic health of citrus growers and must be reckoned with, we know we can move forward with the knowledge that the disease isn't an imminent death threat for the entire industry here in California, whereas five years ago it was considered the number one threat to profitable citrus production by the vast majority of citrus producers. While still significant, many growers would no longer consider it the number one threat. Water availability and affordability, regulatory and statutory burdens, along with competing markets and year-round competition from other commodities would be three issues that may be considered by some growers as greater threats, depending upon an individual grower's circumstances. ACP isn't a threat if it dies from thirst because the tree runs out of water. The upshot here is that the substantial amount of money made available from grower assessments to the CRB or the Citrus Pest & Disease Prevention Division (CPDPD) exclusively for HLB control over the last several years may not be available or chosen to be available in the years ahead. We collectively will have to work smarter with finite resources.

Armed with this knowledge, growers now have much more ability to properly plan for the future. Growers also are going to benefit handsomely from the CRB's ability to hone future research to have a direct benefit to growers. Since we know more, prospective and ongoing research projects that aren't going to result in tangible benefits will be able to be culled out earlier in the process. This will provide greater focus on those projects that will show a greater return on investment to the grower.

***Tamara Tollison is the communications specialist with the Citrus Research Board and also serves as the communications assistant on* Citrograph.**
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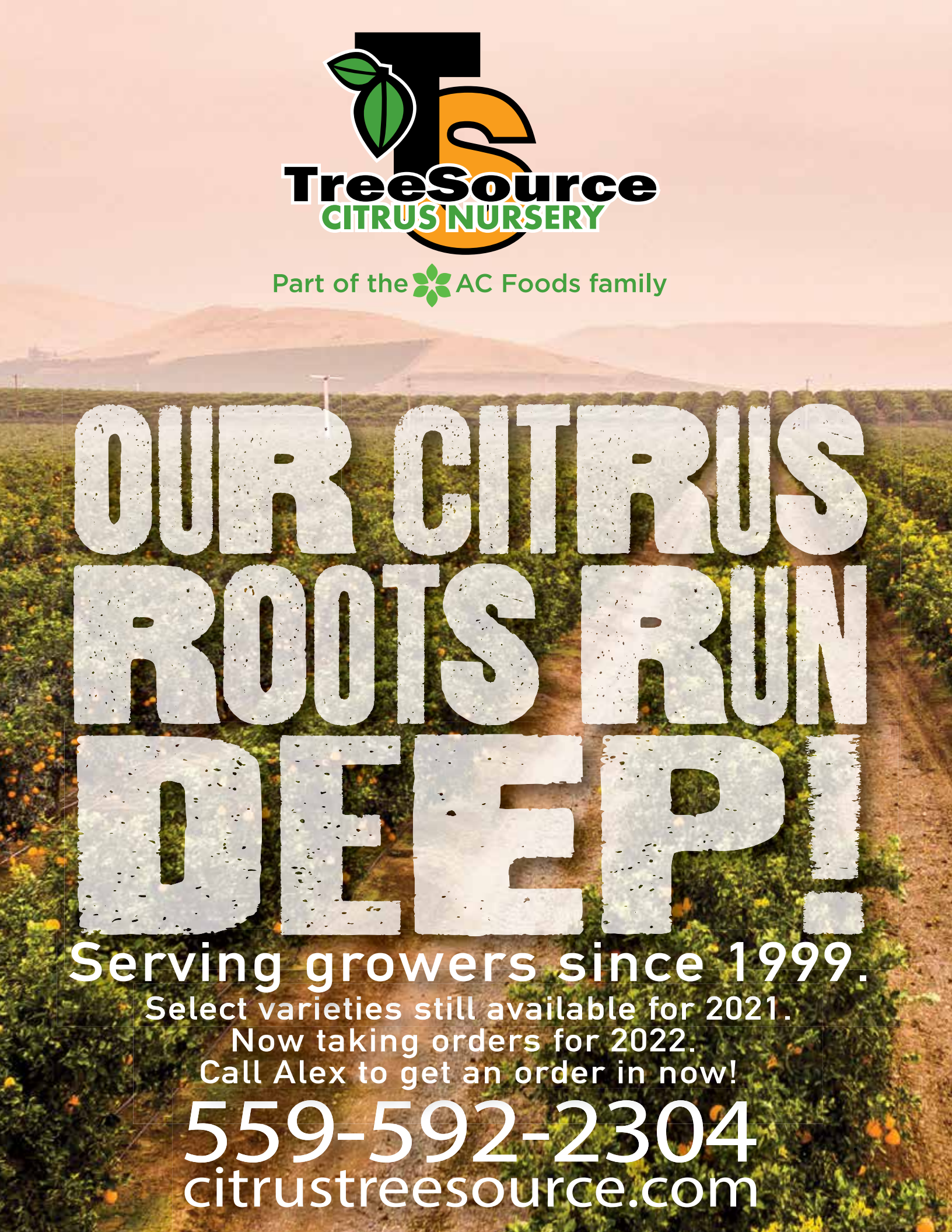
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ANTIMICROBIALS:
A BRIEF INTRODUCTION

Joey S. Mayorquin and Tamara Tollison

For several years, the Citrus Research Board (CRB) has received an increasing number of research proposals outlining the discovery and development of antimicrobial therapies – therapies that can suppress or kill the growth of microorganisms – for a variety of citrus pests. Most notably, many of these proposals outline the development of antimicrobial therapies for huanglongbing (HLB), a disease presumptively caused by the bacterium *'Candidatus* Liberibacter asiaticus' (CLas). The destructive nature of HLB, which has severely impacted the Florida citrus industry, threatens to do the same in California. Accordingly, the need for effective HLB management strategies remains a priority for the California citrus industry. Discussions regarding antimicrobial therapies, however, are not unique to the CRB or to California citrus.

Insect pest and disease antimicrobials have been a 'hot topic' in the research world in the past decade, especially due to their longstanding use in agriculture. The use of antimicrobials in agriculture, particularly in livestock production, boomed during the 1940 and 1950s, and antimicrobials in plant agriculture go back even further when one considers the use of Bordeaux mixture (copper and lime) in the late 19th century for plant disease control (i.e., downy mildew on grapes). Since this time, the number and types of antimicrobials available for use in agriculture have increased substantially. However, the benefits realized in reducing disease pressure have been counteracted by animal and human health, safety and environmental concerns leading to regulatory changes in antimicrobial registration and use and have even resulted in the banning of several antimicrobials.

Despite these regulatory actions and the growing need to address public health and environmental concerns, the global research community continues to explore the discovery, development and use of new antimicrobials for the agricultural industry to manage or mitigate plant diseases that can be as effective as current antimicrobials, but potentially more environmentally responsible and pathogen-specific. Currently, many antimicrobials are being actively pursued by research teams around the globe.

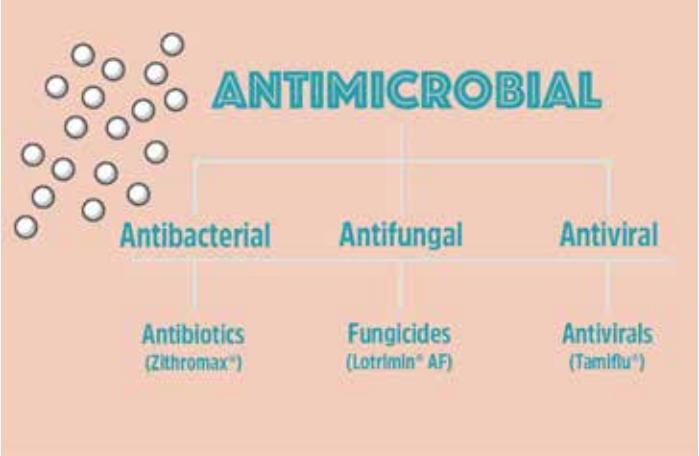

Figure 1. Antimicrobial is a broad term that includes all substances that act against microorganisms (i.e., bacteria, fungi, viruses).

What Exactly is an Antimicrobial?

An antimicrobial is a substance that can suppress or kill the growth of microorganisms (i.e., bacteria, fungi, viruses). Antimicrobials can be developed in a laboratory, but they also occur naturally in widely diverse environments. They are produced by bacteria, fungi, plants, insects and animals. An antibiotic[1] is a substance that either slows the growth of or kills bacteria. Similarly, a fungicide is a substance that can kill a fungus. However, antibiotics and fungicides are completely different from each other. So how can they both be antimicrobials? To put it simply, antimicrobial is a broad term that includes all substances that, in some way, act against microorganisms. Whether the substance is antibacterial (antibiotics, Zithromax®), antifungal (fungicides, Lotrimin® AF) or antiviral (antiviral, Tamiflu®), all these substances fall under the umbrella term of antimicrobial (**Figure 1**).

How Do Antimicrobials Work?

Antimicrobials vary in how they work, which is often referred to as 'mode of action' in the scientific literature. For example, antibiotics can be classified based on whether they interfere with the production of key cell parts like bacterial cell wall, protein or nucleic acid. Antibiotics can be further classified based on what step they target in the formation or development of either the cell wall, protein or nucleic acid (**Figure 2**).

Antimicrobials also vary in their modes of action, which can affect microorganisms directly or by activating host organism defense responses. In addition to modes of action, antimicrobials have varying degrees of specificity, meaning they may affect specific microorganisms (*Liberibacter* species) or a larger group of microorganisms (e.g., gram-negative bacteria).

One class of antimicrobial compounds that has received increasing attention over the years for use in plant production agriculture includes antimicrobial peptides (AMP). AMPs are short proteins that usually are produced by a range of organisms, both animals

[1]*Here we use antibiotic to refer to substances that either slow/inhibit the growth of (bacteriostatic) or kill (bactericidal) bacteria.*

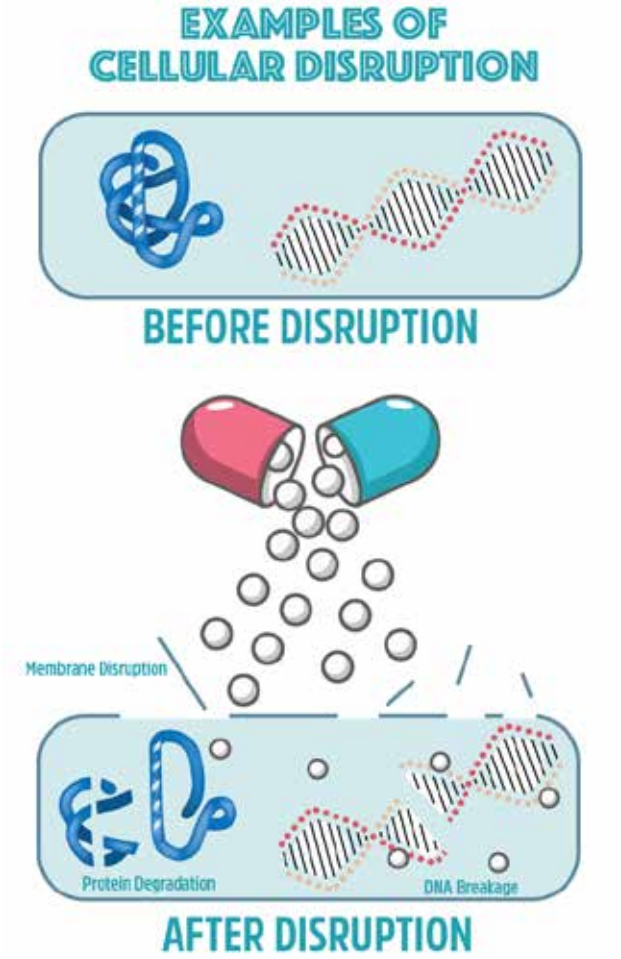

Figure 2. Antibiotics vary in their mode of action (how they work) and can interfere with the production of key cellular parts by disrupting the cell membrane, causing protein breakdown (degradation) or causing damage to nucleic acids (i.e., DNA).

and plants, and function as "in-house" antimicrobials to protect the organism producing them from other organisms. Just like antibiotics can be classified by structure, mode of action and specificity, AMPs also are classified in this manner. There is no shortage of the types of antimicrobials in the natural world.

As a general example, an AMP targeting bacteria may function as a broad-spectrum AMP, affecting different bacterial species (i.e., CLas, *Spiroplasma citri* [Citrus stubborn disease] and *Xanthomonas citri* [Citrus canker]) or it may function as a narrow spectrum, affecting one group of bacteria (*Liberibacter* species only). It also may function by disrupting the bacterial cell wall or by entering the cell and disrupting a cellular process. Although AMPs are naturally derived products – another reason that makes them attractive for use in agriculture – they also can be synthesized in the lab, meaning AMPs can be modified to make them more selective or improve their potency.

How are Antimicrobials Applied?

Antimicrobials can be applied several ways (**Figure 3**). In addition to soil and foliar applications, antimicrobials may be applied

Figure 3. Antimicrobials for agricultural use can be applied in various ways. Some examples include soil and foliar applications, direct injection, laser ablation (requires a second step – usually a spray application – to introduce the antimicrobial compound) and genetic engineering of the plant itself or through the introduction of a genetically engineered microorganism into the plant.

by direct injection into a plant's vascular system or by using a laser to first create an entry point into the plant and then delivering the antimicrobial via another method (e.g., spraying). Beyond these mechanical methods of application, molecular biology allows antimicrobial genes to be incorporated into the host plant's genome and expressed either constitutively (continuously) or under specific conditions. More interestingly, microorganisms can be repurposed, so to speak, to deliver antimicrobials into plants by first modifying a microorganism to produce the antimicrobial and then introducing the modified organism into the plant host.

One of the most widely known examples of this in recent years is the use of citrus tristeza virus (CTV), a pathogen of citrus, as a vector[2] for antimicrobial products. Scientists continue to make progress in modifying the CTV genome to not only avoid causing disease in citrus when introduced, but also to carry the genetic information of the antimicrobial so as the virus replicates within the plant, so too does it produce the antimicrobial product contained within its genome.

What is the Current State of Antimicrobial Research?

Currently, there are a number of research projects investigating the use of antimicrobials to manage various pests of citrus. Many of these projects are devoted to addressing the immediate need for CLas and/or HLB mitigation. They are funded at either the state and/or federal level and are often led by a diverse team of researchers throughout the United States. For the 2020-21 fiscal year, the CRB funded ten research projects for a total of almost $600,000: five projects are investigating the use of antimicrobial therapies to manage CLas/HLB, and five projects are investigating the use of antimicrobials for other citrus pests such as pre- and post-harvest decay fungi (**Table 1**).

At the federal level, the United States Department of Agriculture National Institute of Food and Agriculture (USDA-NIFA) Emergency Citrus Disease Research and Extension (ECDRE) Program awarded more than $33 million from a total of $45 million for FY2020 projects that will work toward combatting HLB with novel antimicrobials and/or delivery methods. The USDA HLB Multi-Agency Coordination (MAC) Group awarded more than $400,000 in FY2020 for two projects – one that will explore cost-effective antimicrobial treatments in managing HLB and another that will bring together industry, academia and government to foster coordinated efforts in the development of HLB antimicrobial therapies.

Efforts to develop antimicrobial compounds to fight HLB are well underway; and the CRB, along with the rest of the US citrus industry, is eagerly anticipating the results from these research projects. The CRB will continue to review and monitor this approach to HLB mitigation with the needs of the California citrus industry in mind.

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[2]Here we use vector to refer to an agent, in this case, a virus that has been modified to carry foreign genetic material to be introduced into a plant host.

Table 1. Antimicrobial Citrus Research Board (CRB)-funded projects for fiscal year 2020-21.

