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On the Cover:
The Citrus Research Board (CRB) is celebrating its 50th anniversary. Since 1968, the CRB has served to ensure a sustainable California citrus industry through its dedicated focus to sound, yet innovative research. We invite you to join us as we toast our first half-century and look forward to the next 50 years of our successful partnership.

Cover designed by CRB communications staff member Tamara Tollison.
IN THIS ISSUE  Fall 2018 | Volume 9 • Number 4  
The Official Publication of The Citrus Research Board

Citrograph’s mission is to inform citrus producers and other industry members of research progress and results that will help ensure the sustainability of California citrus.

9 | A Tribute to Dr. Roger I. Vargas
Karen Ross

12 | Chairman’s View – Fifty Years: A Good Start
Dan Dreyer

14 | From the President’s Desk – 50 Years!
Gary Schulz

16 | Fifty Years of Citrus Research Board History
Carolina Evangelo

18 | Remembering the CRB’s Early Days
Carolina Evangelo

22 | California Citrus Conference to Feature “Best of Best” in Research
Carolina Evangelo

30 | Citrus Research Board Commits to a Strategic Plan
Gary Schulz

32 | Food Safety Reigns as Top Post-harvest Priority
Carolina Evangelo

36 | Economic Impact of California’s Citrus Industry
Bruce Babcock, Ph.D.

40 | Reaching Homeowners During a Critical Time
Mark McBroom

43 | 50 Years as Seen Through Citrograph
Joey Mayorquin, Ph.D., Tamara Tollison and Carolina Evangelo

46 | Results of the National Citrus Research Collaboration Meeting
Melinda Klein, Ph.D.

48 | HLB Research at the UC Davis Contained Research Facility
Kris Godfrey, Ph.D., et al.

52 | Correlating Citrus Tree Health with Microbes
Philippe Rolshausen, Ph.D., et al.

58 | A Microbiota-based Approach to Citrus Tree Health
Xiaochen Yin, Ph.D., et al.

64 | Reducing Breeding Time in Citrus Through Biotechnology
Gloria A. Moore, Ph.D., et al.

70 | The Quest for a Non-vector Psyllid
Michelle Heck, Ph.D., et al.

76 | Update on Disease Forecasting and Management of Septoria Spot of Citrus
James E. Adaskaveg, Ph.D., and Helga Förster, Ph.D.

82 | Purple is the New Orange
Kasturi Dasgupta, Ph.D., et al.

86 | Artificial MicroRNA-based Targeting of the ACP
Yen-Wen Kuo, Ph.D., and Bryce W. Falk, Ph.D.

90 | New Red Navel Selection Interests Growers
Tracy L. Kahn, Ph.D., et al.

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CRB Editorial Correction: Harnessing Hydrogels in the Battle Against Invasive Ants - By: Kelsey Schall

In the Summer 2018 issue of *Citrograph* Vol. 9, No. 3, page 34, the results section and label of Figure 9 incorrectly stated that the difference between high and low hydrogel treatments was in chlorpyrifos concentration. The insecticide contained in the hydrogels was thiamethoxam not chlorpyrifos, and the ultra-low concentration of this toxicant (0.0001%) was the same for hydrogels used in both high and low treatment applications. This version has been corrected to accurately state that the difference between the high and low treatments was in the hydrogel application rate. In the high treatment, 500 grams of hydrogel were applied to each tree, and in the low treatment, 250 grams of hydrogel were applied to each tree.

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Figure 9. Average Argentine ant activity over the two-month sampling period as determined from visual (A) and monitoring vial (B) estimations. Comparisons can be made across the three treatments: high hydrogel application rate (500 grams per tree), low hydrogel application rate (250 grams per tree) and an untreated control. Background colors represent timing of various treatments and sampling periods: pre-treatment monitoring (blue), first treatment (applied on day 0; green) and second treatment (applied on day 7; yellow).
The Mission of the Citrus Research Board:
Ensure a sustainable California citrus industry for the
benefit of growers by prioritizing, investing in and
promoting sound science.

