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Spring 2023 | Volume 14 • Number 2  The Official Publication of The Citrus Research Board

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APRIL 12
CITRUS PEST AND DISEASE PREVENTION COMMITTEE (CPDPC) MEETING.
For more information, visit www.cdfa.ca.gov/citruscommittee

MAY 9
CITRUS RESEARCH BOARD (CRB) MEETING.
For more information, contact the CRB at (559) 738-0246 or visit www.citrusresearch.org

JUNE 6
2023 CITRUS RESEARCH BOARD WEBINAR SERIES.
For more information and additional Webinar Series dates, see page 14.

AUGUST 8
CITRUS RESEARCH BOARD (CRB) MEETING.
For more information, contact the CRB at (559) 738-0246 or visit www.citrusresearch.org

AUGUST 9
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The beginning of the year brings the start of a new research cycle at the Citrus Research Board (CRB). Every year, our Research Department and Research Priority Screening Committee (RPSC) undertakes the immense task of reviewing each new and continuing research proposal submitted in each of our Core Research Program areas. The FY2023-24 Request for Proposals (RFP) was released in February and project pre-proposals currently are being reviewed by the RPSC to determine which projects align with industry needs and should move forward for review by CRB research committee members. In a shift from previous years, the CRB has identified specific research areas and targeted researchers to address certain topics that have been deemed critical. This process will occur in tandem with the regular RFP cycle.
This edition of Citrograph focuses on vectored diseases and highlights the continuous work by CRB-funded researchers to develop solutions in the fight against HLB and Tristeza, among other diseases. I want to highlight two projects focused on mitigating vectored diseases.

Kerry Mauck

At the University of California, Riverside (UCR), Kerry Mauck, Ph.D., currently is wrapping up a research project investigating how an antimicrobial peptide may reduce ‘Candidatus Liberibacter asiaticus’ (CLas) titers in citrus plants and the Asian citrus psyllid (ACP). This federally funded project is being conducted in the California Citrus Research Foundation Biosafety Level 3P facility near the UCR campus and is the first project that will have been completed within the facility. The antimicrobial peptide was discovered by Hailing Jin, Ph.D., at UCR and is derived from Australian finger lime, a citrus relative naturally resistant to CLas. Building on Jin’s earlier work, Mauck is focused on determining what effect this peptide has on CLas-infected ACP when applied to healthy citrus trees. When feeding on treated citrus plants, CLas-infected ACP tended to have lower CLas titers, and fewer of these ACP survived overall. Although data from the remaining experiments are being analyzed, these early results suggest this antimicrobial peptide likely has an indirect effect on ACP but could reduce the survival of adult ACP. More information from this research project can be found on page 30.

Georgios Vidalakis

Georgios Vidalakis, Ph.D., at UCR has been working with an extensive team of researchers throughout the U.S. to develop a novel virus vector for use against several citrus pathogens, including CLas. The virus is called citrus yellow vein associated virus (CYVaV) and has several features (i.e., phloem-limited, graft-transmissible and easy to manipulate in the lab) that make it a suitable candidate for further study and a potential, commercially viable product. This cross-country team is focused on several aspects of research including stabilizing the virus to accept anti-pathogen and anti-ACP compounds, determining its interactions with other citrus viruses and its effects on commercial citrus varieties. The team’s initial results suggest CYVaV is functional in plants and capable of accepting gene inserts that can be used to target the CLas pathogen or ACP. Furthermore, several genes were identified in ACP that if targeted by the vector, could result in ACP death. The team will continue their research to work toward regulatory approval and commercialization of this virus vector. More information from this research can be found on page 44.

Establishment of USDA-ARS Citrus Breeding Facility in Parlier

The California citrus industry welcomed more than $1 million in new federal funding after the 2023 Appropriations bill was passed by Congress in December. Spearheaded by California Citrus Mutual (CCM) and the CRB and championed by California Senator Alex Padilla and Representatives Jim Costa and David Valadao, this funding will establish a citrus breeding program at the U.S. Department of Agriculture-Agriculture Research Service (USDA-ARS) San Joaquin Valley Agricultural Sciences Center in Parlier, California. This new breeding program will develop citrus varieties that are best suited for
California’s changing climatic pressures such as drought, consumer taste preferences and resistance to pests and diseases. The program is an expansion of the existing national USDA-ARS citrus breeding program in Florida and will serve to enhance our efforts to secure longer-term sustainability for California’s citrus growers along with the established University of California breeding program. In addition, the CRB committed $500,000 toward establishing this new program to bring additional representation to California’s industry.

Looking Forward in 2023

In the coming months, the CRB is hosting several grower-focused events to provide educational opportunities on a variety of topics. In June, our annual Citrus Growers Educational Webinar Series will feature four hour-long discussions on valuable research and cultural practices that can implemented in the field. Additional information on this educational opportunity can be found on page 14.

Our Post-harvest Conference will return September 6, 2023, in Visalia, California, and will feature a technical agenda of food safety, post-harvest disease management, fruit quality and maintenance, as well as trade and pest management. This conference is tailored toward packinghouse personnel and post-harvest experts to provide beneficial research updates to this crucial part of the industry. More information will be shared in the next issue of Citrograph and on www.citrusresearch.org.

The CRB is continually working to provide the latest information for growers through our partnerships with numerous universities, researchers and government agencies. We are committed to providing an avenue for grower education through our Citrus Growers Education Webinar Series and Post-harvest Conference, in addition to the research project reports that are always available on our website. We look forward to shepherding in a new group of research projects in the coming months and eagerly anticipate future reports on these valuable projects.

Marcy L. Martin serves as the president of the Citrus Research Board, based in Visalia, California. She also is the executive editor of Citrograph. For more information, please contact marcy@citrusresearch.org

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The Citrus Research Board (CRB) is excited to announce the return of the Citrus Growers Educational Webinar Series in 2023. This successful series has been held annually since 2020 and provides an opportunity for growers around the state to hear the latest citrus research updates where it is most convenient for them—whether that be at home, in the office or in the groves. The webinar series has featured an impressive line-up of extension and industry professionals, as well as many researchers funded by the CRB. Past topics included an update on pesticide laws and regulations, a report on California’s water situation and specific research within the CRB’s Core Research Programs of integrated pest management, citrus clonal protection and new varieties.

The four one-hour webinars are scheduled for June 6, 13, 20 and 27. Each session will highlight valuable research and practical discussions for growers. Continuing education units (CEUs) will be available through the California Department of Pesticide Regulation and Certified Crop Advisers, pending approval. Please check www.citrusresearch.org for updates on topics and speakers.

Caitlin Stanton is the communications coordinator for the Citrus Research Board and also serves as the editorial assistant on Citrograph. For more information, please contact events@citrusresearch.org
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This edition of Citrograph is focused on vectored diseases. With increasing Asian citrus psyllid (ACP) finds and rising inflation affecting citrus production, we recently asked citrus industry members for their insight on these current issues in California.

LOGAN HENDERSON  
AgriCare Inc.  
Strathmore, California

Have the increasing ACP finds in the San Joaquin Valley this past summer/fall and or finds of ‘Candidatus Liberibacter asiaticus’ (CLas)-infected psyllids in some southern California groves affected your approach to psyllid management? Do you have a plan should huanglongbing (HLB) be found in commercial groves near your ranch?

We closely participate in local eradication efforts when ACP finds increase in a given geographical region. These coordinated area-wide treatments have proven to be effective in both Kern and Tulare counties over the last six to seven years, suppressing the population back to near non-detectable levels. The plan as an industry for if/when HLB is found should be quick and decisive action. We intend to be proactive on any tree removals, sprays or necessary actions that are needed to effectively combat the spread of the disease and its vector.

How has inflation affected your grove management plans, especially for pest management or ACP control?

Inflation has caused an expected response by and large. Budget cutbacks in all areas have been observed, which translates to a decrease in the ability to fight all arthropod pests, ACP included. Insecticidal control is mostly limited to when it fits other pest, IPM timings and required regulatory applications.

Would you rather see researchers spend more effort on developing new technologies for citrus or providing better information on how to use the tools/technologies that are already available?
I would like to see effective new technologies for citrus from the pest control standpoint, as this an area that is sorely lacking. Regulatory hurdles can make it difficult to combat pests in new and creative ways on the pesticide front, so technology will continue to be a vital tool in the fight against ACP/HLB.

**LINDEN ANDERSON**
President/Ranch Manager
HMS Agricultural Corporation
Coachella Valley, California

Have the increasing ACP finds in the San Joaquin Valley this past summer/fall and or finds of CLas-infected psyllid in some southern California groves affected your approach to psyllid management? Do you have a plan should HLB be found in commercial groves near your ranch?

The continuing finds are a reminder of the importance of being diligent with our efforts to keep ACP populations low in order to reduce the chances of infection and spread of HLB. Fortunately, here in Coachella Valley, we have had great grower cooperation utilizing coordinated area-wide treatments to achieve that goal. That, along with extreme heat days in the summer—who knew that 120º days actually are a blessing—have allowed us to maintain low populations of psyllids.

Currently, there is a significant time gap between infection and detection of HLB. If a neighboring grove is found to be infected with HLB, there is a high probability that we would also have infected trees. Without the advent of something that can be used to inoculate existing trees to prevent or control HLB bacteria, we will have to determine if the economics of continuing to keep the infected grove in production is feasible. Proximity to other groves, age of trees, productivity, market outlook, future water availability and alternate crops or uses for the land all will be factors in the decision.

**McCall Machado is the communications and event coordinator for the Citrus Research Board. For more information, please contact mcall@citrusresearch.org**

Would you rather see researchers spend more effort on developing new technologies for citrus or providing better information on how to use the tools/technologies that are already available?

It is important to utilize the best tools and techniques to prevent the spread of HLB. However, it is my belief that the long-term solution is the development of resistant rootstock or treatments that prevent or control HLB. That is where I would recommend research be concentrated.

How has inflation affected your grove management plans, especially for pest management or ACP control?

There is no denying that inflation impacts us; however, it really doesn’t change our current pest management plans and what we do for ACP control. These operations are necessary to grow a quality crop; and as always, the goal is to get the best long-term control in the most economic manner. As much as possible, we piggyback our foliar nutrient applications with pest management sprays to reduce application costs; and when possible, we use practices that maintain beneficial insects, thus reducing the number of treatments needed.
Bob McKellar has been farming citrus on his family's land in Ivanhoe, California, for more than 80 years and enjoys teaching the public about the citrus industry.
Within the groves of Ivanhoe, California, curious future farmers can learn how their favorite citrus fruits venture from the tree to their lunch boxes, thanks to the educational opportunities available at Farmer Bob’s World. Bob McKellar’s family has been growing citrus on that same land for more than 80 years, and now they are opening it up to the public to show the world how citrus is produced.

The McKellar family has been at the forefront of communicating directly with consumers. For ten years, they have delivered citrus directly from their groves to homes in Tulare County, in addition to their commercial production. McKellar has remained active in the industry and previously served on the California Citrus Mutual board of directors for many years. Between interacting with consumers through his fruit delivery business and event venues on his property, McKellar came to realize how little people knew about farming. In 2014, he started hosting international groups and school tours to inform a wider audience where their food comes from and how a working citrus farm operates. A year later, Farmer Bob’s World became a non-profit and continued to grow its business by hosting different groups. In 2021, Farmer Bob’s World held its grand opening and now offer tours every Wednesday through Saturday.