CRB ID#	Project Title	Principal Investigator	Affiliation	2020-21 year	2020-21 Funding
5300-190	High performance California-derived CTV-based vectors for the control of HLB and other applications	James Ng	UC Riverside	4 of 3	$0[3]
5300-203	Biological control of huanglongbing by the bacterium *Paraburkholderia phytofirmans* PsJN	Johan Leveau	UC Davis	2 of 3	$2,660
5300-202	Year 2:A screen of nodule-specific cysteine-rich peptides for control of the HLB bacterium in citrus	Michelle Heck	USDA-ARS	2 of 1	$0[3]
5300-207	Independent-mobile RNA (iRNA) expression vector against HLB-initiate operation "Lab 2 Farm"	Georgios Vidalakis	UC Riverside	2 of 2	$189,360
5300-211	Phage hydrolases for *Liberibacter* biofilm control	Jennie Fagen	North Carolina State University	1 of 2	$35,976
5400-155	Epidemiology and management of *Phytophthora* diseases of citrus in California	James Adaskaveg	UC Riverside	3 of 5	$104,000
5400-156	Forecasting and management of Septoria spot of citrus	James Adaskaveg	UC Riverside	3 of 3	$50,000
5400-162	Post-harvest disease management	James Adaskaveg	UC Riverside	1 of 5	$61,062
5400-163	Epidemiology and management of anthracnose dieback and Alternaria Rot in California citrus	Themis Michailides	UC Davis	1 of 1	$67,840
5400-164	Validating the control of cross-contamination in high-pressure washers and fungicide flooders	Steven Pao	CSU Fresno	1 of 1	$79,632

[3]*Project is continuing with funds originally provided during fiscal year 2019-20.*

RESPONSE OF CALIFORNIA CITRUS TO POST-HARVEST PHYTOSANITARY TREATMENTS

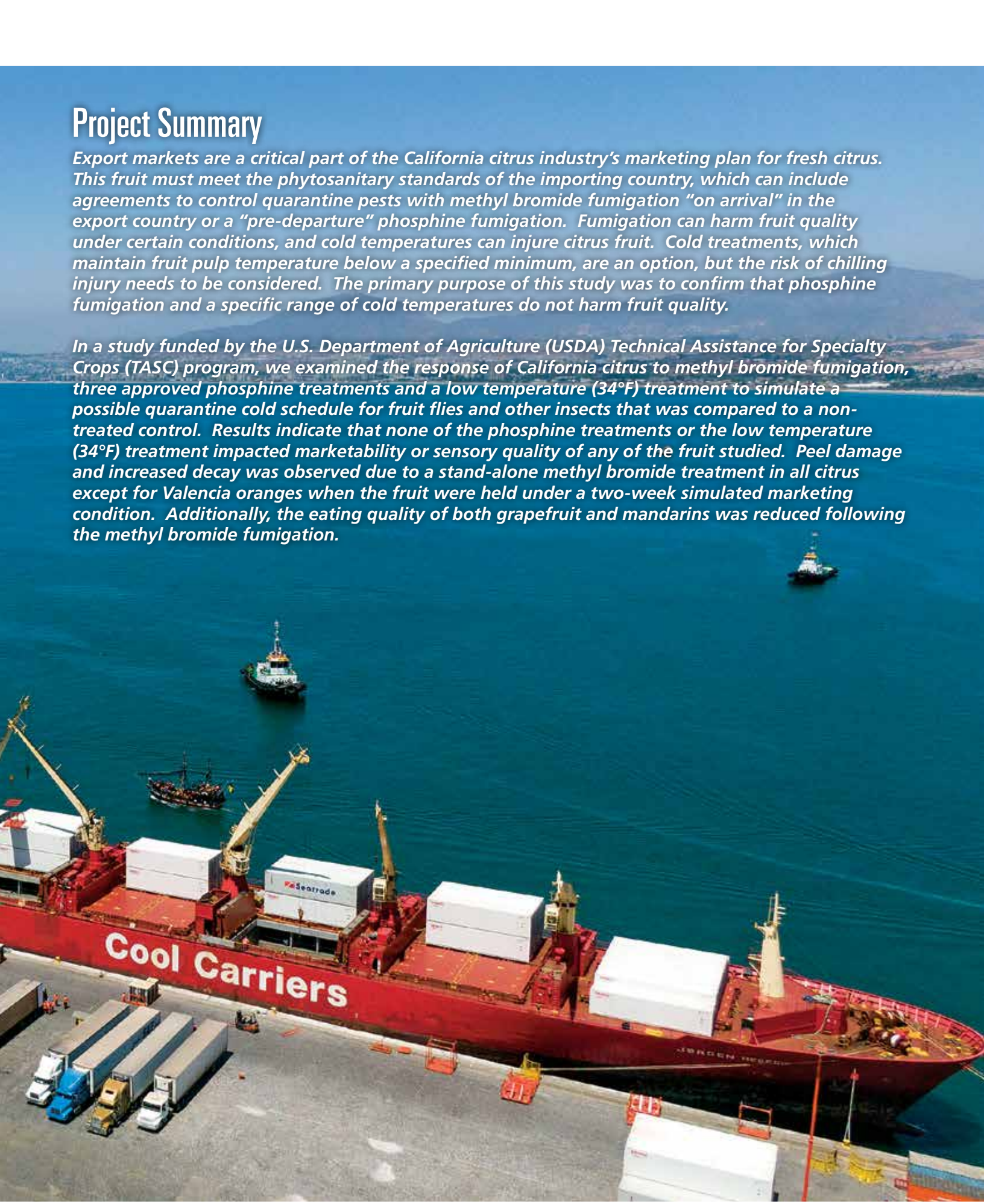
Mary Lu Arpaia, James Cranney, Steve Tebbets, Spencer Walse and David Obenland

Photo provided by Cool Carriers.

Project Summary

Export markets are a critical part of the California citrus industry's marketing plan for fresh citrus. This fruit must meet the phytosanitary standards of the importing country, which can include agreements to control quarantine pests with methyl bromide fumigation "on arrival" in the export country or a "pre-departure" phosphine fumigation. Fumigation can harm fruit quality under certain conditions, and cold temperatures can injure citrus fruit. Cold treatments, which maintain fruit pulp temperature below a specified minimum, are an option, but the risk of chilling injury needs to be considered. The primary purpose of this study was to confirm that phosphine fumigation and a specific range of cold temperatures do not harm fruit quality.

In a study funded by the U.S. Department of Agriculture (USDA) Technical Assistance for Specialty Crops (TASC) program, we examined the response of California citrus to methyl bromide fumigation, three approved phosphine treatments and a low temperature (34°F) treatment to simulate a possible quarantine cold schedule for fruit flies and other insects that was compared to a non-treated control. Results indicate that none of the phosphine treatments or the low temperature (34°F) treatment impacted marketability or sensory quality of any of the fruit studied. Peel damage and increased decay was observed due to a stand-alone methyl bromide treatment in all citrus except for Valencia oranges when the fruit were held under a two-week simulated marketing condition. Additionally, the eating quality of both grapefruit and mandarins was reduced following the methyl bromide fumigation.



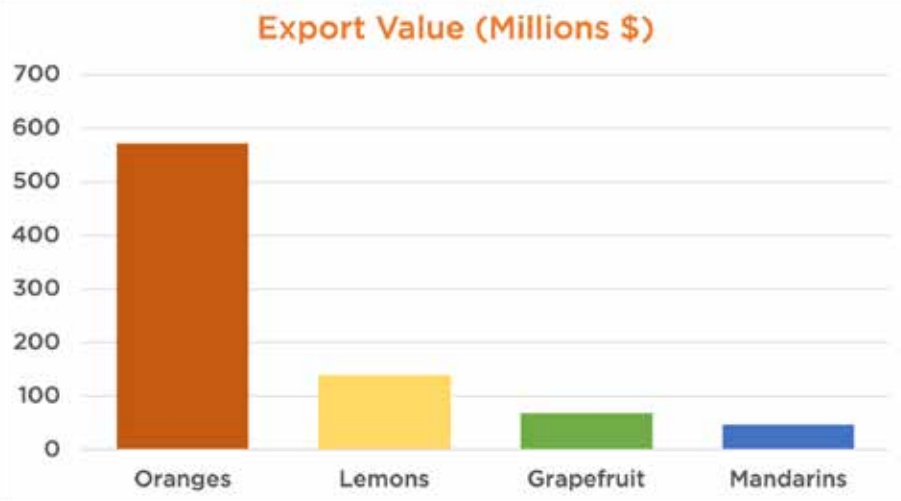


Figure 1. Export value ($ millions) of U.S. citrus for 2019-20 season.

Source: USDA. Economic Research Service. Fruit and Tree Nuts Data (www.ers.usda.gov/data-products/fruit-and-tree-nuts-data) [Accessed January 11, 2021]

Background Information

The value of fresh citrus exports of U.S.-produced citrus in 2019-20 was more than $879 million (USDA-ERS, 2021, **Figure 1**). Of the many types of citrus produced, oranges, for which California is the primary producer, dominate the export market ($570 million). Lemons are the second most important exported citrus ($139 million), followed by grapefruit ($67 million) and mandarins ($46 million). Several of the Pacific Rim countries constitute the major markets for California exports. South Korea is the most important as it is the largest importer of U.S.-produced oranges. Australia also is an important trading partner. Continued access to these markets is challenging. South Korea wishes to phase out methyl bromide treatments for Fuller rose beetle and California red scale, and a treatment that can control bean thrips for fruit exported to Australia is needed.

In the past, methyl bromide was the post-harvest fumigant of choice. Methyl bromide is the primary quarantine fumigant since it is a broad-spectrum biocide and is highly effective. It is inexpensive, easy to use and requires relatively short treatment times. However, methyl bromide is facing increasing regulatory scrutiny because it is an ozone depletor, and some regulators have suggested that it should be phased out. Phosphine fumigation is a potential alternative treatment to methyl bromide for those pests that it controls. Phosphine treatment times are considerably longer than those required for methyl bromide. Beginning in 2018, the California industry began the commercial use of phosphine as a phytosanitary treatment for exported citrus, but questions still existed regarding potential phytotoxicity following fruit exposure. Specifically, this project assessed fruit damage and the potential impact on the eating quality of fruit treated with either phosphine or methyl bromide. Since fruit storage at temperatures below 36˚F for prolonged periods is another non-chemical approach for phytosanitary treatment, we also included a treatment at 34˚F for comparative purposes. Cold temperatures also can contribute to greater insect mortality when used in combination with fumigation, but this approach was not included in this study.

Table 1. Citrus types evaluated.

Citrus type	Source or seasonality	Storage temperature during simulated oceanic transit (except for cold treatment)
Navel Orange	Early, Mid, Late	41°F
Valencia Orange	Early, Mid, Late	41°F
Lemon	District 1, District 2, District 3	50°F
Grapefruit	Early, Mid, Late	46°F
Mandarin	Clementine, Tango (Early, Mid)	41°F

Table 2. List of treatments

Name	Treatment	Timing
Control	None	Not Applicable
Phosphine 1	12 h at 41°F, 1.5 mg liter $^{-1}$	prior to storage
Phosphine 2	48 h at 41°F, 1.5 mg liter $^{-1}$	prior to storage
Phosphine 3	12 h at 54°F, 1.5 mg liter $^{-1}$	prior to storage
Methyl Bromide	2 h at 41°F, 64 mg liter $^{-1}$	applied following 4 weeks
Cold	4 weeks at 34°F	Not Applicable
Phosphine 2 followed by methyl bromide		

What We Did

Prior to conducting this study, we consulted with California citrus exporters to learn the export seasonality for each citrus type and what shipping temperatures and approximate shipping times are currently used. We then evaluated the impact of different post-harvest phytosanitary treatments on various important citrus categories in California (**Table 1**). The storage temperatures listed in **Table 1** reflect this input. We obtained fruit of each citrus type from commercial packinghouses, following packing. For each type, we conducted three tests, spanning the export season for each. For each test, we obtained fruit from three "grower lots," and three cartons of each lot were subjected to the various phytosanitary treatments.

The treatments are listed in **Table 2**. In the second year, we only examined navel oranges and focused on examining their potential response to a sequential treatment of a pre-shipment phosphine treatment followed by a market arrival methyl bromide treatment since this may occur in commercial practice.

The sequence of how we handled the fruit is depicted in **Figure 2**. Fruit were fumigated and stored at the USDA-Agricultural Research Service (ARS) San Joaquin Valley Agricultural Sciences Center in Parlier, California, and the fruit were evaluated and presented to volunteer panelists at the University of California (UC) Kearney Agricultural Research and Extension Center in Parlier. In sampling the fruit for sensory quality, we took fruit that were rated as marketable in our initial assessment. Each individual fruit in every carton was rated for overall marketability or presence of decay. Fruit with any moderate or severe defects were eliminated from the sensory evaluation since it was presumed that these would not reach the consumer and would be culled prior to the purchase point. Volunteer panelists from the USDA-ARS and UC staff evaluated the fruit for sensory quality. For a typical evaluation, we had 20 panelists per day who rated the visual and eating quality of the fruit.

What We Found

Overall, in the nearly two years of fruit evaluation, we found that fruit fumigated with phosphine as a pre-departure

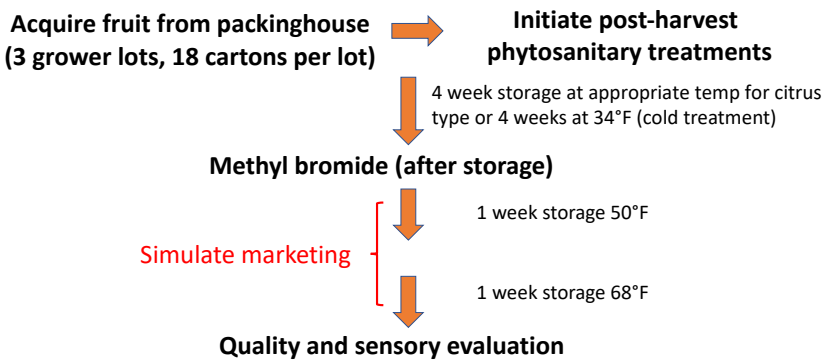


Figure 2. Sequence of experiment.

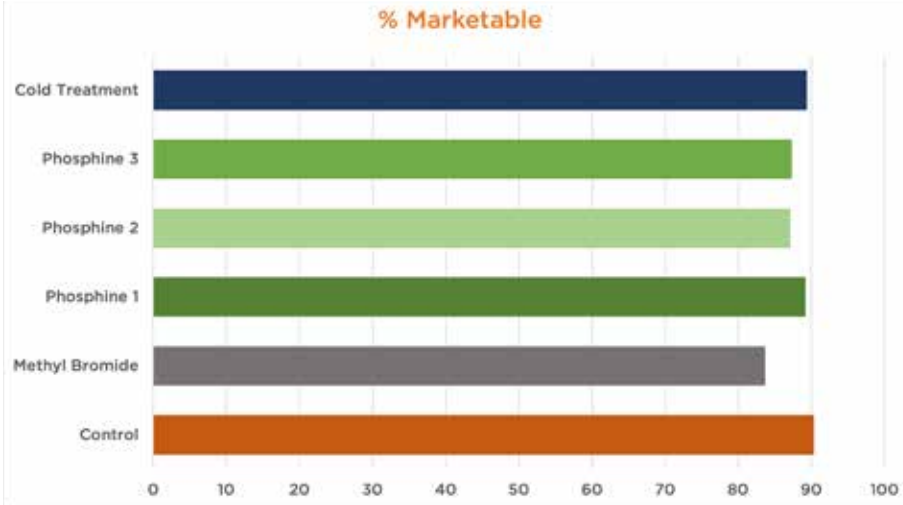


Figure 3. Percent marketability of navel oranges following single treatments.

treatment were not statistically different in terms of marketability, incidence of decay or eating quality from the untreated controls. Fruit fumigated with methyl bromide as an arrival treatment, except for Valencia oranges, had reduced overall marketability and increased decay as compared to the untreated control. Grapefruit and mandarin flavor also were impacted by methyl bromide fumigation. To our surprise, we did not observe a negative impact due to the low temperature (34°F) treatment, since we anticipated that we would observe rind pitting or staining characteristic of chilling injury because of the long exposure (four weeks) to 34°F. We should note that the low temperature (34°F) treatment imposed here approximates guidelines for cold treatments outlined in the USDA-Animal and Plant Health Inspection Service (APHIS) Treatment Manual (2020). One possible reason for this result is that the fruit were not put into cold treatment until seven to ten days following harvest, and previous research has shown that "conditioning" or "curing" can occur if fruit are held above the chilling temperature for several days (Hatton and Cubbedge, 1982; Chalutz et al., 1985). This issue could be the subject of further research if there was interest in understanding how the California industry could utilize low temperature as a quarantine treatment alternative or how colder temperatures could be used in combination with available fumigants to control quarantine insects.

The following discusses the findings for each fruit type.