Citrus Research Board Member List
By District 2017-2018 (Terms Expire September 30)

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<th>District 1 – Northern California</th>
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<tr>
<td><strong>Member</strong></td>
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<tr>
<td>Justin Brown, Vice Chairman</td>
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<td>Greg Galloway</td>
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<td>Etienne Rabe</td>
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<td>John Konda, Secretary-Treasurer</td>
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<td>Keith Watkins</td>
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<td>Jeff Steen</td>
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<td>Justin Golding</td>
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<td>Andrew Brown</td>
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<th>District 2 – Southern California – Coastal</th>
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<tr>
<td><strong>Member</strong></td>
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<tr>
<td>Alan Washburn</td>
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<td>John Gless III</td>
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<th>District 3 – California Desert</th>
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<td><strong>Member</strong></td>
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<td><strong>Member</strong></td>
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## 2018

### October 10
**California Citrus Conference**  
Wyndham Hotel, Visalia, California. See page 22 for details. For more information, contact the CRB at (559) 738-0246 or visit www.citrusresearch.org

### October 25
**California Citrus Quality Council (CCQC) Board Meeting**  
Bakersfield, California. For more information, visit http://ccqc.org

### November 13
**Grape, Nut and Tree Fruit Expo**  
Big Fresno Fairgrounds, Fresno, California. For more information, visit http://agexpo.biz/gntfexpo

### November 14
**Citrus Pest and Disease Prevention Committee (CPDPC) Meeting**  
Ventura, California. For more information, visit www.cdfa.ca.gov/citruscommittee

## 2019

### January 9
**Citrus Pest and Disease Prevention Committee (CPDPC) Meeting**  
Visalia, California. For more information, visit www.cdfa.ca.gov/citruscommittee

### February 12-14
**World Ag Expo**  
International Agri-Center Tulare, California. For more information, visit https://www.worldagexpo.com

### March 10-15
**Joint Conference of the International Organization of Citrus Virologists (IOCV XXI) and 6th International Research Conference on Huanglongbing (IRCHLB VI)**  
Riverside Convention Center, Riverside, California. For more information, visit http://irchlb.org

### March 13
**Citrus Pest and Disease Prevention Committee (CPDPC) Meeting**  
Riverside/San Bernardino, California. For more information, visit www.cdfa.ca.gov/citruscommittee
A Tribute to
Dr. Roger I. Vargas

Roger I. Vargas, Ph.D., research entomologist at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) Daniel K. Inouye US Pacific Basin Agricultural Research Center in Hilo, Hawaii, passed away suddenly on July 10, 2018. He will be missed by everyone who had the pleasure of knowing him.

Vargas was one of the world’s leading experts on Tephritid fruit fly ecology, biological control, integrated pest management and on the development of area-wide programs against these invasive pests of quarantine significance. His scientific impact was considerable, as he authored or co-authored 241 published scientific papers (164 in refereed scientific journals) that collectively have been cited more than 6,300 times.

The dedicated scientist worked closely with the Citrus Research Board (CRB) and was a featured speaker at the CRB’s Post-harvest Conference this past April. Some of his CRB-funded projects included Evaluation of Fipronil in Male Annihilation and Ground Treatments for Control of Mediterranean Fruit Fly Oriental Fruit Fly and Melon Fly; Effects of ACP Cover Sprays against Fruit Flies and Their Natural Enemies; and Evaluation of a Three-lure Detection Trap against Medfly, Oriental Fruit Fly and Melon Fly. His laboratory also had a CRB grant to model the comparative life history, survival and demographics of wild and laboratory lines of Mediterranean, oriental, melon and Malaysian fruit flies and their parasitoids. Vargas was a frequent contributor to Citrograph. His most recent article appeared in the previous (Summer 2018) issue, entitled “New Systems Approaches for Fruit Fly Quarantines.”

Vargas had more than 45 years of research experience. He received a BA in Zoology from the University of California, Riverside, an MS in Biology from San Diego State University and his Ph.D. in Entomology from the University of Hawaii at Manoa. He began work as a research entomologist with the USDA-ARS in 1980. From 1984 to 1990, he served as research leader of the Rearing, Radiation and Genetics Unit in Honolulu. During this time, he researched and developed rearing systems and assisted in the development of improved diets for mass-rearing facilities in Hawaii, Mexico and Guatemala. With Toshiyuki Nishida, Ph.D., a University of Hawaii professor, Vargas published the first abundance, distribution and host preference information (i.e., solanaceous fruits) for the Malaysian fruit fly, discovered in Oahu, Hawaii, in 1983. Vargas also modeled comparative life history, survival and demographics of wild and laboratory lines of Mediterranean, oriental, melon and Malaysian fruit flies and their parasitoids.

A major accomplishment for Vargas was his coordination of a national program “Area-Wide Integrated Pest Management (AW-IPM) of Fruit Flies in Hawaiian Fruits and Vegetables.” Through this program, area-wide control of fruit flies in Hawaii was addressed after more than 100 years of infestation. Vargas’ personal technology transfer efforts for GF-120 Naturalyte Fruit Fly Bait received an “all crops” and organic label in 2006, Amulet C-L was registered in 2007, STATIC™ Spinosad-ME (aka SPLAT-MATTM Spinosad-ME) was registered in 2008/2012 and Warrior II (lambdacyhalothrin) soil drench in 2015 for fruit fly control with the U.S. Environmental Protection Agency.