Visitors to the farm can book a self-guided tour, a tractor-pulled wagon tour or a walking tour with a local orange grower. Each tour includes a visit to the demonstration orchard, an overview of equipment and cultural practices used to produce citrus and the opportunity to pick an orange. McKellar says many of the visitors are curious about the ongoing drought, sunblock on trees, pollination practices and the lifespan of citrus. To demonstrate this longevity, McKellar takes the visitors to his 100-year-old grove, which predates his family owning the land and still regularly produces fruit. A video displaying fruit moving through the packinghouse is shown to provide an overview of the entire production process. From schoolchildren to adults, each guest who visits Farmer Bob’s World comes away with an understanding of how citrus is produced, and their eyes are opened to a way of life to which they had not previously given much consideration.

“It is more and more important, in my view, that we do more to tell our story to those who no longer live on farms, no longer know anybody who’s on a farm or no longer have a relative that is on a farm.”
Bob McKellar

As a veteran citrus grower, McKellar has seen the industry shift in more ways than one. He has seen spraying move from 50-foot hoses
that were applied tree by tree to mechanized options that can spray a row of trees in minutes, as well as the addition of drone imagery and automated wind machines. McKellar champions the researchers and cooperating growers who are developing innovative ways to tackle the most crucial issues in growing citrus. He would like to see further research into growing citrus with limited water resources, as he sees that as one of the top issues for the future of the industry.

Moving forward, McKellar and his staff would like to expand the number of tours and bring in additional groups of people. They are currently constructing a farm animal exhibit and are striving for inclusivity by becoming a Certified Autism Center™ so that all guests will feel welcome. In recent years, Farmer Bob’s World has partnered with organizations like World Ag Expo, the City of Visalia and Visalia Unified School District to promote their tours to a range of consumers. In spring 2023, Farmer Bob’s World will host its inaugural Citrus Festival, which will include several celebrations of citrus and its impact on the Central Valley.

As a long-standing member of the citrus industry, McKellar believes that the advocacy taking place through Farmer Bob’s World is important for the future of California’s citrus.
He and his staff are working diligently to reconnect young people to farm so that they begin to understand where their food comes from and how the next generation can become involved in production agriculture.

To learn more about Farmer Bob’s World and to contribute to this important educational resource, please visit www.farmerbobsworld.com.

Caitlin Stanton is the communications coordinator for the Citrus Research Board and also serves as the editorial assistant on Citrograph. For more information, please contact events@citrusresearch.org.

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As huanglongbing (HLB) detections continue to rise in residential communities, educating elected officials on the threat posed by HLB and the Asian citrus psyllid (ACP) is critical to increase awareness and support for the issue among residents throughout the state.

In California, it is estimated that nearly six in ten residences have a citrus tree on their property. As the Citrus Pest and Disease Prevention Program (CPDPP) works to educate homeowners about the threat posed by HLB and ACP, the CPDPP’s outreach team often engages local city and county elected officials to further communicate with California residents right in their own communities.
Building collaborative partnerships with city council members and staff, county boards of supervisors and county agricultural commissioners’ offices has proven to be a key component of the CPDPP’s outreach strategy. Local elected and appointed officials provide a useful “boots-on-the-ground” perspective that allows the CPDPP’s key messages to be shared through multiple channels and voices.

Educating Elected Officials

In areas throughout the state where HLB is present or in areas where new ACP detections may occur, the CPDPP’s outreach team and key members of the Citrus Pest and Disease Prevention Division staff will offer presentations and deskside briefings with city council representatives, boards of supervisors, city and county staff or other interested officials as a way to educate them on ACP and HLB detection milestones such as the first detection in their city, an expansion of the HLB quarantine area or increased community conversations around pesticide treatments or tree removal requirements. These meetings give officials an opportunity to become more familiar with the program, establish the CPDPP as a resource for future needs and foster understanding of the threat posed by the pest and disease in their communities. In 2022, the CPDPP has hosted deskside briefings or presentations with nearly a dozen entities on the issue.

But the outreach isn’t limited just to the HLB quarantine zone. The CPDPP outreach team also conducts proactive outreach to cities located outside of the current HLB quarantine area or near major commercial citrus growing
regions to educate them on the issue in general, as well as provide elected officials with resources to share with their constituents about how they can take the proper measures to protect their own citrus trees, inspect for signs of ACP or HLB and more.

In addition to hosting briefings and presentations, the CPDPP's outreach team often will connect with elected officials at trade shows or conferences to meet folks in person and engage in one-on-one conversations with California's elected and appointed officials. The benefit of attending these events is that it allows the CPDPP outreach team to have tailored conversations with hundreds of elected officials from municipalities across the state. By meeting these folks in person, we get to learn more about their city or county, speak one-on-one with them about the importance of sharing information on ACP and HLB and build genuine relationships. Some of the events the CPDPP's outreach team attended in 2022 included:

- California Contract Cities Association Annual Municipal Seminar,
- California State Association of Counties Annual Meeting,
- League of California Cities Annual Conference and Expo and
- Southern California Association of Governments Regional Conference and General Assembly.

The CPDPP outreach team added approximately 50 new contacts to our ongoing database and updated the information of more than 200 contacts as a result of these events.

Digital and Social Media Outreach

The impact that HLB can have on California's backyard citrus trees is a complex issue with many layers of terminology, regulatory protocol and regulations. To ensure consistent communication around the complex issue, the CPDPP's outreach team works to provide local elected officials with ACP and HLB messaging guidance in the form of talking points, pre-drafted letters to key stakeholders and more. Messaging guidance always is tailored for each city or county to accommodate the many nuances of ACP and HLB quarantines or address specific scenarios or challenges. As part of this effort and to arm local elected officials with the tools they need to communicate to their constituents about the pest and disease, the CPDPP's outreach team often will send “plug-and-play” content packages, leveraging the program's free informational materials to allow our partners on the local and county levels to create custom content of their own. Through the CPDPP's elected official database, the outreach team will distribute website copy, sample social media posts, flyers and other materials that elected and appointed officials easily can disseminate through websites, newsletters, social media, blogs, community forums and more.

Thanking Our Partners

It's no secret that local elected officials often share vital and important information via their websites or social media pages that impacts the everyday lives of their constituents – and we're thankful for those who choose to share the important messages of the CPDPP as part of that effort. The CPDPP outreach team recognizes California's “Citrus Heroes” as a way to publicly thank city and county officials, departments and staff for actively partnering to disseminate CPDPP information.

Local elected officials are a key component to the outreach efforts put forth by the CPDPP to elevate the important messages we want shared with homeowners and to gain support for activities being conducted by California Department of Food and Agriculture staff. While a variety of statewide initiatives can educate homeowners on threats to California citrus, there is also value in tailoring an approach to fit the features unique to each region, as we've seen in connecting with local elected and appointed officials. By working together and continuing to innovate our approach, we can save California citrus.

Kevin Ball is the outreach subcommittee chair for the Citrus Pest and Disease Prevention Committee. For additional information, please contact kevin.ball@aglandca.com

www.CitrusResearch.org | Citrograph Magazine 27
CITRUS RESEARCH BOARD

POST-HARVEST CONFERENCE

SEPTEMBER 6, 2023 | VISALIA CONVENTION CENTER
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IT'S PLANTING Season

WHEN YOU CALL TO SCHEDULE, PLEASE MAKE SURE YOU HAVE THE FOLLOWING INFORMATION

Location
Property Location/Directions (keep in mind landmarks that will help us find your field).

ACP Number
If you do not have this or need one for a new property, please contact your local Ag. Commissioner's office to obtain one.

Exact Tree Count
Once trees leave the nursery they cannot come back! The contact information for the point person in charge of planting.

Contact Info
For the person in charge of planting. And... don’t forget to have a field forklift on-site for tree deliveries.

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EVALUATIONS OF ANTIMICROBIAL PEPTIDES FOR HLB CONTROL

A project update on work at the California Citrus Research Foundation Biosafety Level-3P facility

Kerry Mauck, Marco Gebiola, Ximena A. Olarte Castillo and Monique Rivera
Project Introduction and Description

Huanglongbing (HLB) is the most destructive disease of citrus worldwide (Wang et al. 2017). HLB is caused by a bacterial pathogen, ‘Candidatus Liberibacter asiaticus’ (CLas), which is transmitted to citrus hosts by the Asian citrus psyllid (ACP, Diaphorina citri). CLas resides within the vascular tissue (phloem) of the citrus host, which is a “highway” for transport of vital sugars, water and other molecules required for the plant to grow and reproduce normally. It is this same transport network that supports growth and reproduction of the ACP, which accesses the phloem using tube-like piercing-sucking mouthparts. As phloem sap is ingested from an infected plant, an ACP can acquire CLas bacteria, which may go on to colonize and reproduce within the insect (a second host). Colonization of the insect host in a way that facilitates further transmission is most successful when CLas is acquired by immature ACP. The infection will carry forward into the flight-capable adult stage, which can transmit CLas to new citrus hosts during feeding.

Since CLas only can be transmitted to citrus trees by grafting and by the ACP vector, insecticides have served as a mainstay control strategy. As of 2022, CLas has not become established in commercial groves in California following its first detection in backyard citrus in 2012. However, the number of infected residential citrus plants continues to rise, with more than 4,000 detections to date. As pathogen prevalence mounts, growers will need new management tools to prevent CLas infections from establishing and, eventually, to treat existing infections.

One option is to target the pathogen in the plant with antimicrobial agents. This was the approach taken by Hailing Jin, Ph.D., and her research team. A recent paper from her team (Huang et al. 2021) documents development of a therapeutic and prophylactic protectant based on a stable antimicrobial peptide (SAMP) discovered in Australian finger lime (Microcitrus australasica). Australian finger lime is resistant to CLas infection and produces a particularly potent version of an antimicrobial peptide.
Figure 2. Preliminary graphic summary of the effects of stable antimicrobial peptide (SAMP) treatments on 'Candidatus Liberibacter asiaticus' (CLas) titer in individual CLas-exposed ACP adults caged on treated plants Citrus medica and C. macrophylla for 12 days. DMSO = the control treatment containing carrier material only, and SAMP = the treatment containing carrier plus antimicrobial peptides.

For replication 1, 92 insects were tested; and for replication 2, 86 insects were tested.

A key question arising from this work is: what effects do SAMPs have on the CLas transmission process? Could SAMPs be effective in reducing CLas infections not only in plants, but also in the vectors? To determine the potential for SAMPs to limit CLas transmission, we collaborated with Jin to perform experiments in the California Citrus Research Foundation Biosafety Level-3P facility (CCRF-BSL-3P). Here, we highlight the results of select experiments to quantify the effect of prophylactic SAMP applications (to uninfected plants) on CLas titers in CLas-positive (CLas+) ACP, ACP survival and immature ACP development.