Oranges

As stated above, we observed no differences due to treatment in marketability, decay or sensory quality for Valencia oranges. Navel oranges, however, showed a slight but statistically significant reduction in marketability when the fruit were fumigated with methyl bromide in the first year (**Figure 3**). When we conducted the sequential fumigation study during the second year, we repeated the Phosphine 2 treatment and methyl bromide treatment alone or in sequence. For the three tests we conducted in the second year, we observed no reduction in overall marketability for

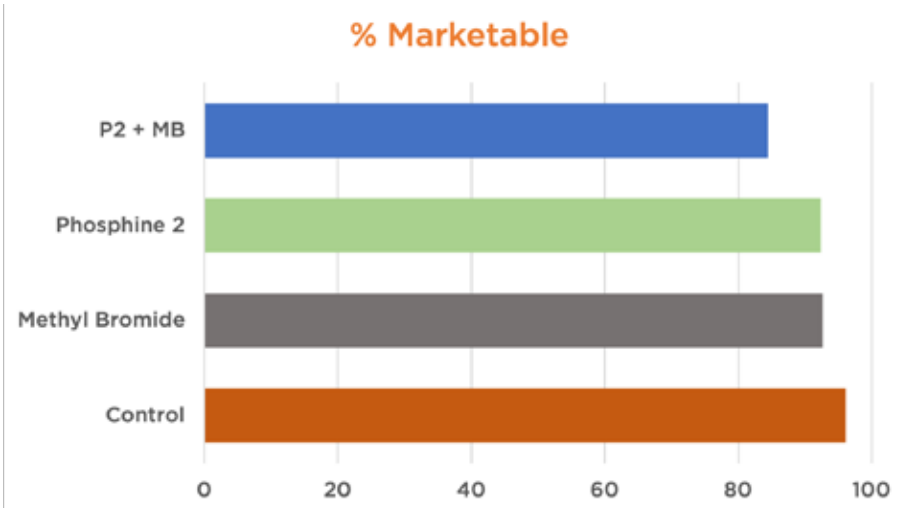


Figure 4. Percent marketability of navel oranges following either single or sequential (Phosphine 2 [P2] + Methyl Bromide [MB]) treatments.

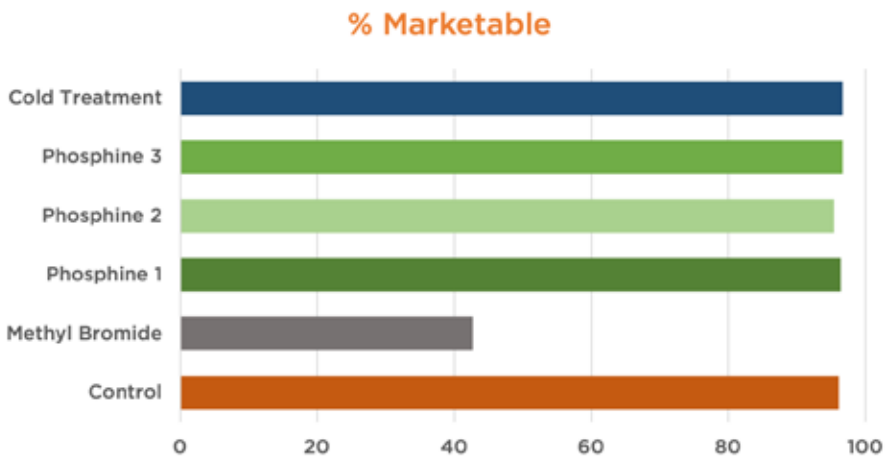


Figure 5. Percent marketability of lemons following treatment.

the treatments as stand-alone fumigations compared to the control, but the sequential treatment (pre-departure phosphine followed by on-arrival methyl bromide) had a statistically lower amount of marketable fruit, which was due primarily to an increase in fruit decay (**Figure 4**). Sensory quality of the fruit was not affected.

Lemons

We conducted tests on lemons representative of each production district. No matter the source of the fruit, we observed moderate to severe peel damage in response to the methyl bromide treatment, thus significantly reducing the percentage of marketable fruit (**Figure 5**). Associated with this peel damage was an increase in fruit decay from about 17 percent following methyl bromide fumigation to about one to two percent for the other treatments . The damage to the lemon peel observed following methyl bromide fumigation is limited to the flavedo tissue (outer colored layer of citrus fruit) as shown in **Figure 6**.

Grapefruit

Similar to lemons, we observed moderate to severe pitting damage on certain lots of grapefruit following methyl bromide fumigation that reduced the overall marketability of the fruit from about 80 percent for all treatments to about 20 percent (**Figure 7**). Enhanced fruit decay also was observed in association with the methyl bromide treatment. For the untreated control, phosphine treatments and the cold treatment, we observed about 15 percent decay, whereas the methyl bromide fumigated fruit had 58 percent decay when evaluated (**Figure 8**). Unlike oranges and lemons, grapefruit presented for sensory evaluation was found to have poorer flavor following the methyl bromide treatment than fruit from the untreated control, the three phosphine treatments or the cold treatment (data not presented). This loss in flavor quality is associated with the fruit being rated higher for the presence of off-flavors.

Figure 6. Lemon showing methyl bromide damage. Note that damage is limited to the flavedo (colored) portion of the peel.

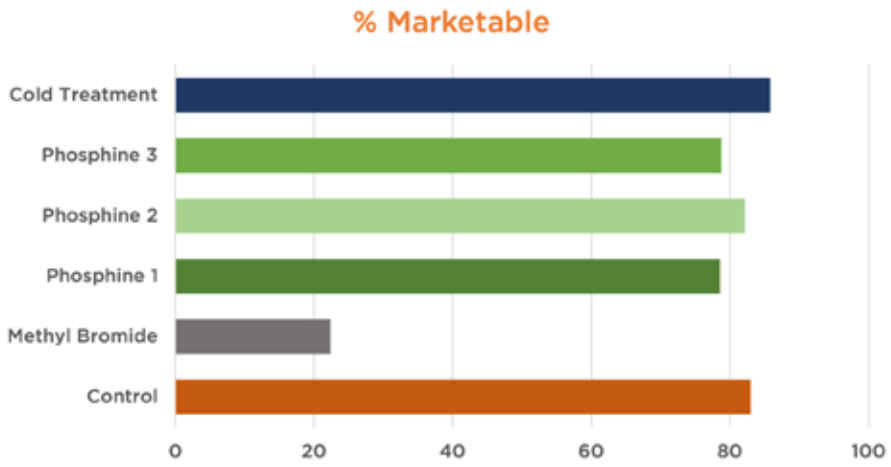


Figure 7. Percent marketability of grapefruit following treatment.

Mandarin

Overall, the visual quality of all mandarins was good at about 90 percent marketable; but as with the other citrus types, fumigation with methyl bromide after four weeks of storage resulted in a slight, but statistically significant reduction in marketability. The results for the sensory analysis of the mandarins were similar to those for the grapefruit with statistically lower flavor and higher off-flavor associated with the methyl bromide treated fruit (**Figure 9**).

Conclusion

This study demonstrated there is no negative impact on fruit quality following a pre-departure phosphine fumigation on either overall fruit marketability or eating quality, which indicates that this fumigant can serve as an alternative treatment to methyl bromide for quarantine pests that respond to phosphine fumigation. Fumigation with methyl bromide as an on-arrival treatment may be injurious to citrus fruit, but the treatment scenarios likely represent the worst case. Our fruit evaluation occurred following an additional two-week simulated marketing scenario that was based on input from industry experts we consulted, but this may have contributed to the poorer performance of the methyl bromide treated fruit. Our results suggest that if a methyl bromide treatment must be applied on-arrival, the fruit should be marketed with minimal delay. The occurrence and severity of damage manifested by peel pitting and an increase in overall decay is dependent on citrus type.

Results from this study indicate that lemons and grapefruit are the most sensitive citrus types, and oranges, especially Valencia (data not shown), are the most tolerant. A sequential treatment schedule in which navel oranges are treated prior to departure with phosphine and then fumigated with methyl bromide upon arrival resulted in more peel damage and decay than fruit fumigated with

Figure 8. Grapefruit showing enhanced decay due to a stand-alone methyl bromide treatment.

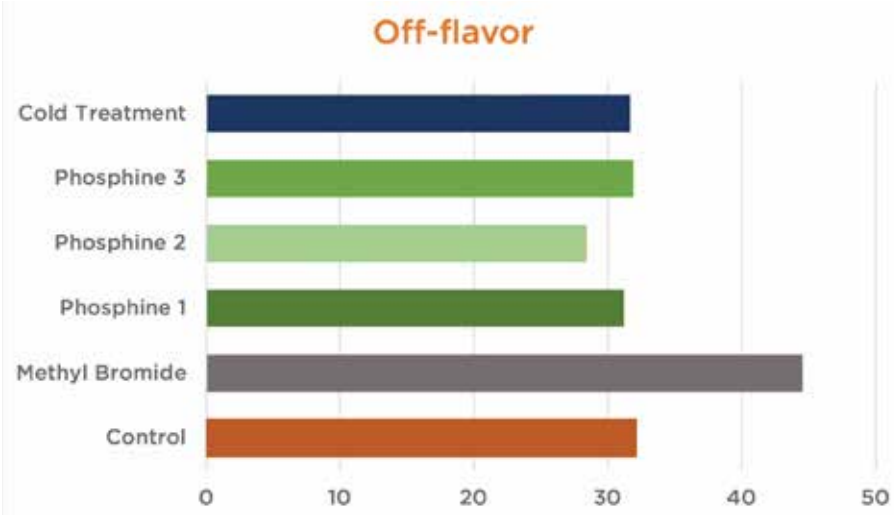


Figure 9. Rating of off-flavor of mandarin following treatment.

either fumigant alone. This suggests a synergistic effect of the fumigants and that a sequential treatment should be avoided whenever possible. Finally, the results of the low temperature treatment (four weeks at 34°F) suggest California citrus could tolerate such a treatment. We believe our results are due to unintended conditioning that occurred following harvest and would warrant further study prior to commercial adoption.

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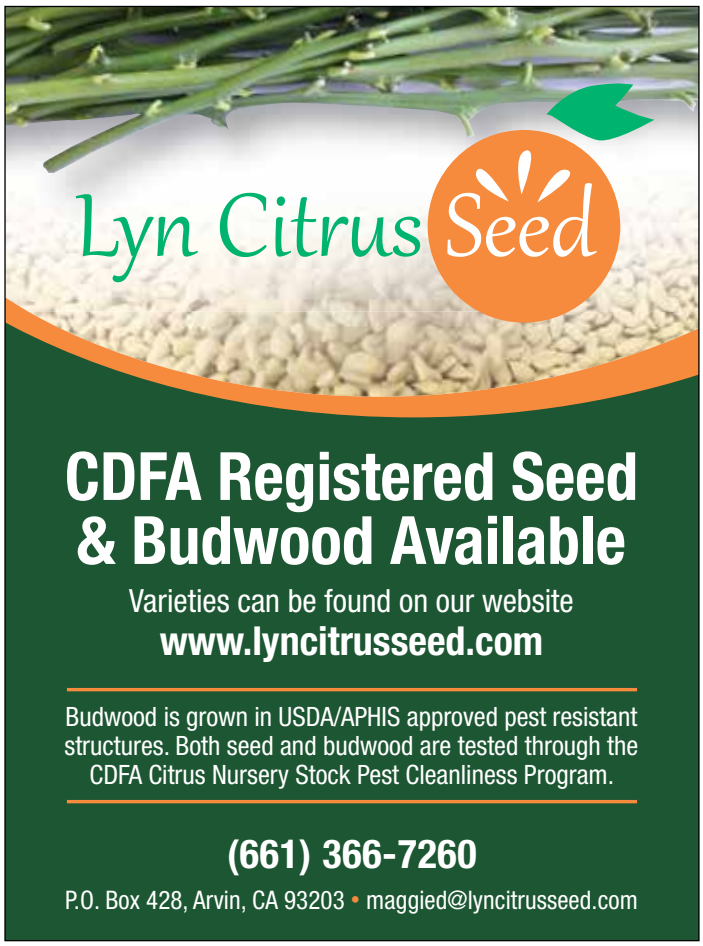

ENGAGING NON-ENGLISH-SPEAKING CITRUS HOBBYISTS IN THE HLB FIGHT

Figure 1. "14 Nasty Citrus Diseases that You MUST Avoid" (Chinese).

Dan Willey

Project Summary

Citrus hobbyists in California have the potential to rapidly spread 'Candidatus *Liberibacter asiaticus' (CLas) via the movement of CLas-infected Asian citrus psyllids (ACP), citrus trees and citrus propagative material, potentially increasing the incidence of huanglongbing (HLB) in the state. This project developed a series of web-based articles and YouTube videos that have reached Chinese-speaking, Vietnamese-speaking and Korean-speaking California citrus hobbyists and taught them how to reduce the spread of CLas.*

Uninformed citrus hobbyists may present a significant threat to citrus in California. Movement of citrus trees, cuttings and fruit has the potential to spread CLas via the vegetation and also the ACP present on that vegetation. Three previous Citrus Research Board (CRB) projects have successfully taught a large number of hobbyists how to avoid the spread of CLas via YouTube videos (**Table 1**).

The focus of the first project (Willey 2016) was to teach hobbyists to graft with pathogen-free citrus budwood from the Citrus Clonal Protection Program (CCPP). Other topics included enhancing ACP biocontrol by managing Argentine ants infesting citrus trees and citrus pruning techniques to avoid spreading ACP and CLas. In the first project, nine English language YouTube videos were published.

Table 1. YouTube videos published in this project and in prior projects to avoid the spread of huanglongbing and Asian citrus psyllid by California citrus hobbyists. Videos are now available in English, Spanish, Chinese and Korean with Vietnamese translations available soon.

Video
Grafting Citrus Trees – Cleft Grafting a Citrus Cocktail Tree
Grafting Citrus Trees with the Patch Bud – Making a Fruit Cocktail Tree
Grafting Lemon Trees – Successfully Grafting Fruit Trees with the T-Bud
Grafting Orange Trees – How to Graft a Tree by T-budding
Grafting Citrus with the Z Grafting Technique
Growing Citrus from Cuttings – Rooting and Grafting Citrus in One Step
Pruning Citrus Trees without Accidentally Killing Them
Ants on Citrus Trees – Organic Ant Control with Tanglefoot Sticky Barriers
How to Keep Ants off Fruit Trees with Liquid Ant Bait
Ordering Citrus Budwood for Grafting Citrus Trees
Grafting Citrus Trees Under a Microscope
Breeding and Growing Citrus from Seed to Overcome a Deadly Disease
Saving Citrus Trees by Removing Unloved Citrus Trees
Grafting Citrus Trees by Chip Budding in a California Nursery
Growing Citrus Trees from Seed – from Harvesting Fruit to Germination
Growing Citrus from Cuttings – How to Root Citrus Rootstocks
Dangers of Moving Citrus Fruit in California
Dangers of Moving Citrus Trees in California
Citrus Tree Care – Fertilizing Citrus Trees and Other Questions Answered by Dr. Ashraf El-Kereamy
Citrus Pests – Your questions answered by Entomologist Dr. Monique Rivera
Citrus Propagation and Disease in California – Your Questions Answered by Dr. Georgios Vidalakis
Asian Citrus Psyllids and HLB – Your questions answered by Victoria Hornbaker and the CDFA
Why does Huanglongbing (Citrus Greening) Spread so Rapidly? Answered by Dr. Neil McRoberts
14 Nasty Citrus Diseases that You MUST Avoid

The goal of the second project (Willey 2018) was to spread the message of the first project to speakers of Spanish, Chinese (Mandarin), Vietnamese and Korean. This involved translating and narrating the videos from the first project into these additional languages, which have large numbers of speakers in California. YouTube channels were created in the additional non-English languages, and the videos were published on the new channels.

These first two projects created some of the most viewed citrus propagation videos on YouTube. Adding the views of the five languages, the most popular of these videos has gained more than 12 million views. Not only has this resulted in a large number of California citrus hobbyists ordering pathogen-free budwood from the CCPP, but it also has created the opportunity to more easily reach many of those viewers with further videos.

The third project (Willey 2019) sought to leverage the success of the first two projects in order to explain the HLB threat in greater depth and to teach and persuade people to adopt behavior that would further reduce the spread of CLas and HLB incidence in California. Video topics included:

- tree removal,
- breeding citrus for HLB resistance,
- citrus variety introduction by shoot tip grafting,
- citrus propagation in a California nursery,
- rooting citrus rootstocks,
- citrus diseases of major concern in California and
- how to avoid spreading ACP and HLB when moving citrus trees and fruit in California.

In addition, a series of five interviews with citrus industry experts was produced to answer more than 50 viewer questions. The 14 videos produced were published in both English and Spanish.

The goal of this project was to further reduce the spread of CLas and the rate of HLB incidence in California by translating

Figure 2. "Interview with University of California, Riverside's Ashraf El-kereamy, Ph.D." (Vietnamese).

Figure 3. "Grafting Citrus Under a Microscope" (Korean).