Vargas frequently was called on as a tephritid expert by agencies including the Animal Plant Health Inspection Service, International Atomic Energy Agency, Secretariat of the Pacific Community, Foreign Agricultural Service and various foreign government agencies for consulting and technical assignments in Australia, Mexico, Central America, Japan, Pakistan, Philippine Islands, Fiji, Mauritius, French Polynesia, Jordan, Israel, Australia, Thailand, Seychelles Islands, PR, Taiwan, Korea and Senegal. From 2010-11, he served as president of the Pacific Branch of the Entomological Society of America and organized the most highly attended Pacific Branch meeting in history. Earlier this year, Vargas was presented with the C. W. Woodworth Award by the Pacific Branch of the Entomological Society of America in recognition of outstanding recent contributions as an economic entomologist.

He will be deeply missed by his ‘ohana in his Research Unit and Center, as well as by his many colleagues and friends nationally and around the world; not only for his contributions as a research scientist but also for his generosity, resilience, wry sense of humor, intelligence and unfailing humanity. Vargas was devoted to his family and is survived by his wife Kathy and his daughters Noelani and Kela. The CRB Board and staff are among those who will greatly miss Roger Vargas, and they join the USDA-ARS, the California citrus industry, the research community and many, many others in extending deepest condolences to the Vargas family.

This tribute included contributions from both the U.S. Department of Agriculture-Agricultural Research Service and the Citrus Research Board.
50 Years of CRB: A Celebration of Science-based Solutions

Karen Ross

So much has changed in the world and in the world of agriculture, over the past 50 years. And so much of that change has been because of science, our growing knowledge base, and our ability to increase our understanding and to apply what we learn about nature.

When you get right down to it, that's the basis of the Citrus Research Board (CRB): science. Learn what you can, use what you know, and share that knowledge for the betterment of this industry, this collection of growers.

As is true with so many of the boards and commissions that are part of the California Department of Food and Agriculture's Marketing Division, the Citrus Research Board is successful because the growers themselves brought the organization into being; they vote for it, they nominate each other as representatives, they identify research needs and gaps, they direct funding decisions, and they often agonize together over the decisions that must be made – decisions that can affect how the citrus industry weatheres such storms as huanglongbing and the Asian citrus psyllid, or tristeza, or exotic fruit flies, diseases and other complex threats.

These are the subjects that seem to dominate the headlines for CRB members, as they should. Certain issues call for such urgency and primacy, especially in the nation's number-one citrus state. But this organization also does the important work behind the scenes of funding foundational research on citrus varieties, integrated pest management, maturity and harvest standards, and other such projects that may not individually call attention, but together they provide a constant basis for stability in the marketplace.

Having served now for nearly eight years as California's Secretary of Agriculture, I have perhaps a unique perspective on how our state's citrus growers have first foreseen, then planned for, then responded to and now maintained vigilance over the threat of huanglongbing. It has been nothing short of a major feature and focus of my tenure. I'm proud of the focused, sustained, science-based approach you have taken to this issue, both as a board and as an industry.

Congratulations to the Citrus Research Board – its current and former members and leaders, and the growers behind them who fund, support and direct its work. Fifty years in pursuit of science-based solutions is an impressive milestone; and as I look ahead, I see an organization that is prepared for what's next.

Karen Ross is the Secretary of the California Department of Food and Agriculture.
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In the early days of the CRB, members used an adding machine during the Board meeting as they developed the year’s budget.

Fifty Years: A Good Start

Dan Dreyer
It is with the deepest gratitude and humility that I serve the California citrus growers as chairman of the Citrus Research Board (CRB) during this very special golden anniversary year.

**Gratitude and Pride in the Past**

The gratitude comes from representing what I feel in my heart is the best citrus industry in the world. This was not by happenstance. Through many, many years of labor, love, dedication, research and plain hard work, California growers have created the world’s best eating oranges; category-leading, delicious mandarins, which represent the fastest growing segment of our business; and incredible lemons and grapefruit.

During the past five decades, the citrus industry has seen many challenges and changes. New markets, new varieties and the development of many innovative technologies are among the positive results that have created pride and excitement.

The export market opening to our spectacular products most certainly has been a boon. The introduction of new varieties and citrus items such as the Cara Cara navel, Tango and other mandarin varieties have been real success stories for those who have chosen to participate.