We carried out two replications of an experiment (Figure 1) to evaluate transmission metrics in tandem using two fast-growing Citrus species that are preferred hosts for ACP: citron (Citrus medica) and alemow (C. macrophylla). Plants of each species were trimmed to induce flush shortly before the start of experiments. Plants were treated with a SAMP solution with adjuvant or a “mock” treatment consisting of the same components minus the SAMPs (labeled as DMSO in all figures). Five days later, age-standardized CLas+ ACP adults were caged on each plant (12 per plant in replication 1, 11 per plant in replication 2) and allowed to feed and reproduce for 12 days before data collection.

Project Results

SAMP treatments did not significantly reduce CLas titers in adult ACP in either replication of the experiment, although there was a trend toward lower titers in ACP feeding on SAMP-treated C. macrophylla in the first replication (Figure 2). Although all CLas+ ACP were from the same cohort, ACP CLas titers within each experiment varied from a few hundred copies of the bacterial target gene (low) to more than one million copies (very high). The graphs shown include values from individuals testing negative for CLas (11 samples from replication 1 and eight samples from replication 2). When these are removed, SAMP treatments still did not significantly reduce titers.

Although SAMP treatments did not significantly reduce CLas titers in adult ACP, there were some negative effects of SAMP treatment on ACP survival. In all cases, fewer ACP survived to the recovery date (12 days) on plants treated with SAMP. Survival differences were
Figure 3. Graphic summary of the effects of stable antimicrobial peptide (SAMP) treatments on adult ‘Candidatus Liberibacter asiaticus’ (CLas)-positive Asian citrus psyllid (ACP) survival by host species. DMSO = the control treatment containing carrier material only, and SAMP = the treatment containing carrier plus antimicrobial peptides. Replication 1 and Replication 2 indicate the two temporal replications of the entire experiment.

Figure 4. Graphic summary of the effects of stable antimicrobial peptide (SAMP) treatments on the development of the psyllid population on plant hosts during the 12-day feeding period. DMSO = the control treatment containing carrier material only, and SAMP = the treatment containing carrier plus antimicrobial peptides.

particularly apparent in replication 1 on C. macrophylla and on both hosts in replication 2 (Figure 3). These results are not consistent with a direct effect of SAMP on survival but are consistent with a prior finding that SAMP treatment activates defense pathways that make plants less suitable for psyllids (Huang et al. 2021). Another interesting finding is that the overall survival rate in the experiment is lower in the replication that had higher titers in the ACP cohort (replication 2) (Figure 3). This suggests that more intense CLas infections take a toll and may predispose ACP to respond more negatively to plant defenses primed by SAMPs.

For the first replication of the experiment, we quantified the numbers and stages of development of all offspring produced by the adult ACP used in experiments (frozen samples from the second replication are still being processed and counted). SAMP treatment did not have a strong effect on the development of the offspring population for C. macrophylla. For C. medica, SAMP induced a slight acceleration in development, with fewer nymphs in the third instar stage and more in the fourth instar stage relative to the DMSO treatment. However, there also were fewer nymphs total on SAMP-treated C. medica plants (100 across all SAMP plants compared to 221 across all DMSO plants), while the number of nymphs on C. macrophylla were similar across treatments (195 SAMP compared to 178 DMSO). This could have
influenced nymph competition, with less competition for resources on the more sparsely colonized treatment leading to faster development.

Conclusions

Overall, these results suggest that SAMPs do not directly alter CLas titers in ACP. SAMP effects on ACP likely are indirect (such as by mediating plant defense responses) and mostly affect the adult stage by reducing survival, especially of individuals harboring CLas infection. Under the conditions of this experiment, where nymphs began feeding approximately ten days after SAMP application, we observed largely neutral effects on immature stage ACP development. Additional data are being collected from completed CCRF-BSL-3P experiments testing whether SAMP applications to infected plants limit CLas acquisition by non-infected immatures. Thus far, our study indicates that despite SAMP's effectiveness in preventing and attenuating infections in the plant, SAMPs may not play a significant role in suppressing infections in ACP vectors to limit transmission.

Glossary

1Antimicrobial peptide: A small protein composed of 5 to 100 amino acids, produced by living organisms that have the ability to kill bacteria, viruses or fungi.

References


Acknowledgements

We are thankful for the assistance of Caroline Roper, Ph.D., and her team (Flavia Campos Freitas Vieira, Ph.D. and Christopher Drozd) who provided cuttings of citron to initiate work in the California Citrus Research Foundation Biosafety Level-3P facility (CCRF-BSL-3P) and assisted with psyllid culture. CLas-infected plant material was generously provided by Kris Godfrey, Ph.D., UC Davis Contained Research Facility. Thanks also to Le’Kneitah Smith, CCRF-BSL-3P director, and Adilene Gomez, CCRF-BSL-3P technician.

Kerry Mauck, Ph.D., is an associate professor with the University of California, Riverside, Department of Entomology in Riverside, California. Marco Gebiola is with the College of Veterinary Medicine at Cornell University in Ithaca, New York. Ximena A. Olarte Castillo is with the College of Veterinary Medicine at Cornell University in Ithaca, New York, and also the Department of Biology, Faculty of Sciences at Universidad Antonio Nariño in Bogota, Colombia. Monique Rivera, Ph.D., is an assistant professor in the Department of Entomology at Cornell University, Cornell AgriTech, Geneva, New York. For more information, please contact kerry.mauck@ucr.edu
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SUPPORTING
HLB
SUPPRESSION
WITH DATA ANALYSIS
THROUGHOUT THE YEAR

Neil McRoberts, Sandra Olkowski and Rick Dunn

Project Summary
Since 2016, the Data Analysis and Tactical Operations Center (DATOC) has provided targeted expert advice to the Citrus Pest and Disease Prevention Committee (CPDPC) and its Operations, Science and Outreach sub-committees. The project’s budget is provided by California Pest and Disease Prevention Division (CPDPD) assessment funds and facilitates collaboration between the University of California and the Citrus Research Board (CRB) to support the CPDPC in the state-wide effort to suppress huanglongbing (HLB) and its vector, the Asian citrus psyllid (ACP).

DATOC’s work is a component of the fourth aim of the CPDPD strategic plan: “Improve data technology, analysis and sharing.” To contribute to that strategic aim, DATOC works on three objectives:
1. **Stakeholder communication**

2. **Situational monitoring/data storage**

3. **Analytical projects**

The current iteration of the project began in 2020 during a period of relative stability in the development of HLB in California. The DATOC team has continued to provide advice to the CPDPC and CPDPD staff at the California Department of Food and Agriculture (CDFA) to improve the transfer of data from surveillance activities to the university and CRB Data and Information Management Department for analysis and use in stakeholder communication.

The state-wide response to the threat posed to citrus production by ACP-vectored HLB is a complex enterprise, involving partnerships among the citrus industry, state agencies, the University of California and an array of private contractors, who provide expertise in areas such as public relations and pest management. Operational decisions about vector management and disease control and outreach messaging rely heavily on up-to-date scientific advice. Both the operational and outreach activities of the HLB/ACP management program are managed by the CPDPC. The overall purpose for the DATOC project is to bring together an expert panel that can provide skills and resources to the industry in support of the CPDPC by making expertise in data analysis, epidemiology and extension available to the committee in a responsive approach that short-cuts traditional research timescales.

Although DATOC’s work is described under three separate objectives, in practice, there is really a seamless integration of stakeholder communication, work on data quality and summaries for situational monitoring, and specific analytical projects connected with the operation of the program. For example, at various times, the issue of whether “buffer” treatments to reduce the ACP population in residential properties adjacent to commercial citrus are cost-effective has been raised at meetings of the Operations and Science subcommittees. The discussion of this question has led to requests to DATOC to examine the available evidence and report to whichever committee has made the request.

Requests for specific pieces of analysis, such as whether residential buffer treatments are effective, fall under the heading “Analytical projects” – i.e., DATOC’s third objective.

Although the example of buffer treatments came from CPDPC subcommittees, DATOC addresses questions posed by other citrus industry stakeholders, such as the local HLB task forces or the Biological Control Task Force. However, each request of this type immediately triggers two lines of activity in addition to the actual work of answering the question that has been posed (Figure 1). First, a discussion takes place among the relevant experts in the DATOC group to identify which sets of data or models might be used to best address the question. This activity relies on our on-going work under Objective 2 to collate data from a range of sources and impose quality control measures to make sure data are reliable for use in analyses. After the initial discussion, the DATOC principal investigator, DATOC coordinator and relevant subject matter experts from the DATOC panel discuss the likely outreach points that will arise from the work, taking account of the different possible results that might come from the analysis. This forward planning allows us to work with CDFA staff and industry stakeholders ahead of work being completed, so that our stakeholder communication efforts (Objective 1) are aligned with the CPDPD’s communications.

Figure 1. How DATOC’s three objectives typically interconnect.
Over the course of its six-year existence, DATOC has helped the CPDPC, its Operations and Science subcommittees, local HLB/ACP task forces and other key stakeholders make evidence-based decisions about a range of key questions. These analyses sometimes have played an important role in helping to define the regulatory context for the CPDPC. For example, after the introduction of tarping regulations for bulk citrus transport, we coordinated a follow-up study at the request of the Science Subcommittee to re-evaluate the risk analysis on which the rules were based. This analysis was requested to allay concerns from some members of the grower community that a uniform requirement for tarping reflected only an academic perspective of the risk. The participatory study, which involved growers, regulatory scientists and university extension scientists, demonstrated that there was broad consensus on the need for uniform mitigation to reduce the risk of ACP movements with bulk citrus.

In another example, at the request of the Science Subcommittee, we carried out a literature review and synthesis of published data on the probability of transovarial (i.e., mother to egg) transmission of *Candidatus Liberibacter asiaticus* (CLas). The review provided supporting evidence that was used to establish the case for treating CLas-positive ACP nymphs as regulatory detections of the pathogen, since the consensus of the available studies was that the transovarial transmission rate is very low, so infected nymphs are overwhelmingly likely to have picked up the bacterium from the tree on which they are living.

There are many online information sources for citrus industry stakeholders and members of the public that are aimed at allowing them to stay up to date with California’s response to the threat posed by HLB. DATOC contributes to that communication effort by maintaining an online dashboard of summaries of the data collected in the regulatory program for HLB management.

The focus of the DATOC dashboard is quantitative information and presenting the available data in such a way that visitors to the website are able to see the full historical record of key indicators of the status of the epidemic, such as the positivity rate for the detection of CLas in ACP samples collected in the statewide detection and delimitation surveys shown in Figure 2.

In 2022, the activities of the CPDPC were reviewed by a specially convened Science Advisory Panel that reported a series of recommendations to the California Secretary of Food and Agriculture. Since the deliberations arising from the review may well have an impact on the future role of (or need for) DATOC, the project is currently in hiatus, pending the outcome of the discussions.

Neil McRoberts, Ph.D., is a professor of plant pathology at the University of California (UC), Davis and the national executive director for the National Plant Diagnostic Network. Sandra Olkowski, Ph.D., was acting DATOC coordinator and is currently a research data analyst in the Quantitative Biology and Epidemiology Group at UC Davis. Rick Dunn is the director of Data and Information Management at the Citrus Research Board.