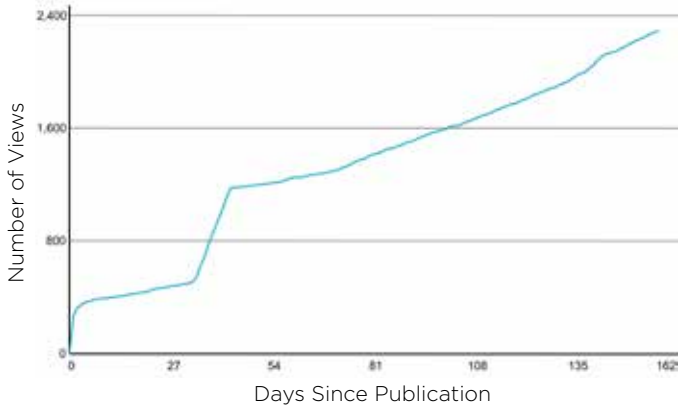

Figure 4. YouTube views of "14 Nasty Citrus Diseases that You MUST Avoid" (Chinese); 162 days since publication.

the remaining videos from the third project into Chinese (**Figure 1**), Vietnamese (**Figure 2**) and Korean (**Figure 3**) and publishing them on those YouTube channels. Translators and narrators were hired, and new versions of the videos were produced in Chinese, Vietnamese and Korean. In total, 40 new videos were published on YouTube.

Figure 4 shows a graph of the YouTube views over time of a Chinese video published early in the project. Shortly after publication, there was an initial burst of views as subscribers of the Mandarin YouTube channel watched the video. In the second month, advertisements targeted at Chinese speakers in California were run, producing a second burst of views. Views continued to increase as YouTube learned more about the video and recommended it to more interested viewers. Similar trends have been observed on the other published videos, varying by topic and viewer interest.

As of December 2020, the new videos were receiving sustained viewership on YouTube, which is likely to continue. It is expected that the publications from this project will reduce the spread of HLB in California by hobbyists and thus help slow the spread of HLB to hobbyists and commercial citrus growers alike in California.

CRB Research Project #5300-208

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Dan Willey is the founder of fruitmentor™, a company that is using Silicon Valley technology to reduce the spread of citrus disease. For additional information, contact **dan@fruitmentor.com**

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Natural Products – Potential for HLB Control

John Ramsey, Laura Fleites, Stacy DeBlasio, Stuart Krasnoff, Kevin Howe, Mark Trimmer, Robert Shatters Jr. and Michelle Heck

Project Summary

The goal of this project was to identify natural, plant-derived antimicrobial[1] compounds that could kill 'Candidatus *Liberibacter asiaticus' (CLas), the bacterium associated with huanglongbing (HLB) in citrus. We identified multiple peptides[2] produced by the legume plant,* Medicago truncatula*, that have antimicrobial activity; and, unexpectedly, we identified a fungal peptide that exhibited more potent antimicrobial activity than the legume-derived peptides. Our findings could offer California citrus growers a new and natural suite of options for preventing or treating HLB and/or preventing the spread of* Diaphorina citri *(Asian citrus psyllid [ACP]), which transmits CLas to citrus trees.*

California citrus growers need effective strategies for managing HLB. Although no infected trees have been found in commercial citrus groves in California, more than 2,200 infected trees have been identified on residential properties (*https://maps.cdfa.ca.gov/WeeklyACPMaps/HLBWeb/HLB_Treatments.pdf*). The presence and, therefore, the likely spread of HLB within the state are a major concern for growers because of the mobility of ACP, the insect vector for CLas. Organic growers, in particular, seek HLB management strategies that do not rely on conventional chemicals. The goal of this project was to identify antimicrobial agents, from plants or other organisms, with different killing modes of action that could effectively control CLas infection in citrus trees and/or prevent ACP from transmitting the bacteria to citrus trees.

Our search for plant-derived antimicrobial agents focused on the Mediterranean legume, *Medicago truncatula* (barrel

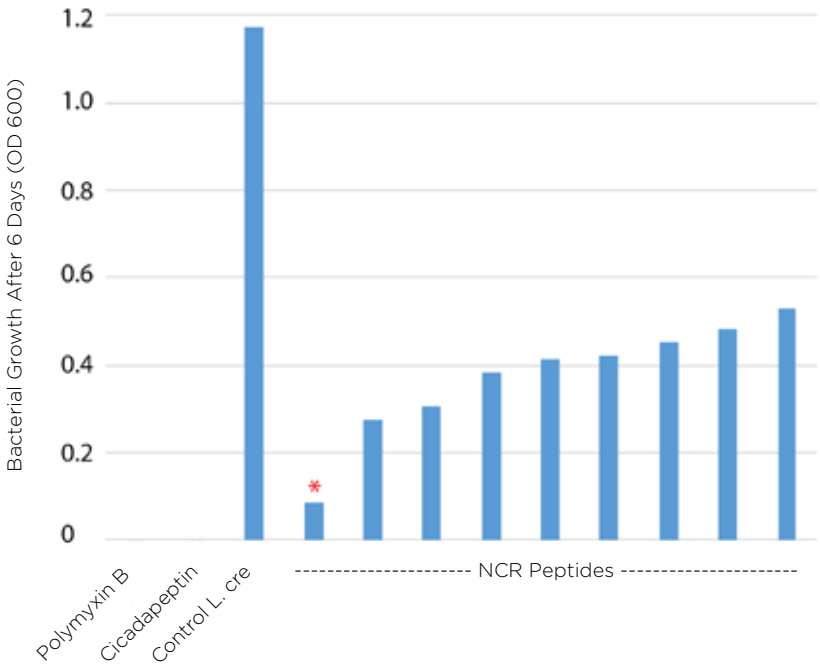

Figure 1. Minimum inhibitory concentration assays with *Liberibacter crescens* (L. cre). grown with a panel of nodule specific cysteine rich (NCR) peptides, no peptide control, polymyxin B and cicadapeptin. The most effective NCR peptides reduced *L. crescens* growth by about 90 percent (one is shown and denoted with an *). Y-axis is the change, after six days of treatment, in optical density (OD600), a method to assess bacterial growth using a spectrophotometer.

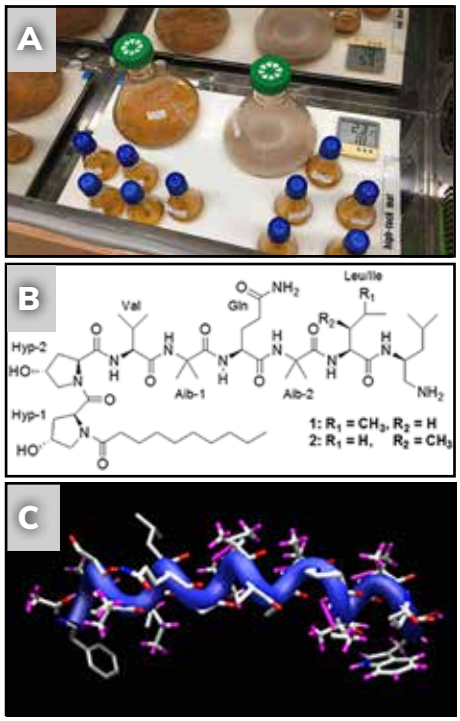

Figure 2. The antimicrobial peptide cicadapeptin is produced by a culturable fungus originally found infecting the Australian cicada. A) Cultures of the *Cordyceps* fungus which produces cicadapeptin, in production in the Heck lab. B) Chemical structure of cicadapeptin variants as determined by mass spectrometry. C) Predicted model of cicadapeptin in an alpha helical formation which is shared by peptides known to disrupt bacterial cell walls.

clover). This legume produces a wide range of antimicrobial peptides, called nodule-specific cysteine-rich (NCR) peptides,[3] that regulate its symbiotic[4] relationship with the soil bacterium *Sinorhizobium meliloti*, an important nitrogen-fixing symbiont that is genetically related to CLas. *M. truncatula* uses NCR peptides to control which plant cells the bacterium can infect and the levels of the bacterium that are allowed to accumulate in the root system, thereby enabling the plant to derive the benefits of the symbiosis with *S. meliloti* while preventing the bacterium from taking advantage of the symbiosis.

More than 600 NCR peptides with antimicrobial sequence characteristics are encoded in the *M. truncatula* genome (Alunni et al. 2007). In addition, 138 of these NCR peptides have been detected in the plant's root nodules induced by *S. meliloti* as part of the symbiotic relationship (Durgo et al. 2015). While previous studies have shown that high levels of *M. truncatula*-derived NCR peptides can kill *E. coli* and other bacteria that infect humans, none have examined whether these can kill bacterial plant pathogens such as CLas.

After further computational analysis of the more than 600 NCR peptides for specific antimicrobial peptide motifs and curation of the peptide list for those with certain physiochemical properties, our lab synthesized more than 300 of these peptides to test their CLas-killing ability. However, because CLas is not culturable in the lab, we tested the NCR peptides against the related bacterial species, *Liberibacter crescens,* which has a very similar genome to that of CLas and thus serves as a substitute (surrogate) for it.

All of the synthesized NCR peptides were tested in a simplified minimum inhibitory concentration (MIC)[5] experiment for their ability to inhibit the growth of *L. crescens*. *L. crescens* was grown in a bacterial medium with a panel of nodule specific cysteine rich (NCR) peptides at 1 milligram per milliliter (mg/ml), no peptide control, polymyxin B, a known antimicrobial peptide, at 10 micrograms (µg)/ml and cicadapeptin, a fungal antimicrobial peptide (*see following paragraph*) at 50 µg/ml. Two NCR peptides reduced the growth of *L. crescens* by more than 90 percent in the MIC assay (one of these is highlighted by an * in **Figure 1**) and several others reduced growth by 50-80 percent as measured six days after incubation due to the very slow growth of *L. crescens* in culture media. The cicadapeptin and commercially available polymyxin B reduced growth of L. crescens by nearly 100 percent (**Figure 1**). Peptide solubility, meaning the ability of the peptide to be dissolved in the solution that was used in the MIC assay, was a strong predictor of success in finding the peptides that displayed antimicrobial activity.

Our MIC experiment also led to a serendipitous discovery. One NCR peptide tested in these experiments was derived from a fungus that infects cicadas and was first reported 15 years ago by another team from

Cornell University and the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) (Krasnoff et al. 2005). We had selected this fungal peptide for our MIC experiment because it previously had shown antimicrobial activity on other free-living bacteria. We were curious about whether peptides produced by insect-infecting fungi would be another source of naturally-derived antimicrobial products. Strikingly, we found it completely blocked the growth of *L. crescens* at low concentrations (**Figure 1**). This particular peptide, called cicadapeptin, was isolated from an Australian cicada infected with the fungi, but the peptide has many structural variants occurring in other cicada-infecting fungi that also could have antimicrobial activity.

Consequently, we now have identified two classes of naturally occurring peptides – NCR peptides from *M. truncatula* and peptides from cicada-infecting fungi – that kill *L. crescens* and, therefore, have the potential to kill CLas. As part of our ongoing work, we are attempting to deliver the fungal and NCR peptides directly to citrus trees, so that we can confirm the peptides do indeed kill CLas. We also are testing whether cicadapeptin can kill ACP. If cicadapeptin plays a role in killing cicadas infected by the fungi, it may be equally potent to kill ACP.

Understanding the role peptides play in symbiotic relationships between plants and bacteria and between fungi and insects gives us a means of repurposing those peptides for our own goals – in this case, controlling HLB. In essence, nature already may have created solutions for the disease. The NCR peptides and fungal peptides we are studying could offer California citrus growers – organic or otherwise – additional options for managing HLB. Ideally, any HLB management strategy would use a combination of peptides from one or both classes to limit the potential for CLas to develop resistance to these natural antibiotics. These peptides also have the advantage of being relatively easy to produce in large quantities on a commercial scale.

To advance this work, our team of USDA-ARS scientists has formed a partnership with AgroSource, Inc., a company with the commercial and manufacturing expertise to turn the antimicrobial peptides we discover into products for citrus growers. Our work with AgroSource, Inc. will encompass testing tree delivery strategies, product safety and specificity.

CRB Research Project #5300-196

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Glossary

[1]Antimicrobial: An agent that kills or stops the growth of a microorganism.

[2]Peptide: A short chain of amino acids; a small protein.

[3]Nodule-specific cysteine-rich peptide (NCR peptide): A type of peptide found in the root nodules of legumes that contains a significant amount of the amino acid cysteine.

[4]Symbiotic: Mutually beneficial relationship between two organisms – in this case, a plant and a microbe.

[5]Minimum inhibitory concentration (MIC): A standard laboratory experiment used to measure the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism.

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To Find CLas in Citrus, Look for the ACP

Greg McCollum

Project Summary

Detection of 'Candidatus *Liberibacter asiaticus' (CLas) – the presumed causal agent of huanglongbing (HLB) – is an important component of HLB disease management.*

The hypothesis tested in this research project was that early detection of CLas can be enhanced by sampling tissues that have evidence of feeding by Asian citrus psyllids (ACP), the insects that transmit CLas. To test the hypothesis, controlled inoculations of citrus with CLas-carrying ACP were conducted in the greenhouse. Diagnostic samples for CLas testing were collected throughout the plant (shoot tips to roots) from citrus plants that had been exposed to ACP. Sampling timepoints ranged from days to months after exposure.

The earliest detection of CLas in citrus was achieved by sampling tissue that had evidence of ACP feeding (presence of ACP adults, eggs, nymphs, leaf notching). CLas could be detected in citrus tissue within two days following exposure to CLas-infected ACP. Pre-HLB-symptomatic detection was confirmed by following the development of HLB symptoms in Lisbon lemon, Tango mandarin and Washington navel trees following exposure to CLas-infected ACP adults. HLB symptoms that developed were obvious within four months after exposure to CLas-infected ACP. This was consistent in sweet orange, mandarin and lemon.

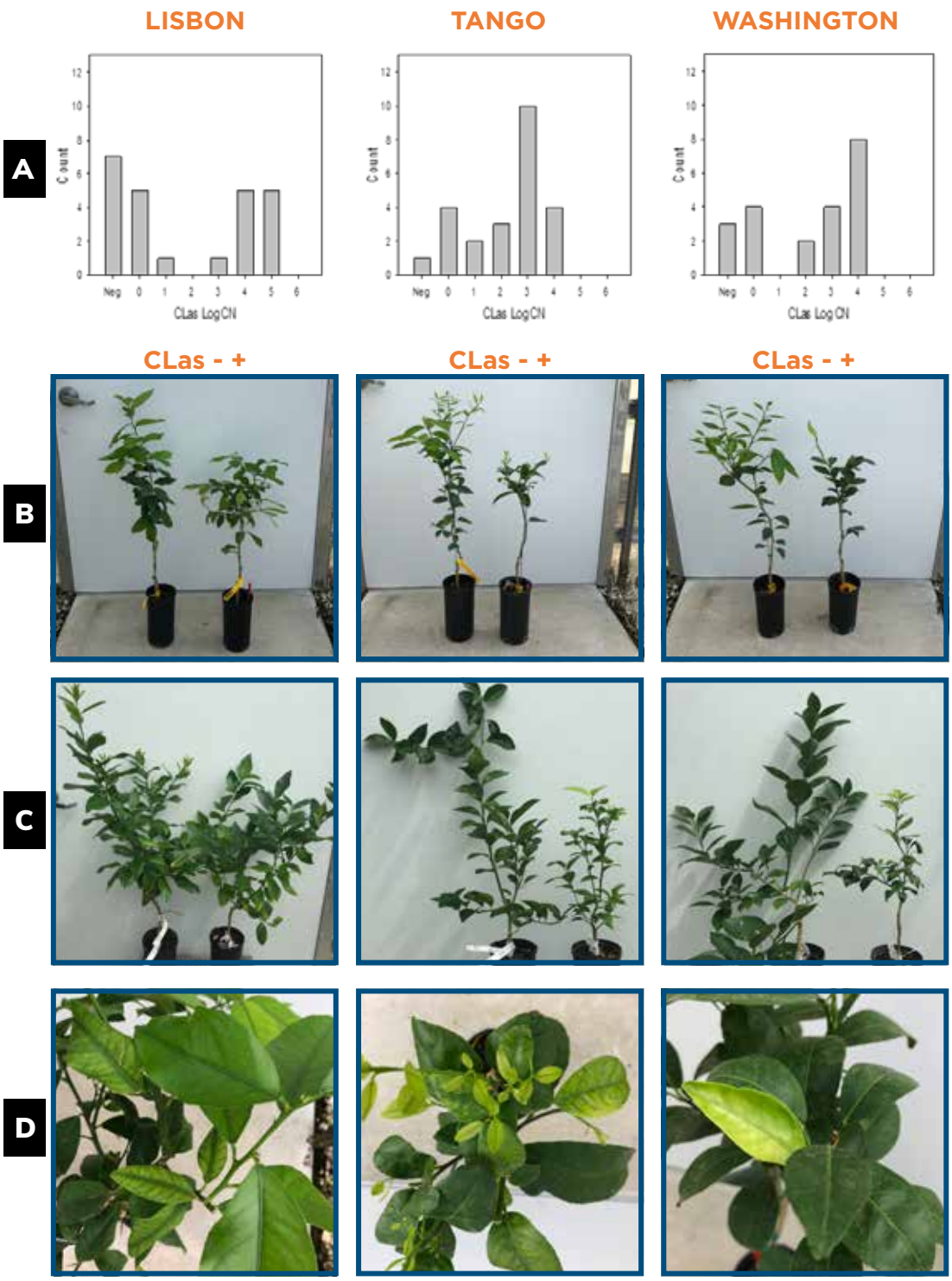

Figure 1. *'Candidatus* Liberibacter asiaticus' (CLas) titer one month post-exposure and examples of huanglongbing (HLB) symptoms for Lisbon, Tango and Washington (columns left to right as indicated at the top of the figure) from one experiment. Typical HLB symptoms on the three scions are shown with plants photographed at four months, six months and nine months (B, D and C) after exposure to CLas-infected Asian citrus psyllid (ACP). Typical HLB symptoms included stunting (B, C) and various forms of chlorosis (D) in each scion. In each photograph, plants on the left are CLas-negative and plants on the right are CLas-positive.