Southeast Asian markets were not open to us 50 years ago. The CRB continues to support our ability to ship fruit abroad through the efforts of the California Citrus Quality Council. Food safety and maximum residue limit issues have become a common part of our vocabulary. Today, over 30 percent of our products are sent west for the rest of the world to enjoy.

**Humility and Hope for the Future**

The humility I feel comes from the knowledge that at this moment in time, we are at the biggest, most dangerous crossroads we growers have ever faced – primarily due to the scourge of huanglongbing (HLB). As we watch the world’s other citrus growing regions struggle to remain viable, as we see their fruit falling from the tree and their groves being pushed, we know we are standing on the precipice. To date, we Californians have held our breath as HLB has gained a toehold only in southern California homeowners’ yards. However, unless we are able to truly do something to head off this killer, our great-tasting, beautiful citrus could become endangered.

HLB isn’t the only serious pest we face. The CRB also is devoting a great amount of attention, time, resources and dollars to California red scale, thrips and the Fuller rose beetle.

Continuing concerns about labor have led the Board to revisit mechanization via hedging, topping and skirting, and harvesting.

Presently, more than 60 percent of our research budget is geared toward pest and disease management projects, with the remainder devoted to development of new varieties and cultural practices that will lead the industry toward continued success.

So where do we go from here? What do the next 50 years hold? Will we be able to control HLB? If not, will the CRB celebrate a centennial?

Many of us will not be here to see what has become of our industry in 2068. However, we always have seen ourselves as the caretakers of our beloved orchards for our children and future generations to come.

So this is where we circle back to the CRB. The Board members and staff of the Citrus Research Board have been toiling ceaselessly to find ways to ensure that each of our businesses remain sustainable. They currently are supervising or directly involved in 60 projects to seek new, better-tasting, more disease-resistant varieties; to manage and/or eliminate the pests that plague our industry; and perhaps most importantly, to find short- and long-term solutions to HLB. In the years to come, we know there will be new challenges – pests, diseases, regulations, water and drought issues, labor and crop protection. Our livelihoods and our futures hang on the work that these fine Board volunteers, staff and researchers are doing, although it is incumbent upon each one of us to remain vigilant.

We deeply appreciate your continued support for the organization and encourage you to consider becoming involved. Please attend one – or more – of our meetings. They’re filled with great information. The knowledge that one gets from participating is invaluable and can only lead to the continued success of the California citrus industry.

In the meantime, on this auspicious occasion of the CRB’s 50th anniversary, let us raise a glass (of juice) to each and every current and former Board representative and staff member, thank them for a job well done and pledge to them our support for the critical work ahead of them. 🍊

*Dan Dreyer is the chairman of the Board of the Citrus Research Board. For more information, contact chairman@citrusresearch.org*
FROM THE PRESIDENT’S DESK

Gary Schulz

Congratulations to all the citrus growers, industry members, staff and others who have made this celebration of 50 years of service by the Citrus Research Board (CRB) possible. Our mission, “To ensure a sustainable California citrus industry for the benefit of growers by prioritizing, investing in and promoting sound science,” is as important today as ever.
It is my privilege to serve as the organization’s president, but I could not do so without our great staff, outstanding Board and growers who support our work.

We remain focused in our efforts to identify the major threats, pests and diseases that stand to jeopardize the future of our vibrant industry, and we are working hard through research to find those solutions. The challenges are HUGE, but I’m confident that we are up to the task.

We enjoy collaborations with many citrus industry organizations such as California Citrus Mutual, Sunkist, the California Citrus Quality Council, the Citrus Pest and Disease Prevention Committee, Citrus Clonal Protection Program, Citrus Research & Development Foundation, U.S. Department of Agriculture (USDA)-Animal and Plant Health Inspection Service, USDA-Agricultural Research Service, Lindcove Research and Extension Center and the many, many universities whose citrus researchers work long and hard getting their projects to a successful goal line.

Ten times, the growers in our industry have supported the continuation referendum required by the marketing order to sustain the work of the CRB. That long-term support means so much to all of us on the Board and staff. We do not take it lightly!

**Goals?**

Goal-setting has been an important part of my life since my early days in the Future Farmers of America. We were taught to set our goals high, work hard to achieve them and never get discouraged by brief setbacks.

I am reminded of the quote from Winston Churchill, “Success is not final, failure is not fatal: it is the courage to continue that counts.” I believe that we will achieve our goals at the Citrus Research Board of uncovering solutions to challenging pests and diseases. Certainly, the Asian citrus psyllid and the disease of huanglongbing are looming large; but with the commitment of our entire industry and all of our industry partners, I am confident we will succeed.