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HIGH-THROUGHPUT SEQUENCING as a Routine Diagnostic Tool

The Final Steps

Tyler Dang, Andres Espindola, Sohrab Bodaghi, Irene Lavagi-Craddock, Fatima Osman, Marcos Ribeiro, Danielle Do Nascimento, Huizi Wang, Kitty Cardwell and Georgios Vidalakis
Project Summary

High-throughput sequencing (HTS) is a powerful technology that combines molecular biology and computer science with the potential to revolutionize the detection and identification of citrus pathogens. The goal of this project is to simplify the HTS data analyses so that an individual with no background in bioinformatics can determine the presence or absence of a specific graft-transmissible citrus pathogen in a sample. This goal can be achieved through a procedure called electronic (E)-probe Diagnostic Nucleic Acid Analysis (EDNA). We have entered the final stages of this project and begun assessing EDNA analysis in parallel with current diagnostics for the Citrus Clonal Protection Program (CCPP) variety index (VI) process. We calculated the statistical limit of detection for E-probes targeting citrus pathogens, designed E-probes for internal citrus controls, organized workshops for regulators and future EDNA users and published EDNA protocols and validation data that will be used as the basis for regulatory acceptance. EDNA analysis has the potential to streamline the diagnostic tests required for citrus variety release from quarantine.

Background

The citrus industry is facing threats from various pathogens that can spread through tree grafting with infected propagative materials. The CCPP is striving to utilize the best available pathogen detection tools in the citrus variety introduction process for the development of pathogen-tested citrus propagative materials. HTS is a powerful tool that combines molecular biology and computer science and is capable of simultaneously detecting and identifying multiple pathogens in a nucleic acid library constructed from the tested plant samples without the need to perform multiple tests. In our HTS studies so far, we have demonstrated that the technology is sensitive and specific and that the sequencing data analyses using EDNA did not require personnel with bioinformatics background or expensive servers running long analyses to obtain diagnostic results (Dang et al. 2019 and 2021).

In this final stage of our HTS studies, we:
- initiated the use of EDNA analysis in the CCPP,
- calculated the statistical limit of detection (LOD) for E-probes targeting specific citrus pathogens,
- designed E-probes for internal citrus controls,
- organized workshops for regulators and future EDNA users and
- published EDNA protocols and validation data that will be used as the basis for regulatory acceptance of the technology.

EDNA analyses for specific pathogens were used in parallel with the current CCPP VI diagnostic and pathogen detection protocols (e.g., biological indexing, polymerase chain reaction and gel electrophoresis) performing equally well in all cases with the currently approved tests. As such, the EDNA results accompanied the request for quarantine release to state and regulatory agencies for 38 VIs.
In the process of calculating the statistical LOD for E-probe sets targeting specific citrus pathogens (Table 1), we discovered that there was a lack of variance (i.e., zero reads) in healthy citrus samples because the only sequences in the sample were those of the citrus accession itself, as no citrus pathogens were present in a healthy sample. Zero reads of the citrus pathogen-specific E-probes in the healthy samples caused problems with the EDNA statistical analyses. We did not use E-probes targeting housekeeping genes of genera in the Rutaceae family because of concerns about the risk of false positives (i.e., that Rutaceae E-probes would have given high number of reads in healthy citrus samples by detecting the housekeeping genes of their relative citrus). We resolved this issue by adding E-probes targeting housekeeping genes from woody hosts such as pistachio (Pistacia vera) and apple (Malus domestica). These reads from apple and pistachio were distinct enough for the completion of the EDNA statistical analyses, but not enough to produce false-positive results for the healthy citrus samples.

We tested the newly developed E-probes mixture (i.e., E-probes targeting pathogens and internal control E-probes) against healthy and infected samples. The data were used to calculate the LOD. The results of our work were presented as a case study in a special issue of PhytoFrontiers on plant pathogen detection principles (Dang et al. 2022a). This peer-reviewed technical publication, in combination with a book chapter describing the step-by-step protocol for the EDNA analysis (Dang et al. 2022b), will be used as part of the documentation for regulatory acceptance. In addition to documentation, we organized five workshops and training sessions for regulators and future EDNA users, in the past two years. These events have been important for understanding the user friendliness of the HTS-EDNA online platform and interest for adoption in citrus diagnostics.

Currently, E-probe validation research has focused on specificity, analytical sensitivity (via computer analysis) and diagnostic sensitivity in which E-probes are tested against confirmed positive and healthy samples and the LOD is calculated. More extensive validation research ultimately will include metrics of robustness, reliability and transferability comparing results among multiple operators in different laboratory settings.

As genomic sequencing and use of EDNA becomes more affordable and well documented, it is anticipated that the technology will become useful for citrus germplasm trade partners around the world, thus strengthening the biosecurity protocols protecting the citrus industry. We anticipate testing HTS-EDNA against known gold standards such as polymerase chain reaction and enzyme-linked immunoassay (ELISA) for several more years to assure that the E-probes work reliably across time. In the near future, we suggest that the cost-benefits of extensive multiplexing for detection of multiple citrus pathogens in a single sample will drive regulatory acceptance and laboratory adoption.

### Conclusion

HTS and EDNA are powerful diagnostic tools that can help us to streamline the citrus variety introduction and quarantine release processes, providing growers access to pathogen-tested propagative materials for citrus grove establishment. We are at the final stages of data collection and presenting our findings to the regulatory agencies. Given the overall acceptance of HTS and EDNA technologies as the future of diagnostics and based on our interactions with regulators, we believe that this technology most certainly will be adopted for mainstream use in citrus disease diagnostics.

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**Table 1. Target pathogens at different stages of the HTS-EDNA design and validation process.**

<table>
<thead>
<tr>
<th>A. TARGET PATHOGENS FOR WHICH VALIDATION IS COMPLETE</th>
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<tbody>
<tr>
<td>1. Citrus tristeza virus</td>
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<tr>
<td>2. ‘Candidatus Liberibacter asiaticus’</td>
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<tr>
<td>3. Citrus exocortis viroid</td>
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<tr>
<td>4. Spiroplasma citri</td>
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<td>5. Citrus vein enation virus</td>
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<tr>
<th>B. TARGET PATHOGENS WITH DESIGNED E-PROBES UNDERGOING VALIDATION</th>
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<tbody>
<tr>
<td>6. Xanthomonas citri</td>
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<tr>
<td>7. Xylella fastidiosa</td>
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<tr>
<td>8. Citrus psorosis virus</td>
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<tr>
<td>9. Citrus leaf blotch virus</td>
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<tr>
<td>10. Citrus tatter leaf virus</td>
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<tr>
<td>11. Citrus variegation virus</td>
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<tr>
<td>12. Citrus concave gum associated virus and Citrus virus A</td>
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<td>13. Hop stunt viroid (citrus isolates)</td>
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<table>
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<tr>
<th>C. TARGET PATHOGENS WITH E-PROBES DESIGNED</th>
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<tbody>
<tr>
<td>14. ‘Candidatus Phytoplasma aurantifolia’</td>
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CRB Research Project #5300-205

References


Tyler Dang, Ph.D., is a post-doctoral scientist in the Department of Microbiology and Plant Pathology at the University of California, Riverside (UCR). Andres Espindola, Ph.D., is an assistant professor with the Institute of Biosecurity and Microbial Forensics (IBMF) at Oklahoma State University (OSU). Sohrab Bodaghi, Ph.D., is an associate research scientist in the Department of Microbiology and Plant Pathology at UCR. Irene Lavagi-Craddock, Ph.D., is an associate project scientist in the Department of Microbiology and Plant Pathology at UCR. Fatima Osman, Ph.D. is an associate project scientist at the University of California, Davis. Marcos Ribeiro, Ph.D., and Danielle Do Nascimento, Ph.D., are post-doctoral researchers with the IBMF at OSU. Huizi Wang, is a Ph.D. student at the Department of Statistics at OSU. Kitty Cardwell, Ph.D., is a professor and the director of IBMF at OSU. Georgios Vidalakis, Ph.D., is a professor and extension specialist in Plant Pathology and director of the Citrus Clonal Protection Program in the Department of Microbiology and Plant Pathology at UCR. For additional information, please contact: georgios.vidalakis@ucr.edu
A NOVEL VIRUS-LIKE AGENT FOR USE AGAINST CITRUS PATHOGENS

Anne Simon, Daniel Nelson, Judith Brown, Kari Debbink, Rafael Simon, Arunabha Mitra and Georgios Vidalakis
Ultimately, the team generated data that highlighted the following suitability of CYVaV:

- to become an expression vector of small size inserts,
- identified potential interactions with other citrus viruses that may affect natural spread of the CYVaV-vector,
- identified ACP genome targets that can be silenced by the CYVaV-vector and
- discovered that different citrus varieties develop different reactions to CYVaV.

Finally, we successfully leveraged non-Citrus Research Board (CRB) funds for the establishment of a start-up company dedicated to acquiring regulatory approval and to commercialize CYVaV-based anti-pathogenic technologies.

**Background**

In 2018, the National Academy of Sciences reported that a promising HLB management strategy for infected trees, which also may confer protection against future infections, was using phloem-restricted virus expression vectors to generate small RNAs or peptides directly into the tissue colonized by ‘Candidatus Liberibacter asiaticus’ (CLas) and fed on by ACP (NAS 2018).

We discovered and described a new species of plant-associated virus-like RNA named citrus yellow vein associated virus (CYVaV) (Kwon et al. 2021; Liu et al. 2021). CYVaV was discovered in association with a citrus disorder named yellow vein, reported once in the 1950s in California (Weathers 1957). Preliminary studies with CYVaV demonstrated that it is a promising virus expression vector¹ that can be used to express anti-pathogenic or anti-vector inserts for disease management. The goal of this two-year project was to build a national cross-institutional team with the scientific expertise necessary to generate preliminary data to leverage large amounts of funds (federal and private) required for the development and regulatory approval of a family of commercial CYVaV-based vectors against tristeza, huanglongbing (HLB) or the Asian citrus psyllid (ACP).

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¹ Preliminary studies with CYVaV demonstrated that it is a promising virus expression vector.
diseases such as tristeza and HLB, including HLB’s insect vector ACP.

This two-year CRB project built the research team and initiated five parallel research tracks, namely:

1. CYVaV biology – Anne Simon, Ph.D., University of Maryland
2. Anti-pathogen products – Daniel Nelson, Ph.D., University of Maryland
3. Anti-ACP small RNAs – Judith Brown, Ph.D., University of Arizona
4. CYVaV interactions with other citrus viruses – Kari Debbink, Ph.D., Johns Hopkins University
5. Field trials for effects in commercial citrus and transmission – Georgios Vidalakis, Ph.D., UCR

The results from this research will provide the foundation for future regulatory approval and commercialization of a disease management tool for the citrus industry. The research team comprised five principal investigators (PIs) at different academic institutions across the U.S. who brought their specific expertise to the project. The PIs and their corresponding research contributions and results are described below.