Introduction

The only way to prevent HLB epidemics in areas where citrus is grown is by excluding ACP and, thereby, transmission of CLas to citrus trees (Bové 2006). When ACP and CLas are introduced into areas where citrus is grown, HLB inevitably spreads. Following invasion of ACP and CLas, HLB no longer can be prevented, only suppressed. The abundance of ACP and the increasing incidence of CLas detection in residential citrus trees in southern California are harbingers of CLas eventually spreading to commercial citrus production areas.

Suppression of HLB epidemics typically involves a "three-pronged" approach that includes:

1. aggressive ACP control to minimize movement of CLas and, thereby, new infections of citrus trees,
2. use of ACP-free and CLas-free nursery stock and
3. identification of CLas-infected trees as early as possible and subsequent prompt removal, thus reducing sources of inoculum for acquisition and transmission by ACP.

The importance of ACP control and use of CLas-free nursery stock are unquestionably essential prongs in the three-pronged approach to suppress HLB epidemics (Gottwald 2010; Grafton-Cardwell et al. 2013). However, the impact of early detection of CLas and tree removal on the spread of HLB is unknown.

Finding CLas infections in citrus trees prior to the appearance of HLB symptoms is an extremely challenging endeavor. Regulatory-based detection, which requires confirmation of CLas, is currently done via quantitative polymerase chain reaction (qPCR). This method has been shown to be highly sensitive and reliable compared to other methods (McGuire and McRoberts 2019). Although the sensitivity of qPCR for CLas detection

Table 1. Incidence (proportion) of '*Candidatus* Liberibacter asiaticus' (CLas) and huanglongbing (HLB) symptoms and numbers of false negative (CLas - / HLB symptoms) and false positives (CLas + /No HLB) for three citrus varieties (Lisbon lemon, Tango mandarin and Washington navel). Forty-five plants of each scion type were used in this experiment.

Scion	CLas Detection by PCR	HLB Symptom Development	False Negative[y]	False Positive[z]
Lisbon	0.93	0.93	0.04	0.02
Tango	0.53	0.63	0.13	0.00
Washington	0.63	0.70	0.11	0.07

[y] False negative – tree tested CLas-negative, but developed HLB symptoms.
[z] False positive – tree tested CLas-positive, but did not develop HLB symptoms.

cannot be exceeded, the mass of a citrus tree that can be assayed in qPCR represents only a very small percent of a total citrus tree, which is a shortcoming of this approach. Obviously, the more individual trees and/or more samples that are tested for CLas, the greater the likelihood of finding the pathogen, if CLas is indeed present in an area.

ACP feed preferentially, and lay eggs exclusively, on very young citrus shoot tips (flush). ACP nymphs, as they mature, feed on the leaf flush and can acquire CLas during development into adults. We reasoned that in the absence of HLB symptoms, shoot tips with evidence of ACP feeding should be selected for CLas diagnostic sampling. Initial findings from greenhouse trials regarding CLas detection in ACP nymphs and the effect of titer levels in adult ACP on CLas transmission to citrus have been reported previously (McCollum et al. 2018). Under controlled conditions, we showed that CLas could be detected in progeny nymphs and the citrus flush upon which they had developed within two weeks following exposure of citrus to CLas-infected ACP (during the exposure period, eggs had been laid, some progeny ACP nymphs present, but not progeny adults). CLas could be detected in progeny nymphs and the citrus flush upon which they had developed; however, the titer in nymphs was typically lower than in progeny adults from the same population. In citrus flush that had been inoculated with CLas by ACP, the titer was highest in the tip and decreased further down the tip. Although CLas eventually could be detected in roots of citrus trees that had been inoculated with CLas-infected ACP, CLas was never detected in roots before shoots tested positive, based on these greenhouse studies. In addition, CLas titers in roots never exceeded those in shoots. This final report describes results that were obtained using Lisbon lemon, Tango mandarin and Washington navel orange trees grown under similar greenhouse conditions.

Results

Experiments were conducted using Lisbon lemon, Tango mandarin and Washington navel scions. In each experiment, trees were confined for four weeks with inoculative ACP adults (about five adults per experimental tree) in cages in the greenhouse until there were abundant nymphs and adults; however, damage to plants was beginning to occur as a consequence of ACP feeding. In the second experiment, the ACP exposure duration was shortened to two weeks, and ACP eggs were present on most trees. Some progeny nymphs were seen, but not progeny adults.

Figure 1 presents CLas titer data and examples of HLB symptoms for Lisbon, Tango and Washington from two experiments. All plants were CLas-negative prior to exposure and populations of those trees were exposed to CLas-infected ACP. In both experiments, detection of CLas preceded the appearance of HLB symptoms. One hundred percent of the trees that tested CLas-positive prior to the appearance of HLB symptoms eventually developed HLB symptoms. All trees that expressed HLB symptoms did so within four months of exposure to ACP, and all trees that developed HLB symptoms were CLas-positive. For all three scions, stunting was the first response observed following infection with CLas. Subsequently, various forms of chlorosis, including blotchy mottle, developed in all the infected trees.

In this study, the only way to validate the detection of CLas prior to HLB symptom appearance (early detection) is to determine if HLB eventually develops in the trees. Following exposure to inoculative ACP and disinfestation by insecticide treatment, trees were rated monthly for HLB symptoms by two independent raters.

The relationship between CLas detection one month after ACP exposure and development of HLB symptoms are presented in **Table 1**. Trees that tested positive for CLas and developed HLB symptoms were classified as true positives whereas trees that tested negative for CLas and never developed HLB symptoms were classified as true negatives. Trees that tested positive but did not develop HLB were classified as false positives, and trees that tested negative but developed HLB were classified as false negatives. As expected, a high proportion of the trees that initially tested CLas-positive subsequently developed HLB. For each scion, the number of false negatives was greater than the number of false positives. The incidence of false negatives demonstrates that even under very controlled conditions it is possible to miss the site of infection when sampling.

In the first year of this project, the systemic movement of CLas in Valencia orange was determined by qPCR one month following a one-month exposure to CLas-infected ACP. Three trees were confirmed to be CLas-positive in shoot tips. Infected trees were subsequently sampled at increasing distances down stems to the roots. CLas only was detected in shoot tips that had evidence of ACP feeding and egg laying (**Figure 2**). These results indicate that by two months following the start of exposure to CLas-infected ACP (one-month exposure plus one-month post-exposure incubation), CLas systemic movement was minimal. In subsequent experiments with Lisbon lemon, Tango mandarin and Washington navel, samples for CLas detection were collected only from shoots on which ACP had fed.

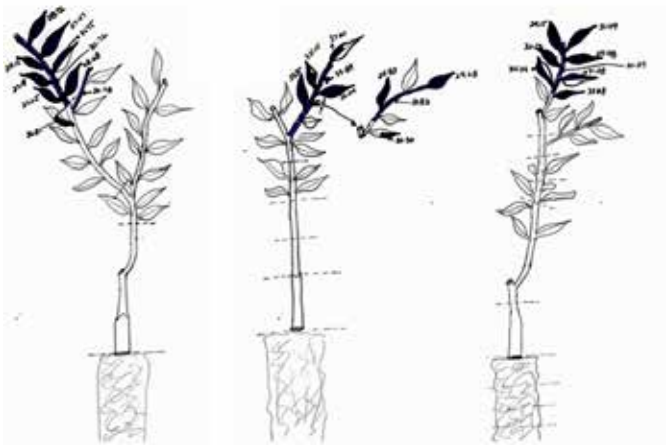

Figure 2. Cartoon illustrating the sites of *'Candidatus* Liberibacter asiaticus' (CLas) detection (dark shading) in three Valencia orange trees. Two months following the start of exposure to CLas-infected ACP (followed by one month of incubation), trees were dissected from shoot tips to roots and samples of each section were assayed by PCR for CLas. Note that at one month following exposure, CLas was only detected in the youngest tissue (as indicated by dark color).

Figure 3 shows the relationship between CLas detection and the development of HLB symptoms. At one month following exposure to ACP, samples were collected from shoot tips and assayed for CLas by qPCR. In addition, at monthly intervals for four months following exposure to CLas-infected ACP, trees were rated for the presence or absence of HLB symptoms by three independent raters. Ratings for all time points were added together and divided by 12 to determine the proportion of rater agreement. The closer the rating was to 1.0, the greater the consensus among raters that the tree was HLB-symptomatic, and the lower the rating, the greater the discrepancy among the raters. Two groups of trees (but not 100 percent of all trees) for each scion were identified based on PCR results: one group that tested CLas-negative at one month and which raters scored negative for HLB symptoms at two, three and four months post-exposure, and one group that tested CLas-positive at one month post-exposure and was consistently rated HLB-symptomatic by the raters at two, three and four months post-exposure. None of the trees, whether CLas-positive or CLas-negative by qPCR, were rated HLB-symptomatic at one-month post-exposure. There was very good consensus among three raters for HLB symptoms in infected trees, but in non-infected trees, consensus was largely absent, except at one-month post-ACP exposure when no trees were rated positive, regardless of CLas detection. In addition, at the final rating time of four months post-exposure to CLas-infected ACP, there was essentially 100 percent consensus among the raters for trees that had tested CLas-positive at one-month post-ACP exposure had developed HLB symptoms.

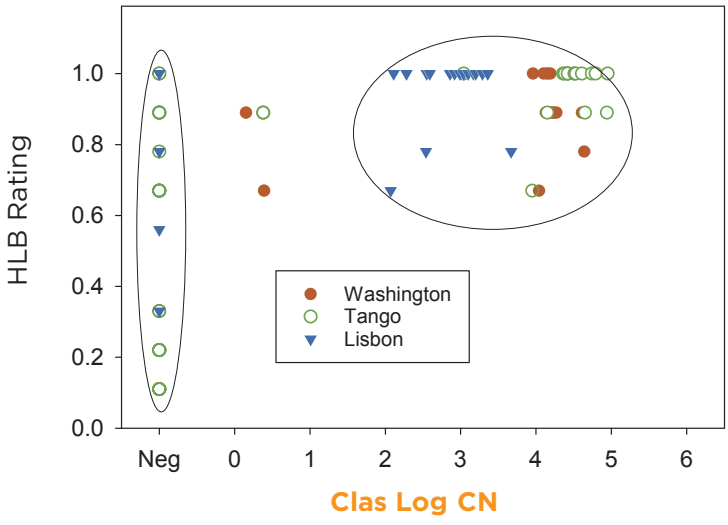

Figure 3. Relationship between *'Candidatus* Liberibacter asiaticus' (CLas) titer in Washington, Tango and Lisbon trees and visual huanglongbing (HLB) symptoms. Each data point represents a tree that consistently tested either CLas-negative or -positive by qPCR at one, two, three and four months post-exposure to CLas-infected Asian citrus psyllid (ACP). Trees were rated either free of HLB symptoms or HLB symptomatic by three raters at the four time points following a three-week exposure to CLas-infected ACP. The ratings were summed, and the proportion of the scores are presented. A score of 1.0 indicates all three raters rated the tree positive at each time point, and a score of zero indicates that no rater scored the tree positive at any time point. Note the great dispersion of ratings for the CLas-negative trees (scores from near zero to 1.0) versus the very great agreement among the raters for trees that were infected with CLas.

Conclusions

Early detection of CLas by qPCR in citrus following exposure to CLas-infected ACP

Results presented in this report and the first-year project report (McCollum et al., 2018) indicate that CLas infection can be detected within one month following exposure of Lisbon, Tango and Washington trees to CLas-infected ACP for one month. ACP eggs are apparent on shoot tips, typically seen one to seven days following exposure to ACP adults. In addition, although CLas can move systemically in citrus, the bacterial titer is highest in shoot tips and decreases with distance from the original site of infection, as illustrated with Valencia orange in **Figure 2**.

Relationship of CLas detection in citrus tissue HLB symptom development

Regardless of scion type (sweet orange, mandarin, lemon), CLas can be detected prior to the appearance of HLB symptoms. In all the experiments in this work, if citrus trees developed HLB symptoms, they appeared within three to four months following exposure to CLas-infected ACP.

Major Conclusions

- By collecting tissue samples from citrus trees with evidence of ACP feeding under these experimental conditions, it is possible to confirm infections essentially immediately following transmission at the site of ACP feeding if psyllids were CLas-positive.
- The duration between CLas infection and appearance of HLB symptoms is approximately four months under these experimental conditions; however, in the orchard, with very few sites of infection, it will continue to be difficult to find infections.

CRB Research Project #5300-176

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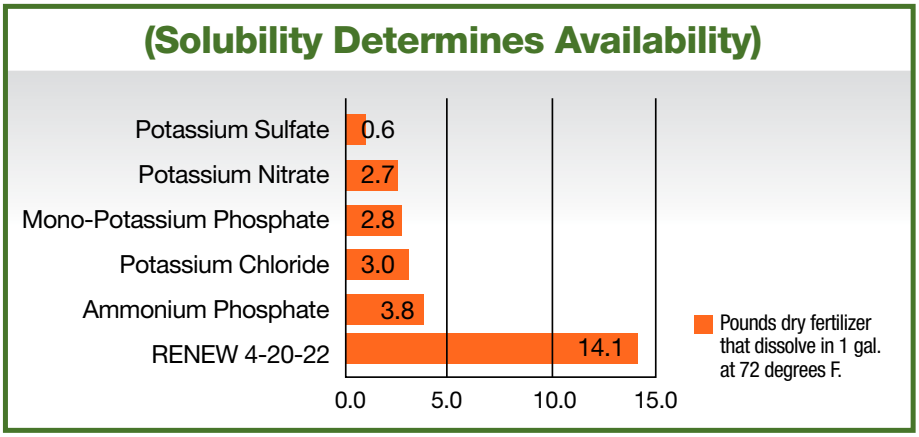

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Figure 1. Experimental set-up. A. Application of treatments to citrons prior to Asian citrus psyllid (ACP) exposure. B. Citrons being exposed to ACP. Plants confined in a screen cage with approximately 1,000 adult ACP from a *'Candidatus* Liberibacter asiaticus'-infected colony. C. ACP adults and eggs on experimental plants.

PROTECTING CITRUS FROM HLB

Greg McCollum

Background

When it comes to huanglongbing (HLB), nothing could be truer than an ounce of prevention being worth a pound of cure. The threat of HLB to the California citrus industry is both real and increasing. It is essential to identify strategies available today that can be implemented immediately to prevent an epidemic that could seriously disrupt commercial citrus production in the state. So far, immediate effective mediations have been to impose quarantine measures to slow the spread of Asian citrus psyllid (ACP) and potentially infected citrus into major citrus producing regions. Surveying for ACP and application of insecticide treatments for local eradication of the insect is another strategy. Citrus trees can be protected from ACP by barriers around the orchard, screenhouses or even by enclosing individual trees in mesh bags. All of these approaches have advantages and disadvantages and may be viable options for some operations, but not all. Unfortunately, as evidenced by the recent finding of a *'Candidatus* Liberibacter asiaticus' (CLas)-infected ACP adult in a commercial citrus orchard in Riverside County, ACP and CLas-infected ACP will find their way into commercial citrus.