Fifty years from now, we will look back at this time and say, “We did it because we didn’t give up. We didn’t give in. We used the ideas of the best and brightest in our industry, and we found the solution to HLB that kept us profitable and sustainable for years to come.”

Churchill also said, “A pessimist sees the difficulty in every opportunity; an optimist sees the opportunity in every difficulty.” The California citrus industry is an optimistic bunch. Here’s to another 50 years of success!

**Cheers!**

*Gary Schulz is the president of the Citrus Research Board, based in Visalia, California. For more information, contact gary@citrusresearch.org*
TBZ is registered due to the work of CRB-funded researchers Joseph Eckert, Ph.D., and Martin Kolbezien, Ph.D.

The early '80s begins the CRB funding of practical projects in entomology led by Beth Grafton-Cardwell, Ph.D., and Joseph Morse, Ph.D., which to date still provide growers with knowledge of IPA, insecticide alternatives and best practices for a variety of fruit pests.

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The California Department of Food and Agriculture (CDFA) requires that funding be allocated to provide support for the continuance of the registration of sodium cyanide for use in generating HCN.

Lindcove foundation block planted.

TBL is registered due to the work of CRB-funded researchers Joseph Eckert, Ph.D., and Martin Kolbezien, Ph.D.

Funding begins on California red scale biological control. By the late 70's, the use of the citrus red scale pheromone was a result of the early research done by Daniel Moreno, Ph.D.

The biology and control of citrus stubborn disease is heavily researched.

1971 — Irvin Eaks, Ph.D., and Charles W. Coggins, Jr., Ph.D., demonstrate that oilosis with cold and foggy weather will release oil. The results from this work are still used worldwide today.

1974 – Albert G. Salter Memorial Award established.

1974-75 — Citrus Variety Improvement Program becomes the Citrus Clonal Protection Program (CCPP).

1975 – The first work on imazalil begins by Eckert.

1975 – The work of John Menge, Ph.D., on the value of citrus mycorrhizae is funded. Menge wins an award from the California Citrus Nursery Society (CCNS).

1977 – Industry Committee on Citrus Additives and Pesticides, Inc. (ICCAP), which formed in October 1967, becomes the California Citrus Quality Council (CCQC).

1977 – Citrus seed tree registration begins.

1979 — Seedling yellow strain of citrus tristeza virus (CTV) is found in the Citrus Variety Collection (CVC) in the UCR foundation block. All trees must be propagated.

2011 – CRB' s Jerry Dimitman Lab in Riverside, California becomes re-accredited.

CRB ramps up its operation to fight HLB with work in field, lab and the early 1900s.

2011 – SOPP is registered based on CRB-funded research.

CRB celebrates its 25th Anniversary.

2012 – CRB holds the first California Citrus Conference in Porterville.

2012 – Based on CRB-funded research, new California Navel varieties are released.

2013 – CRB funds its first HLB project.

2018 – CRB opens a biocontrol lab in Riverside and the Jan 18, 1995, Board meeting.

CRB holds its first California Citrus Conference in Porterville.

2016 – The CRB office moves to Visalia on April 1, 1994.

Based on CRB-funded research, new California Navel varieties are released.

2009 – Laboratory, named in honor of long-time CRB public member, CRB Award for innovation and leadership.

2009 – CRB celebrates its 25th Anniversary.

2003 – Plant nototus projects under the direction of Mikeal Roos, Ph.D., begin receiving CRB grants.

2004–05 – Henry Schneider, Ph.D., studies the decline in navel oranges and links it to trifoliate mite and bud union crease.

2007-08 – Assembly Bill 1528 calls for research on ethylene dibromide (EDB), which the CRB funds.

2009–10 – The late '90s brings the beginning of a new era of molecular biology and genetic engineering. It also brings the beginning of the multi-faceted McKeller Project, a multi-disciplinary project to identify practices that maximize yield and profitability of citrus production. The McKeller Project was jointly funded by the CRB and the University of California.

The early '80s begins the CRB funding of practical projects in entomology led by Beth Grafton-Cardwell, Ph.D., and Joseph Morse, Ph.D., which to date still provide growers with knowledge of IPA, insecticide alternatives and best practices for a variety of fruit pests.

1982-83 – The California Department of Food and Agriculture (CDFA) requires that funding be allocated to provide support for the continuance of the registration of sodium cyanide for use in generating HCN.

1982-03 – The California Department of Food and Agriculture (CDFA) requires that funding be allocated to provide support for the continuance of the registration of sodium cyanide for use in generating HCN.