1. CYVaV biology – Anne Simon, Ph.D., University of Maryland

Simon and her team worked on defining the CYVaV genome structure (Liu et al. 2021) and determining sites where anti-pathogen or anti-ACP genomic sequences can be inserted. Simon’s team discovered five locations in the CYVaV genome that could support the insertion of outside sequences, such as citrus pathogen-targeting sequences. To stabilize the inserts, they made modifications to the CYVaV genome that show no sign of insert loss so far after one year in citrus. They also found that CYVaV can accept multiple inserts, which is important for the larger goal of generating virus-based vector(s) that target multiple citrus pathogens (e.g., citrus tristeza virus [CTV], CLas, etc.) or insect vectors. In an experiment using model plants (i.e., tobacco plants) containing green fluorescent protein (GFP), CYVaV carrying anti-GFP sequences was able to suppress the GFP expression, strongly suggesting that the CYVaV vector is functional in plants. Simon’s team also successfully re-introduced CYVaV into citrus utilizing dodder, a parasitic plant. Dodder was used to connect a tube containing sap from a CYVaV-infected plant (or a CYVaV-infected plant) with a healthy Mexican lime plant, and CYVaV moved via dodder into the healthy plant (Figure 1).

These two discoveries, stable inserts and transmission to citrus and other plants, allowed for further experiments. Simon’s team was able to identify two small RNAs that can target critical CLas genes and, using the surrogate bacterium Liberibacter crescens (Lcr), they are testing to see if the CYVaV-vector can keep the bacterium from growing in papaya, the natural host of Lcr. Simon’s team also developed a CTV-targeting CYVaV-vector that has successfully suppressed CTV replication under laboratory conditions in the model plant system Nicotiana benthamiana.

2. Anti-pathogen products – Daniel Nelson, Ph.D., University of Maryland

The Nelson team has been working with experimental proteins that have antimicrobial properties, known as enzybiotics. The goal of Nelson’s work was to screen and identify the best enzybiotic candidates for expression in the CYVaV-vector system for use against CLas.

Figure 1. Introduction of CYVaV into Mexican Lime from a tube containing sap from a CYVaV-infected plant using dodder, a parasitic plant. Dodder, the yellow string coming out of the plastic tube and wrapped around the plant stem, absorbs the plant sap containing CYVaV from the tube and introduces it into the Mexican lime vascular system.
As CLas cannot be artificially cultured using routine bacterial culturing techniques, the first objective of this project was to cultivate Lcr (a close CLas relative) in the laboratory to evaluate the antimicrobial properties of the enzybiotics. Nelson’s team tested Lcr growth on six growth media conditions and found one recipe worked very well. The second objective of this project was to evaluate anti-CLas enzybiotics, mainly focusing on those that can disrupt bacterial cell walls. Nelson’s team screened several enzymes for antimicrobial activity, and four enzybiotics were identified with antimicrobial activity against a bacterium with a similar cell wall structure to Lcr and CLas. However, as results were generated by Simon’s lab about potential sequence size requirements for CYVaV-vector expression, it became clear that the genes for enzybiotics are too large for CYVaV expression. Therefore, the original enzybiotics idea was not investigated any further. In the future, the CYVaV-vector may be tested for expression of smaller antimicrobial peptides.

Table 1. Citrus varieties and rootstocks tested with CYVaV in the field trial at the University of California, Riverside Agricultural Operations. Inoculum survival and rate of infection per scion/rootstock combination are presented. PGI refers to Post-graft Innoculation.

<table>
<thead>
<tr>
<th>ID</th>
<th>R/S COMBINATIONS (REPLICATED)</th>
<th>6 MONTHS PGI (JUNE 2021)</th>
<th>12 MONTHS PGI (NOVEMBER 2021)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RT-QPCR CYVAV INFECTION RATE (%)</td>
<td>RT-QPCR CYVAV INFECTION RATE (%)</td>
</tr>
<tr>
<td>1</td>
<td>Limoneira 8A Lisbon Lemon/Rubidoux Trifoliate</td>
<td>5/6</td>
<td>83</td>
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<tr>
<td>2</td>
<td>Limoneira 8A Lisbon Lemon/Macrophylla</td>
<td>5/6</td>
<td>33</td>
</tr>
<tr>
<td>3</td>
<td>Parent Washington Navel/Carrizo Citrange</td>
<td>4/6</td>
<td>17</td>
</tr>
<tr>
<td>4</td>
<td>Biogold Giulietta (Shiranui) Mandarin/ Carrizo 5/6 Citrange</td>
<td>6/6</td>
<td>33</td>
</tr>
<tr>
<td>5</td>
<td>Limoneira 8A Lisbon Lemon/Carrizo Citrange</td>
<td>6/6</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>Cara Cara Navel/ Carrizo Citrange</td>
<td>3/6</td>
<td>17</td>
</tr>
<tr>
<td>7</td>
<td>Biogold Giulietta (Shiranui) Mandarin/ Rubidoux Trifoliate</td>
<td>6/6</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Cara Cara Navel/ Rubidoux Trifoliate</td>
<td>5/6</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Tango Mandarin/ Rubidoux Trifoliate</td>
<td>5/6</td>
<td>67</td>
</tr>
<tr>
<td>10</td>
<td>Miho Wase Satsuma/ Carrizo Citrange</td>
<td>5/6</td>
<td>0</td>
</tr>
<tr>
<td>11</td>
<td>Tango Mandarin/ Carrizo Citrange</td>
<td>5/6</td>
<td>33.3</td>
</tr>
<tr>
<td>12</td>
<td>Parent Washington Navel/Rubidoux Trifoliate</td>
<td>4/5</td>
<td>0</td>
</tr>
</tbody>
</table>

*Inoculum survival was determined based on visual inspection of the graft.

3. Anti-ACP small RNAs – Judith Brown, Ph.D., University of Arizona

Brown’s team aimed to determine candidate small RNAs that have a high likelihood of targeting and affecting the biology of ACP. The team also tested multiple CYVaV phloem delivery strategies in experimental tomato plants to determine the most efficient strategy for CYVaV-vector delivery.

The Brown team identified bacteria-mediated and gene gun inoculation as the preferred RNA delivery methods into tomato. They also determined that the effective dose of candidate small RNAs (using the potato psyllid model, PoP), to achieve psyllid knockdown was 100 nanograms per microliter. PoP knockdown persisted for nine days before beginning to decline. Initially, more than 60 genes were tested in preliminary studies to identify psyllid knockdown candidates, and subsequently, 14 psyllid targets were further evaluated with PoP. Based on these results, Brown’s team identified and validated 12 ACP target genes that, when
silenced, resulted in 40-65 percent ACP mortality. Further studies are required for the evaluation of these results and their potential use in the field as anti-ACP technologies.

4. CYVaV interactions with other citrus viruses - Kari Debbink, Ph.D., Johns Hopkins University
Debbink’s team tested whether CYVaV could be enclosed (encapsidated) by the protein shell made by citrus vein enation virus (CVEV), another citrus-infecting virus. This was necessary as previous reports indicated that the yellow vein pathogen interacted with the vein enation pathogen, and symptoms of the two diseases were altered in mix infections (Weathers 1960). This was important to know because CVEV is aphid-transmitted and is present in California. Therefore, if CVEV was to be naturally introduced by aphids into a CYVaV-vector treated plant, the CVEV protein shell could capture the CYVaV-vector. In that case, aphids would be able to transmit the CYVaV-vector beyond the treated plants, raising critical questions for the regulatory approval of the CYVaV-based technology.

Debbink’s preliminary results using tobacco plants as a model system confirmed that CVEV can be encapsidated by CYVaV. Subsequently, analysis was initiated to identify the genetic sequences within the CYVaV genome that facilitates CVEV encapsidation. A series of CYVaV mutants were designed and now require further investigation to pinpoint
the exact sequence involved in the interaction with CVEV. Once identified, this sequence could be manipulated in the CYVaV-vector to prevent CVEV encapsidation.

5. Field trials for effects in commercial citrus and transmission — Georgios Vidalakis, Ph.D., UCR

The first efforts toward this objective involved inoculation of citrus protoplasts\(^1\) with CYVaV-vector to see whether it can replicate within citrus cells. The Riverside team tested a few wild-type and recombinant CYVaV-vector constructs (from the Simon lab) in ‘Daisy’ and ‘Tango’ protoplast cell lines. The preliminary results suggested that recombinant CYVaV-vector constructs can replicate in citrus cells.

A field trial including replicated and non-replicated trees of several commercial citrus rootstock/scion (R/S) combinations was established at the Agricultural Operations, UCR, in October 2020. Trees were graft-inoculated with CYVaV and compared with non-inoculated controls. The Riverside team studied CYVaV mobility and symptom onset at six months and one year after graft-inoculation. At the one-year time-point, for some R/S combinations, laboratory tests indicated a uniform progression of CYVaV infection (e.g., Table 1, IDs 2 and 5). In other cases, results indicated that CYVaV was not evenly distributed in the tree, as the trees tested positive at six months post-graft inoculation (pgi), but tested negative at 12 months pgi (e.g., Table 1, IDs 6 and 9), indicating that further experiments and analyses are required. So far, of the
varieties tested (Table 1), two lemon varieties – Limoneira 8A Lisbon and Perrine Lemon-Lime hybrid – and Tango Mandarin, have expressed yellow vein foliar symptoms (Figure 2).

### Conclusion

This project generated preliminary data that highlighted: (1) original ideas and hypotheses that did not work (i.e., anti-CLas enzybiotics) and (2) areas that require further work (i.e., CYVaV interactions with other citrus viruses, use of CYVaV to silence ACP genome targets and CYVaV-vector field performance). In addition, it generated data that were successfully used to (1) leverage federal funds for further research and (2) set up field trials with commercial citrus varieties to study tree and fruit effects and CYVaV natural transmissibility risks to produce data required for regulatory approval of CYVaV-based technologies. Finally, members of the research team founded a start-up company, Silvec Biologics Inc., to further research and develop CYVaV-based products and work towards the regulatory approval and commercialization of this technology.

CRB Research Project # 5300-207

### Glossary

1. **Virus expression vector**: A lab-generated circular DNA sequence designed to express a particular gene of interest in a host cell system.

2. **Small RNA**: Short RNA molecules that are commonly involved in host defense or “silencing” mechanisms.

3. **Protoplast**: A living plant cell whose cell wall has been removed.