Once CLas-infected ACP invade an area and transmit CLas to citrus, HLB incidence increases. Currently, there are no known effective therapies for HLB, even though considerable resources have been devoted to such efforts. In contrast, other than insecticide treatments to manage ACP, most published reports have focused on treatments to reduce or eliminate CLas from infected tissue. However, fewer have focused on the effects of treatments applied prior to infection to prevent or mitigate HLB symptoms (the ounce of prevention) to inhibit CLas replication and spread following transmission to citrus. In combination with stringent ACP control, it is the prophylactic approach, i.e. preventing establishment of infections immediately following transmission rather than trying to undo symptoms, that eventually will provide the most successful mitigation of HLB.

Objective

The overall objective of the research was to determine if select "antimicrobial" or plant growth regulating compounds could be effective in preventing establishment of CLas infections and mitigating HLB disease development. The hypothesis tested whether any compound with a beneficial impact on the mitigation of HLB would have its greatest effect at the time of CLas transmission into citrus. To test this hypothesis, a greenhouse model system including *Citrus medica* (citron) and ACP from a colony infected with a Florida CLas isolate was utilized. In the experiment described here, three antibiotics currently registered for use in citrus were compared with penicillin (not registered for agricultural use, but reported to reduce CLas titer) for their effects on CLas titers and HLB development following exposure to CLas-infected adult ACP.

Experimental Approach

Plant material, application of treatments and plants during exposure are shown in **Figure 1**. A group of 180 citrons (approximately 15 cm tall initially) was divided randomly into six treatment groups of 30 plants per group (**Figures 1A** and **B**). The six treatment groups were:

1. Water
2. Kinetic® surfactant in water (0.25 percent)
3. Penicillin (0.1 gram/Liter [g/L]) in water plus 0.25 percent Kinetic
4. Firewall™ (0.82 g/L) in water plus 0.25 percent Kinetic
5. Fireline™ (1.8 g/L) in water plus 0.25 percent Kinetic
6. Mycoshield® (1.34 g/L) in water plus 0.25 percent Kinetic

Every group was treated by spraying each plant to run-off (approximately three milliliters (mL) of treatment (**Figure 1A**). Treatments were applied once per week for five weeks. One day after the last treatment was applied, all 180 plants were confined in a single cage (**Figure 1B**). Approximately 1,000 CLas-infectious ACP adults from a colony maintained at the U.S. Horticultural Research Laboratory, Fort Pierce, Florida, were introduced into the cage. The plants were confined with the ACP adults (**Figure 1C**) for 14 days, at which time the plants were treated with insecticide and then maintained free of ACP for the remainder of the experiment.

Forty-five days after ACP exposure, trees were visually rated for HLB symptoms (stunting, leaf curling and chlorosis) by two independent raters. Sixty days after ACP exposure, samples were collected for qPCR, and the plant height, leaf area and dried plant weight (leaves and roots) were recorded.

Table 1. Effects of prophylactic treatments on huanglongbing (HLB) symptoms. Two independent raters scored plants for either no apparent HLB symptoms or HLB-symptomatic 45 days following exposure to *'Candidatus* Liberibacter asiaticus'-infected Asian citrus psyllid and 15 days prior to sampling for qPCR analyses. Scores represent the number of consensus HLB symptomatic (+) or not symptomatic (-).

	HLB symptoms (Consensus)	
Treatment	+	-
Water	0.60	0.07
Kinetic	0.67	0.10
Penicillin	0.70	0.17
Firewall	0.50	0.23
Fireline	0.27	0.70
Mycoshield	0.14	0.73

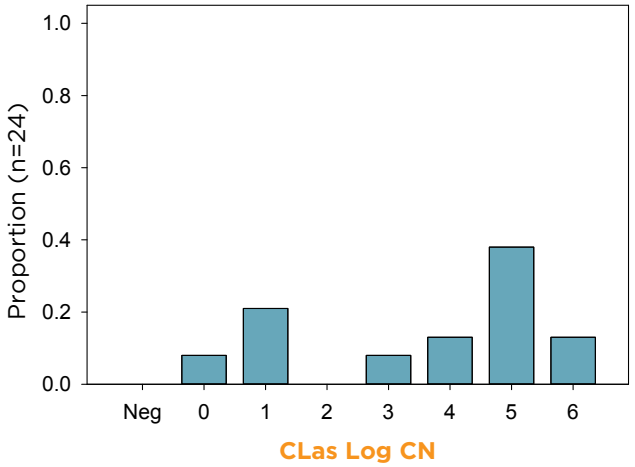

Figure 2. *'Candidatus* Liberibacter asiaticus' (CLas) titers in inoculative Asian citrus psyllids. Titer was determined from a sample of the inoculative Asian citrus psyllid (ACP) population collected on the day exposure started and on the day exposure ended. There was a very high inoculative potential (no negative [neg] ACP in the inoculative population and high proportion of population with CLas titer greater than 10,000 CLas cells per insect).

Results

The objective of this experiment was to determine if a greenhouse model could be used to determine the effectiveness of prophylactic applications of streptomycin and two oxytetracycline products. Treatments were applied as described above to evaluate any efficacy to mitigate HLB development.

The CLas titer[1] in a sample from the population of ACP adults used for transmission to citrons is presented in **Figure 2**. One hundred percent of the sampled ACP adults tested CLas-positive, and 60 percent of the population had CLas titers of 10,000 or greater CLas cells per insect, making this a population with high transmission potential. Forty-five days after exposure to CLas-infected ACP, HLB symptoms in the experimental plants were rated (**Table 1**). The proportion of plants with the lowest HLB symptom ratings, based on the consensus ratings by two raters, were those treated with either of the oxytetracycline products. These were also the only treatment groups that showed fewer or less severe symptoms than all other plants.

Sixty days following exposure, petiole samples for quantitative polymerase chain reaction (qPCR) were collected and subsequently assayed for CLas following standard qPCR protocol (Li et al. 2006). The high incidence of CLas-infected ACP adults and the proportion of the population with titers greater than 10,000 per insect resulted in a 97 percent incidence of infection in plants treated with water. The proportion of CLas-positive trees in trees treated with Kinetic, alone or in combination with either penicillin or streptomycin (Firewall) were each greater than 90 percent. The proportion of CLas-negative trees was highest in trees that had been treated with oxytetracycline (Fireline or Mycoshield) (**Figure 3A**). In addition, in trees that did become infected with CLas, the lowest titers were in those treated with oxytetracycline (Fireline and Mycoshield) (**Figure 3B**). Interestingly, CLas titers in the oxytetracycline-treated trees were less than 1,000 cells per

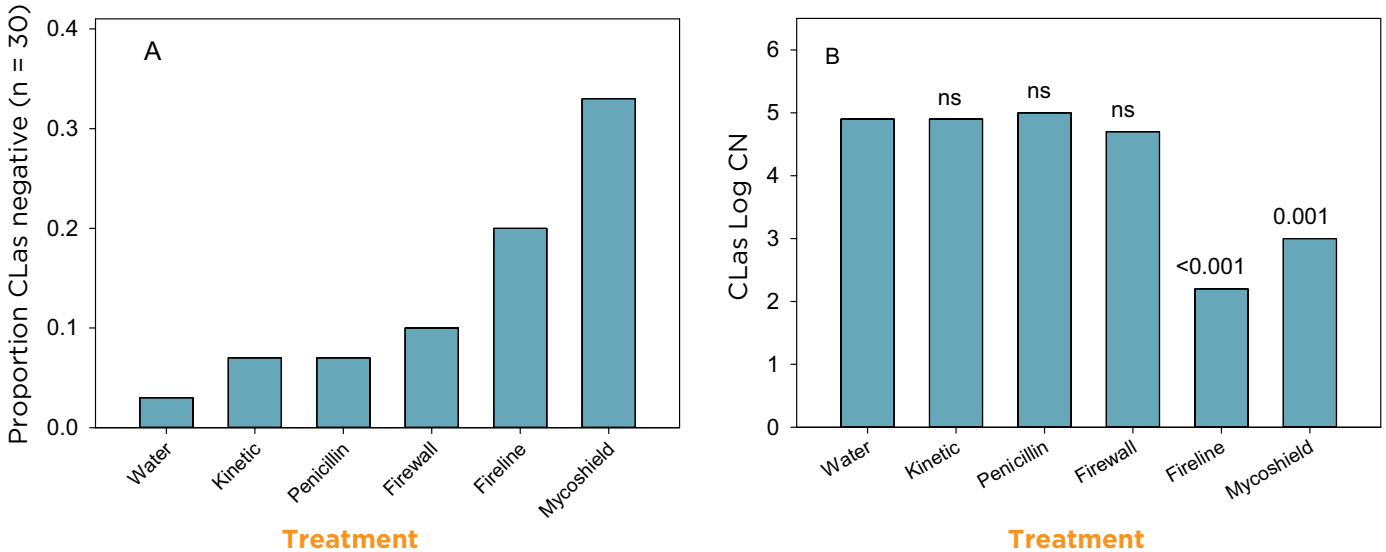

Figure 3. Effects of prophylactic antibiotic treatments on the proportion of *'Candidatus* Liberibacter asiaticus'(CLas) - negative citrus trees (A) and on CLas titer in those trees that became infected with CLas (B). Differences between water-treated and other treatments were either not significant (ns) or significant as indicated by numbers above bars. The smaller the number, the greater the probability of a true difference.

[1]CLas titer is expressed as the number of target copies of the CLas present in a nucleic acid extract either from citrus or ACP. Because CLas titer in an extract can range from none detected (Negative) to as many as several million copies, it is imperative that CLas titer (copy numbers) be expressed as the log of the copy number for meaningful interpretation of the data. The scale shows proportions of individual trees or ACP within each log class.

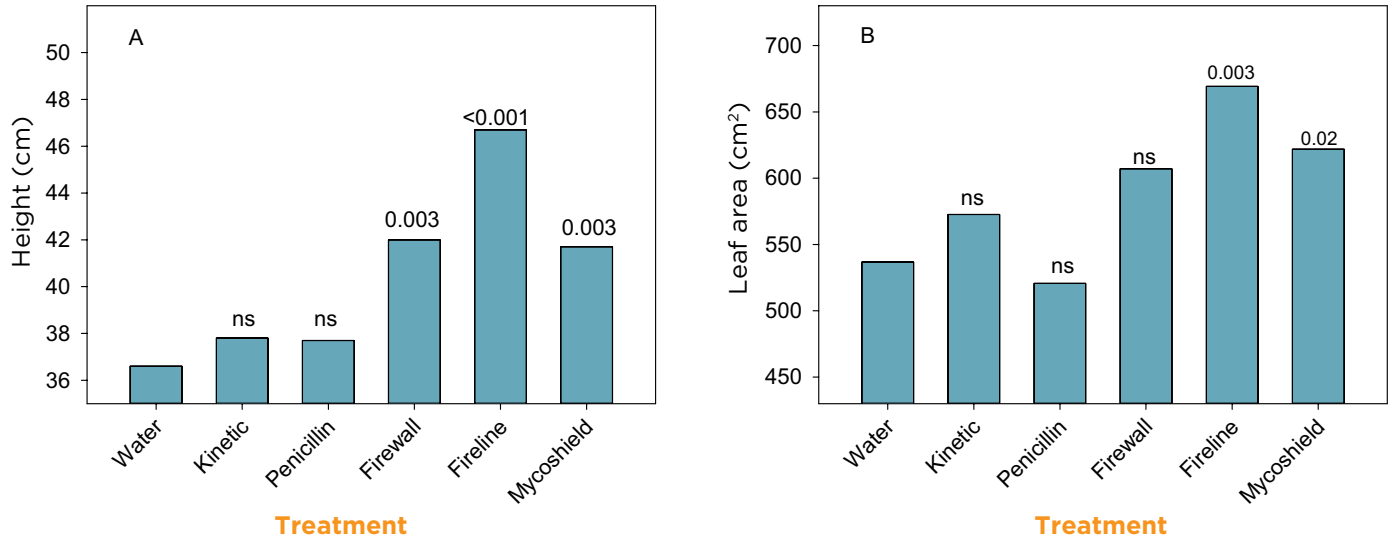

Figure 4. Effects of prophylactic treatments on citron plant height (A) and leaf area (B) following exposure to *'Candidatus* Liberibacter asiaticus' inoculative Asian citrus psyllid. Differences between water-treated and other treatments were either not significant (ns) or significant as indicated by numbers above bars. The smaller the number, the greater the probability of a true difference.

mg of plant tissue. HLB symptoms typically are not apparent in leaves with a titer of less than 10,000 copies per mg of tissue (McCollum et al. 2016).

Plant height and leaf area differed significantly among the treatments. As expected, based on CLas titer, height (**Figure 4A**) and leaf area (**Figure 4B**) were significantly greater than controls for plants treated with either oxytetracycline product. Based on numerous experiments with various citrus types (McCollum 2021), stunting is observed consistently as the earliest HLB symptom following infection with CLas.

Conclusions

This greenhouse model is based on using citron (true citrus), CLas and transmission of CLas to citrus by ACP. Using this model, prophylactic treatment with either of two formulations of oxytetracycline registered for use on citrus can reduce the incidence of CLas infection and titer in infected plants. The effects of oxytetracycline treatment on HLB symptoms were reflected in visual ratings and in plant height and leaf area. Because the results are based on application at higher than label rates of the registered antibiotics, results cannot be extrapolated to the field. However, these results do support the conclusion that some protection against HLB symptom development may be provided by oxytetracycline based on preventing CLas infection and/or reducing CLas titer in infected plants. Additional experiments with the oxytetracycline products should evaluate the effects of label rates, duration of effect, effect of surfactants and others.

CRB Research Project #5300-198

NOTE: *Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.*

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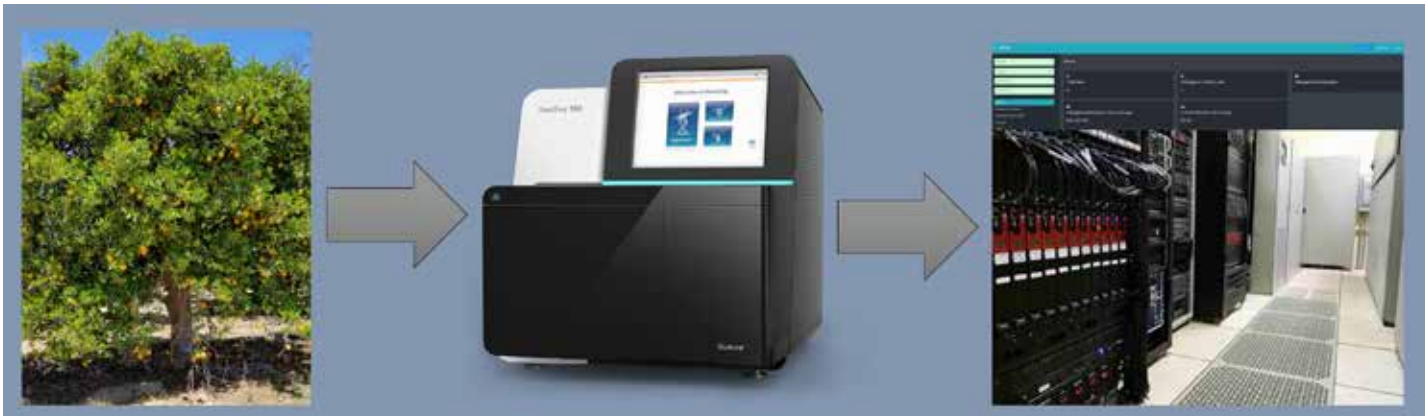
HIGH-THROUGHPUT SEQUENCING: A POTENTIAL DIAGNOSTIC TOOL

Update on Phase 2 Testing for CCPP

Tyler Dang, Andres Espindola, Sohrab Bodaghi, Irene Lavagi-Craddock, Fatima Osman, Kitty Cardwell and Georgios Vidalakis

Project Summary

High-throughput sequencing (HTS) is a powerful technology that combines molecular biology and computer sciences and has a wide range of applications. One of these applications that could help the citrus industry is the detection and identification of multiple citrus pathogens in a sample. A big challenge with HTS is data analysis, which requires in-depth knowledge of bioinformatics[1]. The goal of this project is to simplify the data analysis so that an individual with no background in bioinformatics can determine the presence or absence of a particular graft-transmissible citrus pathogen. We are working towards that goal with a program called E-probe Diagnostic Nucleic Acid Analysis (EDNA), which is a user-friendly web interface that can detect pathogens of interest from raw sequencing data. EDNA eliminates the need for dedicated servers and bioinformaticians to perform the diagnostics. In addition, results are obtained much faster compared to traditional bioinformatic workflows. Full implementation of the EDNA technology in labs involved in citrus diagnostics requires development of a comprehensive collection of EDNA libraries for citrus pathogens. In addition, EDNA technology for plant pathogen detection and identification will need regulatory approval to ensure the method performs as well or better than the current diagnostic methods.