1982-03 – The California Department of Food and Agriculture (CDFA) requires that funding be allocated to provide support for the continuance of the registration of sodium cyanide for use in generating HCN.
Citrus Research Board’s first 50 years. This is only the beginning...
Remembering the CRB’s Early Days

Carolina Evangelo

As the Citrus Research Board (CRB) celebrates its 50th anniversary, several past and present board members and other industry veterans were asked to share their memories of the CRB and to discuss how the organization has evolved over the past half-century.

Ed Civerolo, Ph.D.

Ed Civerolo, Ph.D., retired U.S. Department of Agriculture (USDA)- Agricultural Research Service (ARS) national program leader, said that the CRB’s commitment to supporting research on huanglongbing (HLB) and Asian citrus psyllid (ACP) has been one of its most important efforts. He also credited the CRB for its role in securing federal funding to establish a research project at the USDA-ARS San Joaquin Valley Agricultural Sciences Center in Parlier, California, dedicated to citrus tristeza and citrus tristeza virus, which also pose a serious threat to citrus crops. This project has expanded over time to include research on citrus stubborn disease and HLB with CRB support.
James Gorden

“There’s a lot more high-tech science now,” said James Gorden, past CRB chairman and CRB diagnostic laboratory technical advisory committee chair. “We had to re-educate ourselves as we became more heavily engaged in science.”

He recalled the first CRB meeting he attended in the early 1970s.

“I was just kind of a young whippersnapper,” he said, describing those years as “a fond memory,” even if he can’t recall much about the business conducted at those meetings.

“It was an opportunity to interact with leaders in the industry at that time,” said Gorden about those meetings, which were held at the University of California, Riverside (UCR). “In those days, pretty much everything the CRB sponsored was through UCR.”

John Kirkpatrick

“I’ve always been interested in science and all the scientific things connected with agriculture,” said John Kirkpatrick, past CRB chairman and a citrus grower. He explained that he joined the CRB in the early 1980s because “it provided me and those who served with me on the board with insight into the cutting edge of agronomic technology as it relates to the citrus industry. You were right there – and that’s kind of exciting.

“I always enjoyed the drives to Riverside with other members,” he added.

Beth Grafton-Cardwell, Ph.D.

For the past 28 years, Beth Grafton-Cardwell, Ph.D., director of the Lindcove Research and Extension Center in Exeter and a research entomologist with UCR, has responded to invasive pest and disease issues affecting the citrus industry. The CRB has provided most of her funding.

“When I started, growers were using organophosphate insecticide, but some of the pests were developing resistance,” said Grafton-Cardwell. “That led to me screening and getting registration for new insecticides to replace organophosphate insecticide. A lot of the newer insecticides are more human-friendly and better for pest control.”

Initially, Grafton-Cardwell focused on invasive pests such as the glassy-winged sharpshooter and citrus leaf miner. More recently, her attention has turned to the ACP and HLB.

“Not only has the Citrus Research Board supported my research and extension (grower education efforts), they have provided funding for a mobile lab for teaching purposes, for publications and workshops,” she said.

In 2006, Grafton-Cardwell became director of the Lindcove facility. “The Board has been incredibly supportive,” she said. “They have supplied state-of-the-art facilities and equipment for all the research here.” She also noted the CRB-funded lab for fruit quality research, as well as the construction of screenhouses.

“It’s been an absolute pleasure working with the Citrus Research Board, and we have a fantastic relationship,” she added.

Philip LoBue

Philip LoBue, President of LoBue Packing House in Exeter, explained why he has served on the board of the California Citrus Quality Council (CCQC).

“They’ve addressed the issues that have been facing the industry and as the issues changed, their focus has changed,” he said.

LoBue is most interested in export issues. “That’s where I have spent most of my time,” he said. “As a packinghouse manager, those were the issues in front of me.”

Joseph Morse, Ph.D.

Joseph Morse, Ph.D., professor emeritus of entomology at UCR, is currently living in retirement in Thailand.

“I greatly enjoyed every one of my 36 years working with the Citrus Research Board,” he said. “I always felt the CRB gave me just as much, if not more, than I gave them. Truly enjoyable interactions with board members, Pest Control Advisors (PCAs), other researchers and citrus growers taught me a great deal about the practicalities of farming and pest management decisions in the context of exotic pest introductions, increased regulations, more selective and sometimes less effective control materials, and a global marketplace with shifting export markets.”

“The work was always interesting and challenging – different pest populations and dynamics in the various growing regions of California, some differences in citrus varieties, some pest species evolved over time (pesticide resistance, other adaptations), a lot of year-to-year variability due to environmental impacts, and changing pesticide or management practices as new species were introduced,” he continued.
“The CRB is unique in my opinion because it has a singular focus, i.e. promoting research that benefits the industry both in the short- and long-term,” said Morse.