### References


Anne Simon, Ph.D., is a professor of Cell Biology and Molecular Genetics at the University of Maryland. Daniel Nelson, Ph.D., is a professor of Veterinary Medicine at the University of Maryland. Judith Brown, Ph.D., is a professor of Plant Sciences at the University of Arizona. Kari Debbink, Ph.D., is an associate scientist in the Department of Molecular Microbiology and Immunology at Johns Hopkins University. Rafael Simon, Ph.D., is co-founder and president of Silvec Biologics, Gaithersburg, Maryland. Arunabha Mitra, Ph.D., is a post-doctoral researcher in the Department of Microbiology and Plant Pathology at the University of California, Riverside. Georgios Vidalakis, Ph.D., is a professor and University of California extension specialist in Plant Pathology with the Department of Microbiology and Plant Pathology at the University of California, Riverside and also is the Director of the Citrus Clonal Protection Program. For more information, please contact Georgios.vidalakis@ucr.edu
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IMPROVING TISSUE SAMPLING FOR CONSISTENT DETECTION OF CLAS

Subhas Hajeri, Lucita Kumagai, Sandra Olkowski, Raymond Yokomi and Neil McRoberts

Project Summary

Early detection and prompt response are key factors in both the eradication and suppression of the huanglongbing (HLB) epidemic in California. Due to the lack of visible symptoms, low titer and uneven distribution of ‘Candidatus Liberibacter asiaticus’ (CLas) in an infected tree, selecting the best leaves to sample from a mature tree with more than 200,000 estimated leaves is a major hurdle for early detection by quantitative polymerase chain reaction (qPCR). Phloem-limited plant pathogens move in a source-to-sink fashion along with sugars and nutrients. The purpose of this two-year research project was to evaluate the titer of CLas in source tissues (mature leaves) and various sink tissues (new flush, reproductive tissues and roots) and determine the best tissue to test at the right time of the year for early CLas detection. Optimizing the sampling protocol will increase the accuracy of detection and lead to earlier removal of infected trees, which will help contain the spread of HLB and protect the multi-billion-dollar citrus industry in California. In this study, we found that the peduncle tissue sample was the most reliable tissue type overall for accurately detecting CLas in trees known to be infected. Based on the results of this study, single peduncle samples were more reliable than single or quadrant mature leaves or other sink tissues. Quadrant peduncle samples were 10-20 percent more sensitive than other source tissues (single or quadrant mature leaf samples). No statistically significant relationship was found between season and tissue types for positive or negative qPCR results.
Background

HLB is the most serious disease of citrus (Bové 2006; Gottwald 2010). It is presumptively caused by the bacterium CLas and transmitted by the Asian citrus psyllid (Diaphorina citri Kuwayama) in the United States. qPCR is the current standard regulatory method for CLas detection, and it can detect the DNA from a single copy of bacterium in a sample. However, detection of CLas in early stages of infection in a mature tree is a major challenge due to uneven distribution of the pathogen and the difficulties of sampling large dense trees and scouting for symptomatic foliage. Clearly, sampling for the right tissue is a major hurdle for early detection of CLas by qPCR.

Multiple studies have characterized the distribution of phloem-limited pathogens within citrus. Bar-Joseph et al. (1979) found higher concentrations of CTV in the bark of the peduncle compared to that in the bark of branches of the same age. Similarly, based on our preliminary data from young shoots tested for Spiroplasma citri (causal agent of citrus stubborn disease), the titer of this phloem-limited bacterium is higher in the peduncle of young fruit than either the bark of a young shoot or petiole of a young leaf (Hajeri and Yokomi, unpublished). Moreover, fruit tissues such as columella and receptacle are used routinely for reliable detection of S. citri by the Citrus Pest Detection Program (CPDP) in Tulare, California (Mello et al. 2010). In Florida, Tatineni et al. (2008) and in Texas, Kunta et al. (2014) observed a relatively high titer of CLas in fruit peduncle. Recently, in Texas, the Kunta lab has shown that the CLas titer not only was greater in the roots, but also was uniformly distributed and detected more consistently throughout the year compared to leaf samples (Louzada et al. 2016, Park et al. 2018 and Braswell et al. 2020). Recent greenhouse studies indicate new flush can be used for reliable detection of CLas (McCollum, unpublished). In all the cases, the sink tissue such as the new flush, fruit peduncle and roots had higher titers of phloem-limited citrus pathogens. In addition, Irey et al. (2011), concluded that seasonality of sampling is critical in Florida after he observed that the greatest percentage of HLB positives based on qPCR occurred during July through January and coincided with the period of maximum leaf HLB symptomatology.

Despite valuable data available from these previous studies, a large-scale comparative study of CLas in source and sink tissues was missing. The overall goal of this project was to identify the most reliable tissue to sample, matched with its ideal season, for consistent detection of CLas in California. To achieve the goals of the project, we examined the relative titers of CLas from source tissues (old mature leaves) and sink tissues (new flush/young leaves, fruit peduncle/bark tissues and feeder roots) for two years (Figure 1) by qPCR. All the CLas research data were captured from southern California residential trees following California Department of Food and Agriculture (CDFA) regulatory guidelines. Once the tree was confirmed CLas-positive by the CDFA, the tree was removed, and no further testing was conducted. However, to monitor the distribution of phloem-limited bacteria in the same infected tree over several seasons, we analyzed S. citri infected trees (another phloem-limited citrus bacterial pathogen), since positive trees are not removed. There were major differences in these two pathosystems such as the survey for S. citri being conducted in commercial citrus settings of central California while the survey for CLas was done in residential citrus settings in southern California.

Stubborn

About 50 mature trees (from Kern and Tulare counties) that previously tested positive for S. citri were included in this study to monitor the relative bacterial titer in four tissues and evaluate the effect of seasonality on titer. Data were
Figure 2. A) Titer of *Spiroplasma citri* in four types of tissues (young and mature old leaves, fruit peduncle/bark and feeder roots) in fall, winter, spring and summer. Cycle threshold (CT) values are on the X-axis. A lower Ct value is an indicator of higher titer. B) Reliability of detecting *S. citri* in each season. Four tissues (petioles of mature old and young leaves, peduncle of fruits and feeder roots) were compared for reliability of detecting *S. citri* in four seasons. Reliability of detecting *S. citri* in specific tissues was calculated based on percent positive trees identified by a specific tissue type. Data were collected over a one-year period.

Figure 3. Distribution of Ct values for detection of ‘Candidatus Liberibacter asiaticus’ (CLas) in all CLas-positive qPCR assays in this study. Note: *n*=2,116

Huanglongbing

Regarding CLas testing, 765 samples were taken from 408 residential citrus trees (*Table 1*), carried out between November 7, 2019, and October 26, 2021, in six counties in southern California, from within and outside HLB quarantine zones. We collected and tested the four tissue types (*Figure 1*) from each targeted tree every season for two years. The CDFA field staff collected the samples, and the CDFA’s Plant Pest Diagnostics Center (PPDC) in Sacramento prepared and freeze-dried the tissues for the CPDP Lab. DNA was extracted from the freeze-dried samples and qPCR was performed (Zheng et al. 2016) to test for the presence of CLas. qPCR Ct values were recorded and used for comparative analyses between different tissue types. A majority of the samples (approximately 82 percent) in the study (*Table 2*) were taken from trees that previously tested CLas-positive by the CDFA (samples for this study were collected just prior to tree removal and destruction). Some samples (about 18 percent) were collected from residential trees that presented a higher risk of exposure to CLas (such as CLas-positive ACP sites, inconclusive ACP sites, inconclusive trees with qPCR Ct of more than 37, trees adjacent to HLB-infected trees and dog-alerted trees) (*Table 2*).

During the two-year research period, 346 trees were tested only once, while the remaining trees were tested repeatedly, up to ten different times due to negative test results. The possible correlation between...
Table 1. Summary of qPCR results for all tissue samples collected from November 2019 – October 2021

<table>
<thead>
<tr>
<th>TISSUE SAMPLES</th>
<th>NUMBER</th>
<th>% POSITIVE (CLAS+/TOTAL)</th>
<th>MEAN CT VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDFA original sample*</td>
<td>765</td>
<td>45.2% (346/765)</td>
<td>26.5</td>
</tr>
<tr>
<td>CDFA quadrant leaf*</td>
<td>642</td>
<td>25.2% (162/642)</td>
<td>25.7</td>
</tr>
<tr>
<td>(Inconsistently collected throughout study period)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDFA mature leaf*</td>
<td>765</td>
<td>28.3% (217/765)</td>
<td>25.6</td>
</tr>
<tr>
<td>CPDP mature leaf</td>
<td>765</td>
<td>29.7% (227/765)</td>
<td>24.9</td>
</tr>
<tr>
<td>Young leaf</td>
<td>765</td>
<td>30.7% (235/765)</td>
<td>25.2</td>
</tr>
<tr>
<td>Single peduncle</td>
<td>765</td>
<td>33.7% (258/765)</td>
<td>24.8</td>
</tr>
<tr>
<td>Quadrant peduncle</td>
<td>314</td>
<td>54.8% (172/314)</td>
<td>26.0</td>
</tr>
<tr>
<td>(Consistently collected from 4/2021-10/2021)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Root</td>
<td>765</td>
<td>34.5% (264/765)</td>
<td>26.7</td>
</tr>
</tbody>
</table>

*Not part of this research project but collected and tested by the CDFA in parallel.

CDFA original sample: Mature leaf samples collected during regular survey by the CDFA.
CDFA quadrant leaf: Quadrant mature leaf samples collected by the CDFA in parallel to research samples.
CDFA mature leaf: Single samples (20 mature leaves) per tree.
CPDP mature leaf: Same as CDFA mature leaf but split between Sacramento and Tulare labs.
Single peduncle: Peduncle of fruits collected by the CDFA and tested in the Tulare lab.
Quadrant peduncle: In year two of the project, peduncle of fruits collected by the CDFA and tested in the Tulare lab.
Roots: Feeder roots collected by the CDFA and tested in the Tulare lab.

Table 2. Types of trees or exposure-risk categories tested during the study.

<table>
<thead>
<tr>
<th>DESCRIPTION</th>
<th># TREES</th>
<th># TREES WITH POSITIVE STUDY TISSUE</th>
<th># SAMPLING EVENTS</th>
<th># SAMPLING</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known Positive</td>
<td>336</td>
<td>303</td>
<td>337</td>
<td>304</td>
</tr>
<tr>
<td>ACP+ site</td>
<td>27</td>
<td>11</td>
<td>152</td>
<td>15</td>
</tr>
<tr>
<td>Inconclusive (ACP)</td>
<td>4</td>
<td>2</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>Inconclusive (Plant)</td>
<td>27</td>
<td>10</td>
<td>176</td>
<td>12</td>
</tr>
<tr>
<td>K9 Alert</td>
<td>10</td>
<td>2</td>
<td>56</td>
<td>3</td>
</tr>
<tr>
<td>K9 Survey</td>
<td>2</td>
<td>1</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Misc. Category</td>
<td>2</td>
<td>0</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>408</td>
<td>329</td>
<td>765</td>
<td>339</td>
</tr>
</tbody>
</table>

Known Positive: Tree confirmed CLas-positive by the CDFA during regular survey.
ACP+ site: ACP sample from a residential property tested positive for CLas.
Inconclusive (ACP): ACP sample from a residential property tested inconclusive for CLas.
Inconclusive (Plant): Plant sample from a residential property tested inconclusive for CLas.
K9 Alert: Residential tree alerted by HLB detection dog (F1 K9).
K9 Survey: Trees part of K9 survey but not alerted by HLB detection dog.
Figure 4. Distribution of CLas-positive (CLas+) qPCR results by sample tissue type. CDFA mature leaf and CPDP mature leaf are the same mature leaf petiole tissue shared by the Sacramento and Tulare labs respectively. Simultaneously, petiole of young leaf, peduncle of fruit and feeder roots are other tissue types sampled by CDFA staff and tested by the CPDP in Tulare. Note: n=765 sampling events from 408 unique trees (Nov 2019 to Oct 2021).