The general workflow for determining the over-all health of a tree. Samples collected from the field can be tested by high-throughput sequencing. The data are analyzed using an online platform called e-probe Diagnostic Nucleic Acid Analysis (EDNA) and provides information about which citrus is present in the sample.

Background

The citrus industry is under constant threat from various citrus pathogens and diseases. As a result, it is imperative for the Citrus Clonal Protection Program (CCPP) to use new diagnostic tools to introduce pathogen-tested citrus propagative materials. High-throughput sequencing (HTS) generally is accepted in the international scientific and regulatory community as a tool that will revolutionize plant pathogen diagnostics and become the gold standard. HTS is a powerful tool for simultaneous detection and identification of multiple pathogens in a plant sample without the need to perform multiple tests. In our previous study (Dang et al. 2019), we showed that HTS was sensitive and specific for citrus pathogen detection. From our experience with the data, we have found that HTS analysis requires personnel with an in-depth background in bioinformatics analysis, access to dedicated severs and long analysis wait times to obtain diagnostic results on a particular sample.

A promising tool that can manage HTS data analyses is the E-probe Diagnostic Nucleic Acid Analysis (EDNA) *in silico*[2] technology. If successfully implemented with regulatory approval, we anticipate utilizing EDNA in the CCPP variety indexing (VI) process for citrus variety introductions, which will allow us to progressively phase out multiple individual laboratory tests (PCR, qPCR, gel electrophoresis, etc.) currently required for the detection of graft-transmissible citrus pathogens.

EDNA is a user-friendly online interface that uses raw sequencing data containing a mixture of sequences from the citrus plant, associated microbes and pathogen(s) of interest. The files can be uploaded to the online platform, where the user can choose which pathogens to test. This is achieved by using anywhere from ten to thousands of "electronic probes" (e-probes[3]), ranging from 20 to 60 nucleotides[4] in length, to specifically search for genomic sequences of the pathogen of interest in the raw data. The number of e-probes that bind to the target pathogen is then calculated, and a diagnostic result is provided. Currently, there are e-probes designed and validated for citrus tristeza virus (CTV), citrus exocortis viroid (CEVd) and *'Candidatus* Liberibacter asiaticus' (CLas). Discussions with regulatory agencies for approval to use e-probes for pathogen diagnosis have begun. In addition, our collaborators at the Institute of Biosecurity and Microbial Forensics (IBMF) at Oklahoma State University

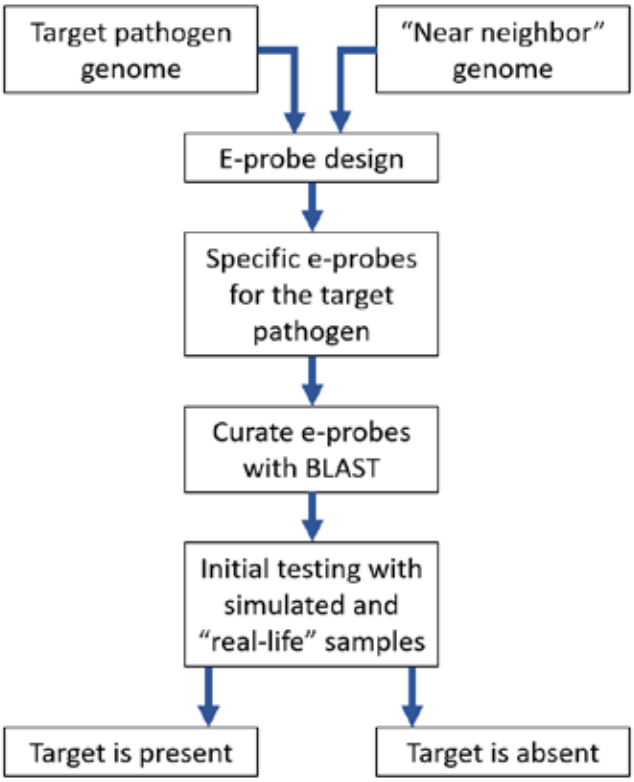


Figure 1. Graphic representation of the e-probe design workflow. The workflow for designing e-probes begins with mining the genomes of the target pathogen and the "near neighbor" genomes. The genome sequences are cross-referenced to remove sequences that overlap or show similarities, and specific e-probes for the target pathogens are created. To further curate the e-probes, a computer program (BLAST®) is used. The curated e-probes are then tested with simulated Illumina data and "real-life" samples to ensure they are working correctly.

have launched MiFi: Microbe Finder (*http://bioinfo.okstate.edu/index*), the online platform for this technology – not only for citrus, but also for various other crops and diagnostic uses.

In this project, we intend to build a library of e-probes for 14 known graft-transmissible citrus pathogens. Because of the flexibility of the EDNA platform, more e-probes can be developed and added to the libraries as new citrus pathogens are discovered (**Table 1**).

The workflow for designing e-probes begins by mining the pathogen genome[5] sequences and closely related organisms (near neighbors) from databases such as GenBank (**Figure 1**). If genome resources in public databases are limited, a genome of the organism will be generated by Illumina sequencing. The genome of the pathogen and its near neighbors are cross referenced, any regions that show high similarities are removed, and the unique sequences to the pathogen are used for e-probe design. The raw e-probes are curated by a computer program (BLAST®) to ensure the e-probes do not match with genomic sequences of other organisms, then tested against simulated Illumina data that have been "spiked" with different amounts of the pathogen and finally tested with "real-life" healthy and diseased citrus samples. The final phase is to train the e-probe with a statistics model and further refine the diagnostic determination so we can present our data to the regulatory agencies for approval.

Based on our initial finding with the e-probes designed for CTV, CEVd and CLas, we have determined optimal lengths for the different organisms summarized in **Table 2**. The number of e-probes are determined by the pathogen genome size. The larger the genome, the greater the number of e-probes that can be generated to ensure they are able to detect the pathogen from the raw HTS data and vice versa. To fully validate the method, the three developed e-probes for a

Table 1. List of citrus pathogens in this project's pipeline.

Pathogen	Genome Mining Phase	Sequencing Phase	Design Phase	Validation Phase	Statistical Model Training Phase	Regulatory Approval in Progress
Citrus tristeza virus					X	X
Citrus exocortis viroid					X	X
'Candidatus Liberibacter asiaticus'					X	X
Citrus psorosis virus				X		
Citrus leaf blotch virus				X		
Citrus vein enation virus				X		
Citrus tatter leaf virus				X		
Citrus variegation virus		X				
Xanthomonas citri				X		
Xylella fastidiosa				X		
Hop stunt viroid	X					
Spiroplasma citri			X			
Citrus concave gum associated virus		X				
'Candidatus Phytoplasma aurantifolia'	X					

Table 2. The optimal lengths and number of e-probes generated for citrus tristeza virus (CTV), citrus exocortis viroid (CEVd) and *'Candidatus* Liberibacter asiaticus' (CLas).

Organism	Pathogen	Genome Size	Optimal Length	Number of E-probes
Virus	Citrus tristeza virus	19,296 base pair (bp)	30	860
Viroid	Citrus exocortis viroid	371 bp	30	6
Prokaryote	*'Candidatus* Liberibacter asiaticus'	1,268,237 bp	40	5,904

Table 3. Results for different diagnostic assays compared to the e-probe Diagnostic Nucleic Acid Analysis (EDNA). P-values less than 5.0E-2 denote the sample will be called positive for the pathogen. Alternatively, if the p-value is greater than 5.0E-2 or a p-value is not computed (n/a), the sample will be called negative for the pathogen.

#	Sample	RT-qPCR	Bioinformatic workflow	Bioindexing	EDNA Results	
					p-value	Diagnostic
1	SY568	CTV +	CTV +	CTV+	2.32E-161	Positive
2	14A-3-8	CTV+ (CPsV, Viroid)	CTV+ (CPsV, Viroid)	n/a	1.00E-110	Positive
3	ippn122	CTV+ (Viroid, CTLV)	CTV+ (Viroid, CTLV)	CTV+ (Viroid, CTLV)	1.67E-32	Positive
4	Healthy Navel	Negative	Negative	Negative	n/a	Negative
5	Healthy Mandarin	Negative	Negative	Negative	n/a	Negative

citrus virus (CTV), viroid (CEVd) and bacterium (CLas) were tested with "real-life" samples, and the EDNA results were compared against traditional bioinformatic analysis, RT-qPCR and biological indexing (**Table 3**). Samples 1-3 tested positive for CTV for all methods, while the healthy controls (samples 4-5) were negative across all methods. This indicates that the EDNA is specific and potentially works as well as the traditional and regulatory approved detection methods. With any new technology, there will be challenges that require time to fully optimize each e-probe to work properly. The current status of HTS as a tool for plant pathogen diagnostics looks very promising.

Our team had scheduled a face-to-face workshop with U.S. Department of Agriculture (USDA) regulators to work on the EDNA technology, which had to be canceled due to the COVID-19 pandemic. Alternatively, we participated in the virtual meeting of Western Extension Research Activity20 (WERA20) "Virus and Virus-Like Diseases of Berries, Fruit and Nut Trees and Grapevines" and discussed the EDNA technology for citrus with several scientists, agricultural experiment station directors and regulators across the country. The implementation of HTS-based technologies for plant pathogen diagnostics and the release from quarantine of plant propagative materials based on HTS diagnostics were discussed. Also, the main U.S. plant regulatory agency, the USDA-Animal and Plant Health Inspection Service-Plant Protection and Quarantine (APHIS-PPQ), informed us about the function of two new USDA HTS-dedicated labs in the PPQ Science and Technology (S&T), as well as the USDA Germplasm Field Operations that have begun working on developing and validating HTS-based assays for routine plant pathogen diagnostics. This clearly signaled that the regulatory agencies are becoming more receptive of this technology. Subsequent to the WERA20 meeting, we initiated direct discussions with USDA HTS laboratories, and we have begun presenting our findings for regulatory approval of the EDNA-HTS technologies in citrus diagnostics.

Conclusion

HTS is a powerful tool that still requires development and refinement to become routinely used. Because sequencing costs have decreased significantly, many scientists and regulators are working toward the implementation of HTS technology in routine diagnostics (for both plants and animals). The technology will increase laboratory efficiency, potentially phasing out the need for individual pathogen

tests. This will facilitate faster introduction and distribution of pathogen-tested budwood of new and established citrus varieties to citrus growers for citrus grove establishment. The combination of HTS and EDNA analysis is user-friendly and allows personnel with little or no bioinformatic background to analyze complex data. We will continue to work to get approval for this exciting technology that shows promise for use as a routine diagnostic tool for the CCPP and other citrus diagnostic programs.

CRB Research Project #5300-205

Glossary

[1]Bioinformatics: A field of study combining biology, computer science, statistics and related fields to analyze and utilize large volumes of complex biological data.

[2]*In silico*: Produced by computer modeling or simulation.

[3]Electronic probe (e-probe): Sequence specific probes that search the uploaded sequencing data for the pathogen of interest, *in silico*.

[4]Nucleotides: Chemical compounds that serve as the 'building blocks' of DNA and RNA.

[5]Genome: The genome of an organism is all of its genetic material.

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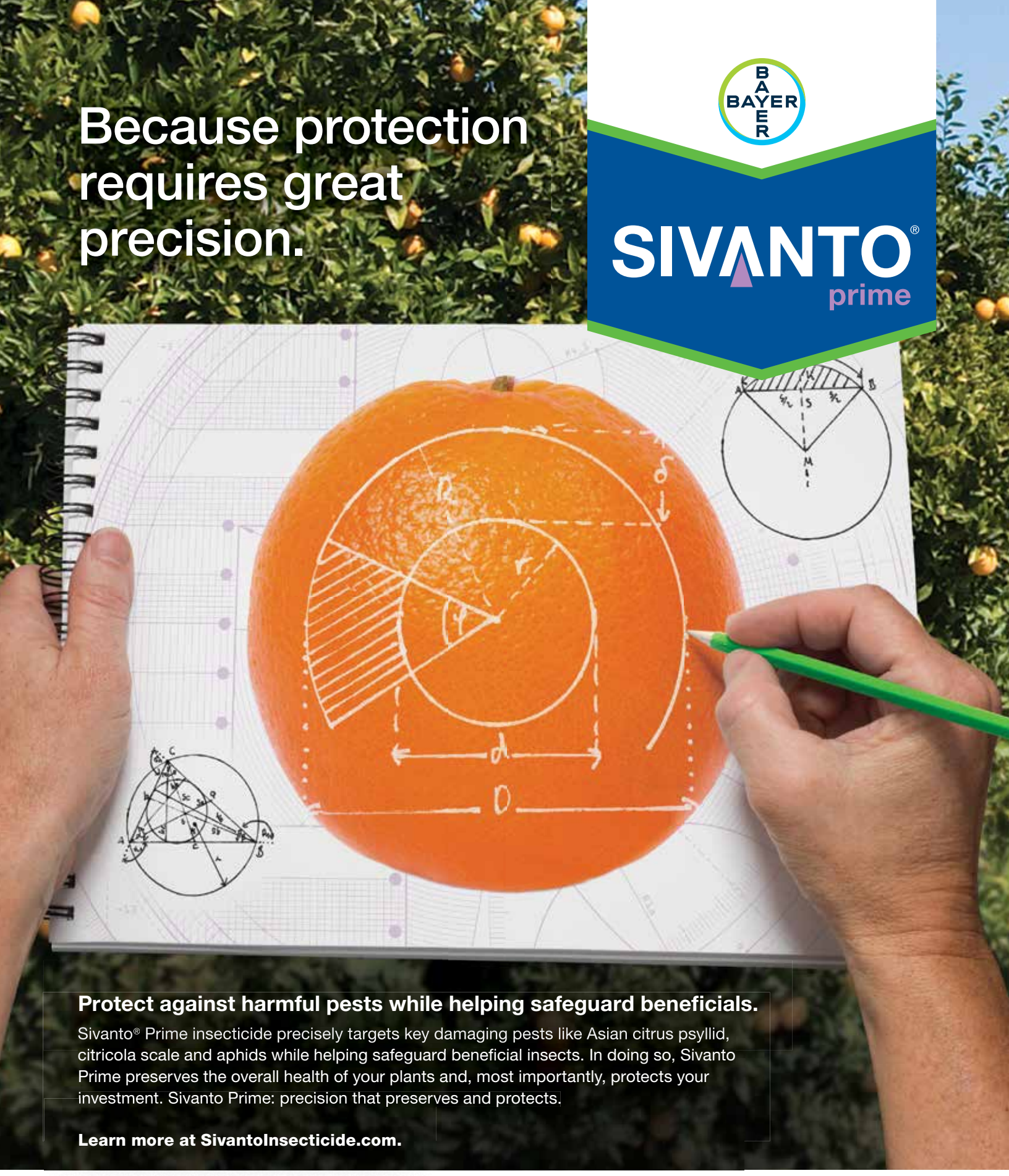