Joel Nelsen

“The bonds between Citrus Mutual and the Citrus Research Board have grown stronger in the past decades as growers recognized they had two tools that could work together to enhance their future and our collective economic environment,” said California Citrus Mutual President Joel Nelsen.

“Research detailing the costs of doing business, our economic impact and production costs are vital components for addressing challenges,” he said. “Our ACP/HLB Advisory Committee is a prime example. The combined focus on grower needs – such as finding a cure, early detection tools and using vital federal research dollars in conjunction with operations to find the bug and the disease before it finds the commercial industry – is a prime example.”

Donald Roark

Past CRB Chairman Donald Roark, who served on the board from 1974 to 2016, explained why he was a board member for so many years.

“I was interested in agricultural research,” he said. “I was fresh out of college and was encouraged by some of the group to get involved because they thought somebody with a newer skill set could understand the research.”

Roark recalled the early days of the CRB, when the structure of the organization was much different.

“When the board was started in the late ’60s, the administration was done as a caretaker for the Navel and Valencia Orange Committee, a federal marketing organization with a full-time administrative staff,” he said. “Sometime in the ’80s or early ’90s, we transitioned and hired a district manager of our own and ran it pretty much in the form it is now.”

The CRB focused on three basic areas: citrus research, the California Citrus Clonal Protection Program and the CCQC, according to Roark.

“In some ways, the basic premise of it hasn’t changed, but the budget has changed and the issues are somewhat different,” said Roark. “The CCQC is now involved in providing technical information for trade issues, such as exports and quarantines. In the research part, one of the larger changes that has taken place is the difference in research. In many cases, it is now basic research. Initially, it was all applied research.”

He explained that “basic research” may include a science such as molecular biology that at some point may lead to a new technique or product. “Applied research” focuses on existing products and techniques to determine their effectiveness.

“I enjoyed working with a lot of interesting and motivated people on research and on the grower level,” said Roark. “I was pleased with the accomplishments that evolved from the activities of the board. We used data to change maturity standards that were in effect for 100 years to standards based on taste. We built two screenhouses where we continue to keep trees available for budwood for distribution to growers, protected from insects. We responded to a couple of forced quarantine restrictions so the industry could continue to supply those markets.

“The research board was involved in moving forward with the EPA registration of a couple of chemicals – one was a fungicide and one was a growth regulator to keep navel oranges from dropping prematurely,” he added. “We actually passed a referendum to increase our assessment to make this happen. The companies that were producing them did not have high enough sales. They were never going to get their money back in their opinion. The two products are both still in use.”

James Stewart

James Stewart, pest control advisor and partner with Ag IPM Consultants in Exeter, described CRB-funded research as having been “very meaningful over time,” even though the focus of the research has changed over the years.

“The advent of the HLB problem has taken most of the money,” he said, recalling how funds needed to be diverted from other CRB projects, such as a picking machine for citrus growers that “would take several photos of a tree and had an arm that could go up and pick the fruit. It could pick certain sizes of fruit and it could work at night.

“But a picking machine won’t do you any good if you don’t have any trees,” he added.

Carolina Evangelo is the director of communications for the Citrus Research Board in Visalia, California, where she also serves as co-publisher and project manager of Citrograph. For additional information, contact carolina@citrusresearch.org
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The Citrus Research Board (CRB) has announced that the 2018 California Citrus Conference will be held at the Wyndham Hotel in Visalia, California, on Wednesday, October 10. The entire citrus industry is invited to participate in this free event, which will once again bring together a who’s who of citrus research, including those on the front lines to combat the Asian citrus psyllid (ACP) and huanglongbing (HLB).

“Once again, we are delighted to showcase the best of the best CRB-funded research at this year’s conference,” said CRB Chairman Dan Dreyer. “We are proud to offer this opportunity for people in the citrus industry to network, learn about critical citrus research developments and hear updates from industry partners.”