Figure 5. Distribution of CLas-positive (CLas+) qPCR results by sample tissue type, including peduncle quadrant samples. CDFA mature leaf and CPDP mature leaf are the same mature leaf petiole tissue shared by the Sacramento and Tulare labs respectively. Simultaneously, petiole of young leaf, peduncle of fruit and feeder roots are other tissue types sampled by CDFA staff and tested by the CPDP in Tulare. Similarity, quadrant peduncle samples (ped_quad) per tree collected at the same time by CDFA staff and tested in Tulare lab. Note: n=314 sampling events from 212 unique trees (Apr to Oct 2021).

To evaluate how reliably each tissue sample type identified infected trees, we used the known-positive trees as a reference standard. Reliability is expressed here as the sensitivity (Se) of the tissue type, which is the proportion of qPCR-positive samples out of the total known-positives. The results of this calculation are presented in Table 3. These data shows how erratic the CLas distribution is within an infected citrus tree. Overall sensitivity of a mature leaf is only about 65 percent (based on this study's testing of known-positive trees previously identified as positive through the CDFA's regulatory protocol). Relative to mature-leaf source tissue, both peduncle (single and quadrant) and root sample tissues had an observed higher probability of detecting a known-infected tree.
Using only mature-leaf source tissue could potentially result in false negative sampling (i.e., negative results from a CLas-positive tree) of between 9.8 - 22.8 percent (Table 3). Considering peduncle tissue relative to mature leaf source tissue – a single peduncle sample could potentially increase detection sensitivity of sampling by about 2-10 percent, and then there potentially could be an additional gain of up to 22 percent on top of that by using a quadrant peduncle sample (Table 3). Although single peduncle tissue had a relatively high sensitivity, quadrant peduncle samples had the highest detection sensitivity overall and also in every season in which they were collected, which suggests that this tissue type may be more reliable and more consistent in the seasons this was tested.

Peduncle and root sample tissues had consistently higher numbers of CLas-positives across each season (Table 3). The single peduncle and root tissue samples did switch places in different seasons (e.g., root was higher in summer, peduncle was higher in fall), so we used regression modeling to determine if these observations could be generalized to populations outside the study. However, there were no statistically significant relationships between season and tissue type.

### Summary of Major Findings

1) **What is the most reliable sink tissue for consistent detection of pathogen?**

In this study detecting CLas in trees known to be infected, peduncle samples were the most reliable tissue type overall (Table 1). Single peduncle samples were more reliable than single or quadrant mature leaves, or other sink tissues (Table 1). Quadrant peduncle samples were 10-20 percent more sensitive than source tissue (single or quadrant mature leaf samples) (Table 3). In comparison specifically to root tissue, peduncle tissue had higher detection reliability (i.e., sensitivity) overall and also consistently higher reliability in each season tested (Table 3).

2) **What is the effect of seasonality on the pathogen titer?**

Lower CLas titers were detected in roots than in other tissues, especially in the summer; but importantly, this did not result in lower CLas-positive detection by qPCR for root tissue (Table 3). There was no statistically significant relationship between season and tissue type for a positive/negative detection by qPCR.

The next course of action is to share the research data with United States Department of Agriculture-Animal and Plant Health Inspection Service-Plant Protection and Quarantine.
to incorporate the fruit peduncle and bark tissues as part of a regulatory sampling protocol.

CRB Research Project #5300-204

References


Subhas Hajeri, Ph.D., is a program director and plant pathologist at the Citrus Pest Detection Program in Tulare, California. Lucita Kumagai is a senior plant pathologist at the California Department of Food and Agriculture in Sacramento. Sandra Olkowski, Ph.D., was acting DATOC coordinator and is currently a research data analyst in the Quantitative Biology and Epidemiology Group at the University of California, Davis (UC Davis). Raymond Yokomi, Ph.D., is a research plant pathologist at the U.S. Department of Agriculture-Agricultural Research Service, San Joaquin Valley Agricultural Sciences Center, Parlier, California. Neil McRoberts, Ph.D., is an associate professor at UC Davis and director of the Western Plant Diagnostic Network. For additional information, contact shajeri@cc tea.org

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SURVEYS OF
CITRUS TRISTEZA VIRUS
IN CALIFORNIA CITRUS ORCHARDS

Raymond Yokomi and Subhas Hajeri

Project Summary

California citrus orchards were surveyed to estimate the incidence and strains¹ of Citrus tristeza virus (CTV) present. The objective was to document the presence of CTV in commercial orchards and determine what CTV strains would be most appropriate to develop as a future CTV viral vector (CTVvv) to help mitigate huanglongbing (HLB) in California. Surveys conducted between 2020-22 in portions of Santa Barbara, Ventura, Riverside, San Bernardino and San Diego counties estimated county-wide CTV incidence ranged from 3.6 to 34.9 percent. CTV was not detected in the Madera, Imperial, San Luis Obispo or Monterey county orchards surveyed. More than 95 percent of CTV detected in these surveys were the T30 strain alone and in mixtures with other strains including VT, RB and S1 and, rarely, T36. In addition, 3.1 percent of the CTV detected did not fall into any genotype categories. CTV in Tulare, Kern and Fresno counties previously was estimated to range from 0.2 to 1.3 percent with an overall average of 0.7 percent in surveys conducted by the Central California Tristeza Eradication Agency (CCTEA) from 2010-13. These data indicate T30 is the most common CTV strain in the state and would be the most appropriate strain to develop as a CTVvv for California citrus. Since T36 was rarely detected in California, it may be another strain to consider as a CTVvv to inoculate existing trees to reduce the chances of same strain cross protection.
Background

Citrus tristeza virus is a graft-transmissible virus and occurs in nearly all citrus-growing regions in the world. CTV can cause tristeza quick decline of citrus trees grown on sour orange rootstock and virulent strains induce debilitating stem pitting in the scion regardless of rootstock (Moreno et al. 2008). Most CTV strains in California cause mild, asymptomatic infections in commercial citrus cultivars grown on rootstocks other than sour orange (Yokomi and DeBorde 2005).

Florida CTV strains of T36 and T30 have been developed as a virus vector (CTVvv), which was modified in the lab to carry small, foreign genetic elements that are activated during viral replication in plants to produce antimicrobial peptides (AMPs) or RNA interference (RNAi) genetic sequences that can be directed against *Candidatus Liberibacter asiaticus* (CLas) and the Asian citrus psyllid (ACP) (Dawson et al. 2015) within phloem tissue. Florida now has field trials under an Environmental Protection Agency (EPA) Experimental Use Permit (EUP) to evaluate CTVvv clone expressing spinach defensins for biocontrol control as a microbial biopesticide to control HLB.

Although more than 4,000 CLas-infected trees have been detected and removed from residential properties across four southern California counties, HLB has not yet been detected in any commercial citrus orchard in California. However, ACP populations can be found in commercial citrus orchards and are spreading to other parts of the state. Eradication of CLas-infected trees and frequent orchard spraying to control ACP are unsustainable as long-term control strategies. The CTVvv system offers an efficient citrus production and delivery system for AMPs and RNAi to help control HLB. To achieve this, however, federal and state regulatory approvals are required since the U.S. Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) considered CTVvv as a genetically engineered virus that is a microbial biopesticide and, therefore, regulates its use. To this end, this study was conducted to estimate the incidence in commercial groves of CTV strains in the major citrus-growing counties in California not previously surveyed. This data could be incorporated in an Environmental Assessment of CTV, which will be required for regulatory approval for future field release of CTVvv in California. In addition, this study will inform selection efforts of the best CTV strains to develop as a CTVvv for use in California citrus to mitigate HLB.
Survey Acreage

The commercial citrus acreage in California is 268,137 acres, and Figure 1 shows the acreage per county (CDFA CASS 2022). The major citrus growing areas in the state can be divided into regions that include the San Joaquin Valley (SJV), coastal-intermediate, interior and desert regions (Figure 2).

These climatic regions influence the seasonal growth cycles of citrus and the abundance of aphid populations that result in CTV spread by aphid vectors (Yokomi et al. 2010). The focus of this research was to survey the major citrus counties in California, excluding Tulare, Kern and Fresno counties, because 2010-13 CTV data from the Central California Tristeza Eradication Agency (CCTEA) using hierarchical sub-sampling method (HS) and ELISA (Barnier et al. 2010) were available for this report. To obtain an estimate of the CTV incidence in the remaining major citrus-growing counties, new surveys were conducted from 2020-22 using the same sampling protocols. These surveys included commercial orchards in Ventura, Riverside, San Diego, Madera, Imperial, San Bernardino, San Luis Obispo, Santa Barbara and Monterey counties. These counties have a combined citrus production area of 62,572 acres and constitute 28.5 percent of the state’s citrus plantings. Combining this data with that previously collected from 233,926 acres of citrus in Tulare, Kern and Fresno counties, our report covers 96.7 percent of citrus grown in California.

CTV Testing Methodology

The HS method samples 25 percent of the citrus trees per orchard (Gottwald and Hughes 2000). Briefly, every fourth group of four trees was sampled by collecting three leaves from each of the four trees and combining the 12 leaves into one sample. The leaf petiole tissue samples were placed in a small envelope and double-bagged in Ziploc® bags filled with desiccant for transport to the laboratory. CTV was detected using ELISA4 with universal CTV polyclonal antibodies and MCA132 monoclonal antibodies. CTV-positive samples were further tested to identify viral strains using real-time polymerase chain reaction (PCR) with CTV strain-specific primers and probes (Yokomi et al. 2010). The number of HS samples collected per orchard depended on orchard size. A minimum orchard size was 20 acres when possible. It often was not practical to sample over the entire orchard.

CTV Incidence in the 2020-22 Survey

In the 2020-22 survey which excludes Fresno, Tulare and Kern counties, 3,415 HS samples were collected from 64 orchards, 13,656 trees over 1,539 acres (Table 1). Samples were collected from individual orchards scattered across each county. There were 1,010 HS samples positive for CTV for an estimated CTV incidence from the nine counties of 8.4 percent. The estimated CTV incidence was highest in San Diego and San Bernardino counties at 34.9 and 31.3 percent, respectively (Figure 3A). The CTV estimate from the interior region of Riverside County was 16.1 percent, whereas CTV in the Coachella Valley in the desert region of Riverside and Imperial Counties was zero percent (Table 2). This shows how climate can affect CTV incidence. CTV incidence in Ventura was 6.3 percent. Santa Barbara County had a low level of CTV at 0.9 percent, and no CTV was detected in samples.