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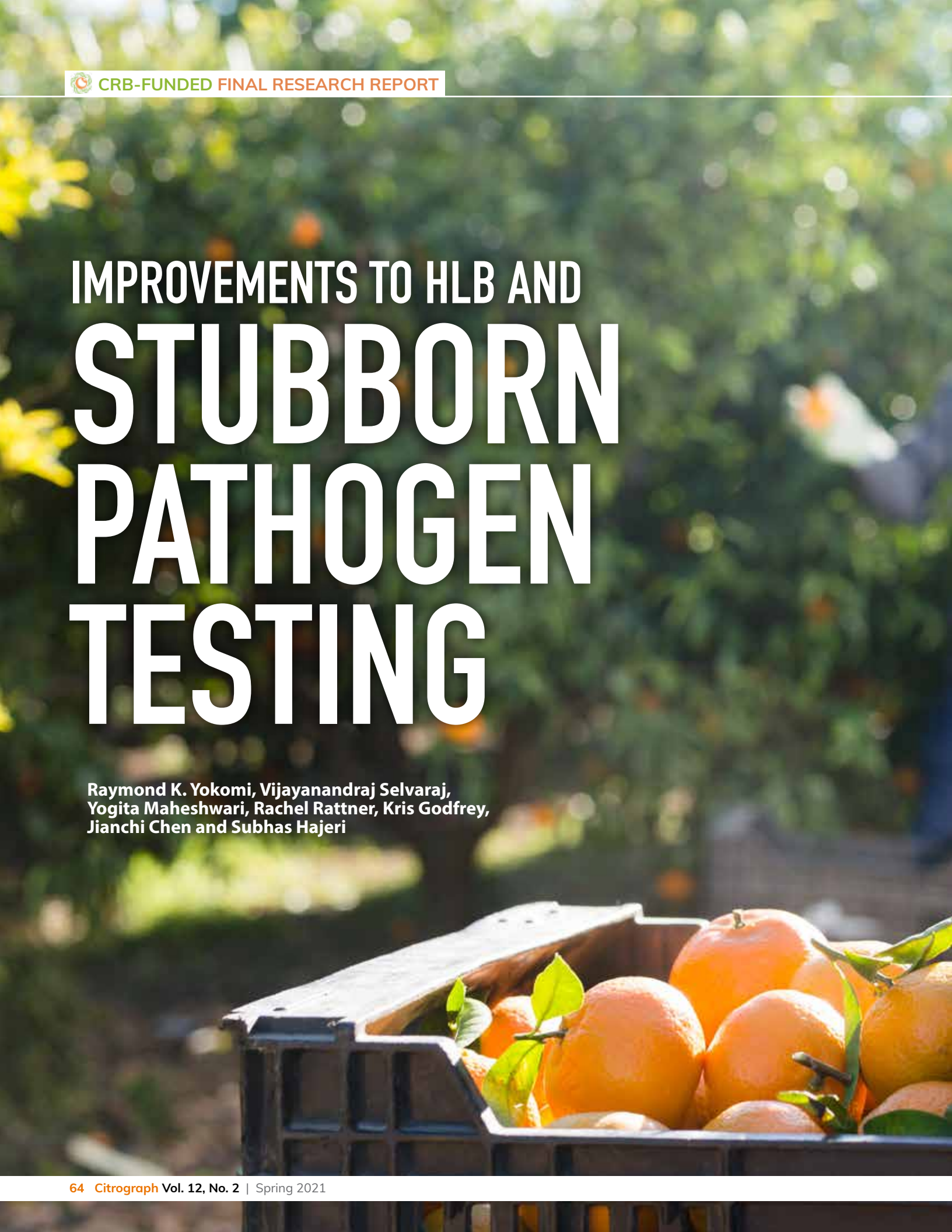
IMPROVEMENTS TO HLB AND STUBBORN PATHOGEN TESTING

Raymond K. Yokomi, Vijayanandraj Selvaraj, Yogita Maheshwari, Rachel Rattner, Kris Godfrey, Jianchi Chen and Subhas Hajeri



Project Summary

Huanglongbing (HLB) control in California is based, in part, on testing citrus trees and eradicating 'Candidatus *Liberibacter asiaticus' (CLas)-positive trees. This is complicated by the presence of* Spiroplasma citri*, which is the causal agent of citrus stubborn disease (CSD), since CSD symptoms can resemble those of HLB. These trees will test negative for CLas, but this may raise concern that this is a false negative result. The objective of this study was to develop a protocol to detect and differentiate both pathogens in a single test. Using specific primers and different fluorescent probes for each pathogen, a duplex qPCR[1] assay was developed that detects and differentiates both pathogens simultaneously. Because conventional qPCR assays provide only a relative quantity of the pathogen target, a duplex droplet digital (ddPCR[2]) assay was developed that measures the exact number of DNA targets in the sample and can serve as a confirmation test. These tests can be used by regulators and diagnosticians to improve reliable detection of CLas. In addition, whole genome sequencing of seven strains of* S. citri *were determined and examined for new gene targets to improve detection of* S. citri.

Figure 1. Comparison of a confirmed Washington navel tree affected with huanglongbing (HLB) (*left*) and Citrus stubborn disease (CSD) (*right*). (A) HLB-affected tree is from suburban southern California; (B) Blotchy mottle leaves and distorted fruit symptomatic for HLB. (C) Stubborn-affected tree in a commercial orchard in central California; (D) Chlorotic leaves with shortened internodes and (E) smaller misshapen fruit, with stylar-end greening typical of CSD.
Photos A and B provided by Magally Luque-Williams, California Department of Food and Agriculture.

Introduction

HLB is a devastating citrus disease caused by CLas. Testing symptomatic trees for CLas and eradicating infected trees as soon as possible to limit pathogen spread by its vector, *Diaphorina citri*, the Asian citrus psyllid (ACP), is a critical part of managing HLB in California. ACP is well established on citrus in residential properties in four counties in southern California where HLB is spreading. Fortunately, HLB has not yet been found in any of California's commercial citrus orchards.

In contrast, CSD, caused by *S. citri*, is vectored by the beet leafhopper (BLH) and is endemic in citrus orchards in the southwestern United States. This complicates survey efforts for HLB, because CSD symptoms can be easily confused with those of HLB (**Figure 1**). Tree-to-tree spread of CLas occurs rapidly by ACP, but *S. citri* spreads much slower since citrus is a non-host of BLH. However, the BLH uses citrus as a temporary habitat during migratory flights where it can infect citrus if it is infected by *S. citri*.

Thus, disease dynamics are different between HLB and CSD. Whereas CSD is managed by good cultural practices such as weed control, good fertigation and avoidance of close proximity to BLH habitats when possible, HLB is managed, in part, by eradication. Current orchard scouting for HLB-symptomatic trees will encounter trees with CSD, which can lead to misidentification. In contrast, the familiarity of CSD symptoms can lead to complacency that the symptomatic tree is not HLB-affected. Since both CLas and *S. citri* are bacterial pathogens that reside in citrus phloem, sample methods and tissue processing procedures for molecular testing can be harmonized for dual detection of these pathogens.

Multiplex qPCR and ddPCR

A sensitive duplex qPCR technique was developed for simultaneous detection and differentiation of citrus trees infected by CLas or *S. citri*. This assay uses primers with sequences of the multicopy CLas RNR[3] gene (Zheng et al. 2016) and the multicopy *S. citri* ORF1[4] Virus 1 gene (Wang et al. 2015) for specific identification of CLas or *S. citri*, respectively. These gene targets were selected because of their greater copy number in the pathogen's DNA resulting in increased diagnostic sensitivity. The duplex qPCR was optimized and shown to be as sensitive for detecting CLas and *S. citri* as a qPCR for each pathogen alone (singleplex) (Maheshwari et al. Submitted). These experiments were performed and validated with citrus tissue infected with CLas and/or *S. citri* obtained from the Contained Research Facility at the University of California, Davis.

Citrus and other woody plants contain more compounds that inhibit or interfere with PCR reactions than annual plants. These inhibitors can cause diagnostic tests to fail even when sufficient target DNA is present. This problem is increased in multiplex qPCR assays since more primers and probes are included. Therefore, a duplex ddPCR was developed for absolute quantification expressed as copies of both CLas and *S. citri* targets in the same sample. In the ddPCR assay, tolerance to inhibitors is achieved by partitioning the sample reaction into thousands of droplets, each containing a small amount of sample DNA and reaction mixture. This increases the ratio of target DNA to the primer/probe set while decreasing the ratio of inhibitors in a sample droplet. The sensitivity of the duplex ddPCR was shown to be as low as two copies of CLas DNA and 0.75 copies of *S. citri* DNA (**Table 1**), which is roughly equivalent to the results obtained in singleplex ddPCR test. Although the sensitivity of the ddPCR was similar to the single target qPCR tests, the ddPCR result was more precise since it measured the exact copy number of target DNA in the sample. In contrast, qPCR measured a relative quantity of the pathogen that needs to be compared with known quantities of the pathogen as a control.

The U.S. Department of Agriculture-Animal and Plant Health Inspection Service-Plant Protection and Quarantine program and the California Department of Food and Agriculture now routinely conduct CLas diagnostics using CLas RNR primers/probe in conjunction with the diagnostic CLas 16S rRNA primers/probe. The Citrus Pest Detection Program of the Central California Tristeza Eradication Agency is well positioned to use the duplex qPCR and duplex ddPCR for non-regulatory tests when tree symptomology is not adequate for distinguishing HLB and CSD.

Sequencing of *S. citri*

Even though we used ORF1 of the *S. citri* Virus 1 for the project, we had another project that focused on sequencing the *S. citri* genome in search of new targets for *S. citri* detection. Since the first whole genome sequence of *S. citri* was for the Moroccan *S. citri* strain R8-A2T (Davis et al. 2017), we sequenced seven diverse strains of *S. citri* from the U.S. (Yokomi et al. 2019, 2020). We are presently testing new primers to improve *S. citri* diagnostics for CSD. In addition, the new data on *S. citri* genomics provides new knowledge to better understand *S. citri*/CLas interactions that could help develop new control strategies to mitigate HLB.

Conclusions

Disease mitigation efforts are stymied when a citrus tree that appears to have HLB or HLB-like symptoms tests negative for CLas. To help determine that this is not a false negative test for CLas, the duplex qPCR assay includes a test for *S. citri* that may be responsible for the HLB-like symptoms. The duplex test, therefore, provides added confidence for HLB diagnostic tests and potentially has a future use as a regulatory tool. This test uses the same sample, which saves time and costs. Since qPCR measures a relative amount of the pathogen target, a ddPCR was developed that measures the copy number of pathogen target for better pathogen quantification at very

Table 1. Sensitivity of droplet digital PCR for detection of *'Candidatus* Liberibacter asiaticus'*(CLas) and *Spiroplasma citri*** in a singleplex test versus a duplex test from dually infected citrus leaf tissue. NA means not detected.

	Copies of DNA target			
	Singleplex		Duplex	
DNA conc. (ng)	CLas	*S. citri*	CLas	*S. citri*
2 x 10-0	3526	803	3732	752
2 x 10-1	386	78.1	375	74.4
2 x 10-2	39.7	8.7	39.4	7.0
2 x 10-3	3.5	0.94	2.0	0.75
2 x 10-4	0.03	NA	0.56	NA

*CLas Ribonucleotide reductase (RNR) gene
**S. citri* Virus 1 Open Reading Frame1 (ORF1) gene

low concentrations. In addition, the new data generated on *S. citri* genomics should provide a better understanding of interactions between CLas and *S. citri* when dual infection in trees occurs.

CRB Research Project #5300-191

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Glossary

[1]duplex qPCR: A qPCR assay that detects two different genetic sequences (such as from two different pathogens) in the same sample.

[2]ddPCR: Droplet digital Polymerase Chain Reaction (ddPCR) is a highly precise and sensitive method for DNA detection and quantification. Measurements are performed by partitioning each sample reaction into droplets, each of which is analyzed.

[3]RNR: Ribonuclease reductase gene (nrdB) of *'Candidatus* Liberibacter asiaticus' (CLas). Primers for this gene are used in some CLas regulatory tests due to the presence of multiple copies (leading to greater sensitivity) and greater specificity relative to related bacterial species.

[4]ORF1: Open Reading Frame 1, which is a short sequence of DNA that is part of a gene in *Spiroplasma citri*. There are multiple copies of ORF1 sequences in *S. citri* genomes.

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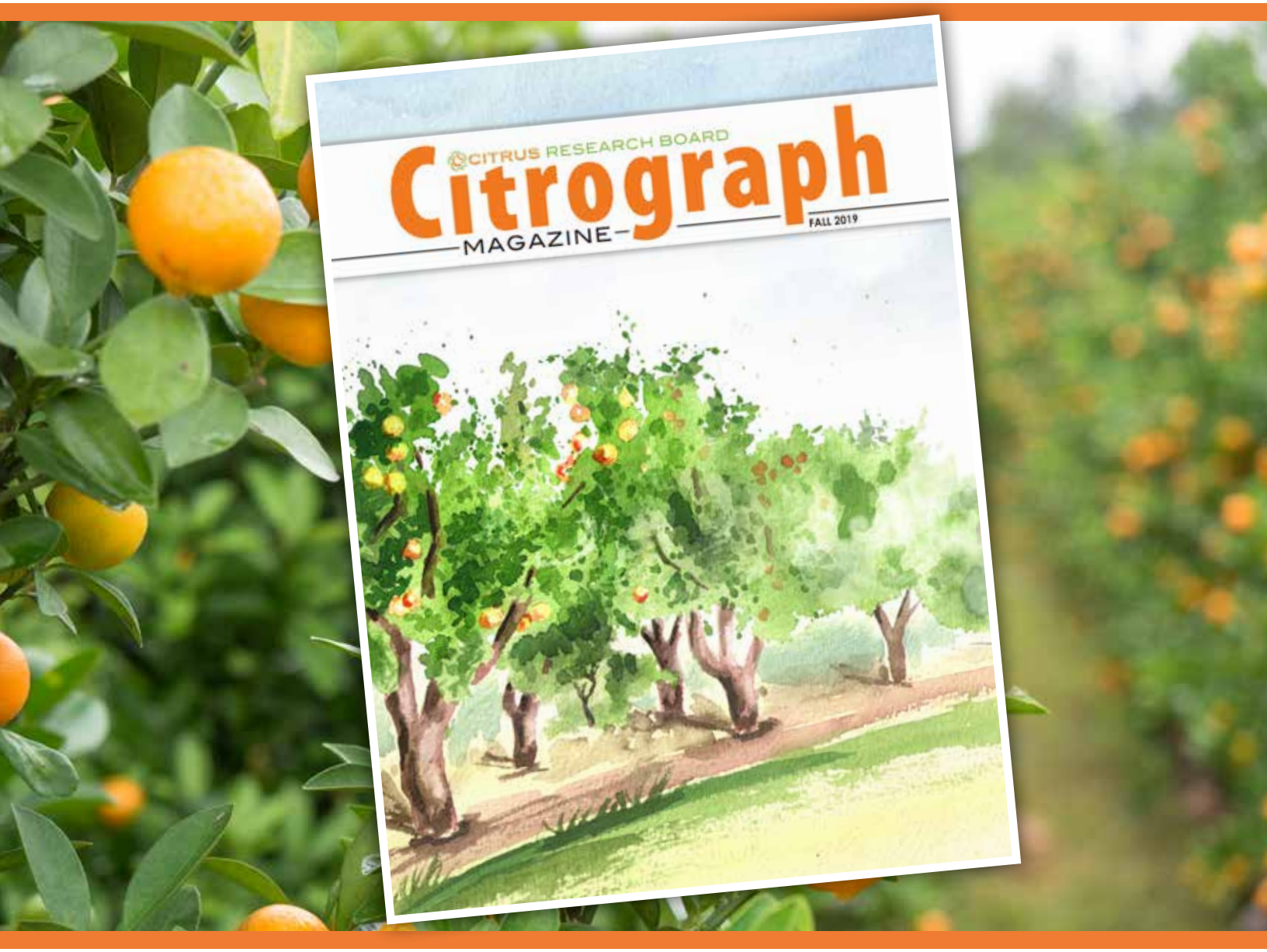

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A Taste of CITRUS HISTORY

BARK DISEASES OF CITRUS TREES IN CALIFORNIA*

HOWARD S. FAWCETT

INTRODUCTION

Frequently, when the bark is irregular in contour, gum pockets from 1 to 2 inches in length will be formed. The gum accumulates at certain places, forming definite pockets. The pressure is usually relieved by a break in the bark before the pockets become large. A few deeper gum pockets of considerable size have also been found,

Fig. 1.—Pythiacystis or brown-rot gummosis on lemon trunk. The tree is completely girdled, the dead bark resulting from several infections starting near the soil surface. The ridges of exuded gum are characteristic.

Diseases and pests have been beguiled the California citrus industry since the nineteenth century. A rogue's gallery of insects, from the cottony-cushion scale to the Asian citrus psyllid, and an array of viruses have thrived in the orange and lemon groves of the Golden State. To overcome pests and diseases, in 1907 the University of California opened a Citrus Experiment Station in Riverside, the heart of citrus country.

Originally located on the slopes of Mt. Rubidoux, the Citrus Experiment Station brought together a world-class group of botanists, entomologists and other scientific experts. Scholars at the Citrus Experiment Station regularly published bulletins issued to citrus growers across the state, warning about the dangers of disease and providing recommendations for coping with viruses or insect infestations. The image here comes from one such bulletin issued by a researcher at the Citrus Experiment Station, a document that is now part of the Citrus Roots Collection at the University of La Verne. This bulletin pictured lemon trees infested with a type of gummosis, which was related to the blight that devastated Irish potatoes in the 1840s. A variety of gummosis also killed one of the parent Washington Navel orange trees in Riverside. By spreading information on problems such as bark diseases, scholars helped orange and lemon growers in California follow the best scientific practices to safeguard the future of the industry.

To learn more about the history of California citrus, please contact *bjenkins@laverne.edu*

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