<table>
<thead>
<tr>
<th>COUNTY</th>
<th>CITRUS ACREAGE</th>
<th>SAMPLE DATE</th>
<th>NO. ORCHARDS SAMPLED</th>
<th>ACRES SAMPLED</th>
<th>PERCENT AREA SAMPLED</th>
<th>NO. TREES SAMPLED</th>
<th>NO. HS</th>
<th>NO. HS POSITIVE</th>
<th>EST. CTV INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>San Diego</td>
<td>7,435</td>
<td>6/8/2021</td>
<td>8</td>
<td>113</td>
<td>0.8</td>
<td>1,920</td>
<td>480</td>
<td>394</td>
<td>34.9</td>
</tr>
<tr>
<td>San Bernardino</td>
<td>2,747</td>
<td>6/7/2021</td>
<td>3</td>
<td>49</td>
<td>1.5</td>
<td>576</td>
<td>144</td>
<td>112</td>
<td>31.3</td>
</tr>
<tr>
<td>Riverside</td>
<td>12,682</td>
<td>6/8/2021</td>
<td>13</td>
<td>278</td>
<td>1.2</td>
<td>2,940</td>
<td>736</td>
<td>275</td>
<td>11.0</td>
</tr>
<tr>
<td>Ventura</td>
<td>23,025</td>
<td>10/12/2021</td>
<td>15</td>
<td>360</td>
<td>1.4</td>
<td>3,816</td>
<td>954</td>
<td>217</td>
<td>6.3</td>
</tr>
<tr>
<td>Santa Barbara</td>
<td>1568</td>
<td>6/16/2022</td>
<td>7</td>
<td>36</td>
<td>0.9</td>
<td>1,344</td>
<td>336</td>
<td>12</td>
<td>3.6</td>
</tr>
<tr>
<td>Madera</td>
<td>6,253</td>
<td>8/18/2021</td>
<td>5</td>
<td>368</td>
<td>5.6</td>
<td>960</td>
<td>240</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Imperial</td>
<td>5,984</td>
<td>5/10/2021</td>
<td>5</td>
<td>115</td>
<td>1.7</td>
<td>960</td>
<td>240</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>San Luis Obispo</td>
<td>1,879</td>
<td>3/5/2021</td>
<td>3</td>
<td>39</td>
<td>1.7</td>
<td>384</td>
<td>96</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Monterey</td>
<td>989</td>
<td>11/13/2020</td>
<td>5</td>
<td>181</td>
<td>13.6</td>
<td>756</td>
<td>189</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Other counties</td>
<td>2,449</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Totals</td>
<td>65,011</td>
<td>64</td>
<td>1,539</td>
<td>13,656</td>
<td>3.415</td>
<td>1,010</td>
<td>8.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HS: Hierarchical Subsampling
from Madera, San Luis Obispo and Monterey counties. HS samples with MCA13 reactivity were rarely encountered, possibly because titer of MCA13-reactive strains in the HS samples were diluted by co-infection of T30. Therefore, MCA13 data were not included in this report.

Among the cultivars sampled in the 2020-22 survey, oranges had the highest infection level at 34.6 percent (Table 3). Infections in grapefruit, mandarins and lemon were 5.9, 2.4 and 0.1 percent, respectively. No infection was detected in Minneola or sweet lime, probably due to small sample size. These infection rates are the result of the relative CTV titer in host tissue, vector abundance and varietal susceptibility to vector transmission of CTV.

CTV Incidence in Central California

CCTEA surveys conducted from 2010 to 2013 in Fresno, Tulare and Kern counties found overall CTV incidence at 0.2, 1.3 and 0.4 percent, respectively (Figure 3B). The overall average estimated CTV incidence in these three SJV counties was 0.7 percent (Table 4). The large number of samples was taken and processed during a four-year period and included 48, 56 and 100 percent of the citrus acreage in Tulare, Fresno and Kern counties, respectively.

CTV Strains

In the 2020-22 survey, T30 was the predominant strain detected at 61.6 percent alone or 95.2 percent in mixtures with other strains (Figure 4A). The RB and S1 strains were present in mixtures with other strains in 24 percent of the CTV detected. Although the RB and S1 strains characterized in California are mild, some CTV isolates in these

Table 2. Estimated incidence of citrus tristeza virus (CTV) in different areas of Riverside County.

<table>
<thead>
<tr>
<th>REGION</th>
<th>RIVERSIDE CO.</th>
<th>ACRES</th>
<th>NO. ORCHARDS</th>
<th>HS SAMPLES</th>
<th>PCA</th>
<th>EST. PERCENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desert</td>
<td>Coachella</td>
<td>134.5</td>
<td>4</td>
<td>192</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Interior</td>
<td>Arlington Heights</td>
<td>46.4</td>
<td>4</td>
<td>256</td>
<td>194</td>
<td>30.0</td>
</tr>
<tr>
<td>Interior</td>
<td>Temecula</td>
<td>25</td>
<td>2</td>
<td>96</td>
<td>37</td>
<td>11.5</td>
</tr>
<tr>
<td>Interior</td>
<td>Hemet</td>
<td>72</td>
<td>3</td>
<td>192</td>
<td>44</td>
<td>6.3</td>
</tr>
<tr>
<td>Interior totals</td>
<td></td>
<td>143.4</td>
<td>9</td>
<td>544</td>
<td>275</td>
<td>16.1</td>
</tr>
<tr>
<td>County totals</td>
<td></td>
<td>277.9</td>
<td>13</td>
<td>736</td>
<td>275</td>
<td>11.0</td>
</tr>
</tbody>
</table>

HS: Hierarchical Subsampling
PCA: Polyclonal antisera for CTV detection

Figure 3. Estimated citrus tristeza virus (CTV) incidence in the major citrus-growing counties in California. A. In a 2020-22 survey, no CTV was detected in samples from Madera, Imperial, San Luis Obispo or Monterey counties. B. 2010-13 survey of Tulare, Kern and Fresno counties conducted by the Central California Tristeza Eradication Agency.
Table 3. Estimated citrus tristeza virus (CTV) incidence per cultivar sampled in the 2020-22 survey from Ventura, Riverside, San Diego and Santa Barbara counties.

<table>
<thead>
<tr>
<th>CULTIVAR</th>
<th>ACRES SAMPLED</th>
<th>NO. HS SAMPLES</th>
<th>NO. HS WITH CTV</th>
<th>ESTIMATED CTV INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange</td>
<td>348</td>
<td>1,052</td>
<td>859</td>
<td>34.6</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>175</td>
<td>560</td>
<td>120</td>
<td>5.9</td>
</tr>
<tr>
<td>Mandarin</td>
<td>250</td>
<td>256</td>
<td>24</td>
<td>2.4</td>
</tr>
<tr>
<td>Lemon</td>
<td>571</td>
<td>1,451</td>
<td>7</td>
<td>0.1</td>
</tr>
<tr>
<td>Minneola</td>
<td>30</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sweet lime</td>
<td>70</td>
<td>48</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Overall totals</td>
<td>1,444</td>
<td>3,415</td>
<td>1,010</td>
<td>8.4</td>
</tr>
</tbody>
</table>

HS: Hierarchical Subsampling

Table 4. Estimated incidence of citrus tristeza virus (CTV) in Tulare, Kern and Fresno counties from surveys conducted by the Central California Tristeza Eradication Agency from 2010-13.

<table>
<thead>
<tr>
<th>COUNTY</th>
<th>CITRUS ACREAGE</th>
<th>SAMPLE DATE</th>
<th>NO. ORCHARDS sampled</th>
<th>ACRES SAMPLED</th>
<th>PERCENT AREA SAMPLED</th>
<th>NO. TREES sampled</th>
<th>NO. HS</th>
<th>NO. HS POSITIVE</th>
<th>EST. CTV INCIDENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tulare</td>
<td>114,948</td>
<td>2010-2013</td>
<td>7,775</td>
<td>28,737</td>
<td>48</td>
<td>1,597,080</td>
<td>399,270</td>
<td>20,553</td>
<td>1.3</td>
</tr>
<tr>
<td>Kern</td>
<td>53,904</td>
<td>2010-2013</td>
<td>7,896</td>
<td>13,476</td>
<td>100</td>
<td>2,364,460</td>
<td>591,115</td>
<td>9,441</td>
<td>0.4</td>
</tr>
<tr>
<td>Fresno</td>
<td>35,074</td>
<td>2010-2013</td>
<td>2,990</td>
<td>8,768</td>
<td>56</td>
<td>757,076</td>
<td>189,269</td>
<td>1,834</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>203,926</td>
<td></td>
<td>18,789</td>
<td>50,981</td>
<td></td>
<td>4,718,616</td>
<td>1,179,654</td>
<td>31,828</td>
<td>0.7</td>
</tr>
</tbody>
</table>

HS: Hierarchical Subsampling

Conclusions

CTV incidence in California was highest in the interior region, which includes San Bernadino and western Riverside counties and coastal-intermediate regions of San Diego and Ventura counties. This reflects the long history of CTV in southern California. No CTV was detected in the desert regions of Coachella and Imperial Valley, presumably due to the high summer temperatures detrimental to CTV, even though samples were taken from new shoots in May when temperatures were mild. CTV incidence was low in central California and can be attributed to many factors. It is geographically separated from southern California by the Tehachapi Mountains. Also, CTV is regulated by management programs such as suppression of MCA13-reactive CTV by the CCTEA (Barnier et al. 2010) and regulatory programs such as planting pathogen-free trees through the National Clean Plant Network and the Citrus Clonal Protection Program.

T30 was found to be the predominant genotype of CTV in all surveys in this report. Yokomi and DeBorde (2005) have shown that CTV T30 genotypes are efficiently transmitted by Aphis gossypii (the melon or cotton aphid) which is present in California. The VT, S1 and RB genotype strains were detected as CTV mixtures in samples tested. Over time, citrus trees become co-infected by multiple strains. Cross-protection only prevents co-infection of the same genotype strain and does not cross protect infection by other genotype strains (Folimonova 2013). These data show T30 is common and widespread in citrus in California. Therefore, if the industry deploys CTVvv in new propagations by California citrus nurseries, a mild T30 strain should be used. To protect existing trees, T36 should be considered as it is rare and would not be excluded by cross protection. Since CTV was not detected in the citrus orchards in desert region, another consideration would be a heat stable CTVvv.

CRB Research Project #5300-210
Glossary

1. **CTV strains**: Genetic variants or subtypes of CTV based on genomic analysis in which each group has at least seven percent difference at the nucleotide level.

2. **Antimicrobial peptides (AMPs)**: Small molecules composed of 5 to 100 amino acids, produced by living organisms that can kill microbes such as bacteria, viruses or fungi.

3. **RNA Interference (RNAi)**: A biological process by which RNA molecules inhibit gene expression by activating native antiviral responses that “cut up” and destroy matching RNA.

4. **ELISA**: Enzyme-linked immunosorbent assay, an immunological assay commonly used to measure antibodies, antigens and proteins in biological samples.

5. **MCA13**: A monoclonal antibody that reacts to many strains of CTV, especially those known to induce severe stem pitting in citrus.

References


Raymond Yokomi, Ph.D., is a research plant pathologist at the USDA-ARS San Joaquin Valley Agricultural Sciences Center and Subhas Hajeri, Ph.D., is program director/plant pathologist at the Citrus Pest Detection Program, Central California Tristeza Eradication Agency, Tulare, California. For more information, contact ray.yokomi@usda.gov
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