Scheduled speakers include:

- **Michelle Heck, Ph.D.**, research molecular biologist for the U.S. Department of Agriculture (USDA)-Agricultural Research Service in Ithaca, New York, will speak about psyllid bacteria;
- **Bruce Babcock, Ph.D.**, professor in the School of Public Policy at the University of California, Riverside, and the author of the CRB’s newly released study, *Impact of Regulations on Production Costs and Competitiveness of the California Citrus Industry*, will present on the economics of California citrus;
- **Frank Byrne, Ph.D.**, associate researcher in the Department of Entomology at the University of California, Riverside, will address imidacloprid use for California citrus;
Silvio A. Lopes, Ph.D., who works in Research and Development at Fundecitrus – Fundo de Defesa da Citricultura in São Paulo, Brazil, will discuss the management and impact of ACP/HLB in Brazil and how California growers can learn and benefit from Brazilian research and experiences;

David Bartels, Ph.D., USDA-Animal and Plant Health Inspection Service-Plant Protection and Quarantine (USDA-APHIS-PPQ), will speak about the mapping of citrus pest diseases;

Daniele Zaccaria, Ph.D., assistant cooperative extension specialist at the University of California, Davis, will talk about water conservation and ground water management;

Tim Eyrich, vice president of research at Southern Gardens Citrus in West Palm Beach, Florida, will report on the latest research in the fight against HLB;

Victoria Hornbaker, Interim Citrus Program Director with the California Department of Food and Agriculture, will present an update from the California Citrus Pest and Disease Prevention Program;

Joel Nelsen, president of California Citrus Mutual, will provide an update about the work his organization is doing for the citrus industry; and

Christina Loren, meteorologist for RFD-TV, will discuss weather issues that affect the citrus industry.

Scientific research posters will be on display at the conference, and researchers will be available during poster sessions to answer questions. The conference also will include prize giveaways. (Attendees must be present to win.)

Continuing education units have been applied for and will be available to conference attendees, pending approval by the California Department of Pesticide Regulation.
Although the conference is a free event, registration is recommended. To register or to obtain more information about the conference, please visit www.citrusresearch.org. Registered attendees will receive a hot all-American breakfast buffet, a morning break, lunch, an afternoon break and conference materials in addition to admission to all sessions.

Early registration is strongly advised, since conference attendance has increased each year and space is limited.

The Wyndham Visalia, located at 9000 W. Airport Drive in Visalia, also served as the site of the successful 2017 California Citrus Conference. A block of the hotel’s rooms is available for conference attendees at the special rate of $99. For reservations, call (559) 931-2117 and mention the conference.

**Conference Schedule**
(Tentative)

- **6:30 AM** - Conference registration opens/complimentary all-American breakfast buffet
- **8:00 AM** - Morning conference session begins
- **10:00 AM** - Morning break/scientific poster touring
- **10:30 AM** - Morning conference session continues
- **12:00 PM** - CRB 50th anniversary luncheon (complimentary)
- **1:45 PM** - Afternoon conference session begins
- **3:30 PM** - Afternoon break/scientific poster touring
- **3:45 PM** - Afternoon conference session continues
- **5:00 PM** - Conference concludes

**Industry booths were packed with attendees during last year’s conference.**

**An attendee studied the scientific research posters, which were sponsored by Dow AgroSciences at last year’s California Citrus Conference.**

Carolina Evangelo is the director of communications for the Citrus Research Board in Visalia, California, where she also serves as co-publisher and project manager of Citrograph. For additional information, contact carolina@citrusresearch.org
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(TENTATIVE) CONFERENCE SCHEDULE

6:30 AM CONFERENCE REGISTRATION OPENS
8:00 AM MORNING CONFERENCE SESSION BEGINS
NOON CRB 50TH ANNIVERSARY LUNCHEON (COMPLIMENTARY)
1:45 PM AFTERNOON CONFERENCE SESSION BEGINS
5:00 PM GIVEAWAYS ANNOUNCED (MUST BE PRESENT TO WIN)
CONFERENCE CONCLUDES

CONFIRMED SPEAKERS

SILVIO A. LOPES, PH.D., FUNDECITRUS, BRAZIL
MICHELLE HECK, PH.D., USDA-ARS, NEW YORK
CHRISTINA LOREN, RFD-TV METEOROLOGIST, TENNESSEE
BRUCE BABCOCK, PH.D., UC RIVERSIDE
FRANK BYRNE, PH.D., UC RIVERSIDE
DAVID BARTELS, PH.D., USDA-APHIS-PPQ, TEXAS
DANIELE ZACCARIA, PH.D., UC DAVIS
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STAY TUNED TO WWW.IRCHLB.ORG FOR MORE CONFERENCE DETAILS
In 2016 and 2017, the Citrus Research Board (CRB) expended a great deal of time and effort in the external review of our Asian citrus psyllid and huanglongbing (HLB) research projects by two different groups of scientists so that we could receive fresh, objective opinions. The final reports may be found on the CRB web site at: www.citrusresearch.org/?s=external+review

Subsequent to the delivery and digestion of the 2017 report, with the help and facilitation of Ed Stover, Ph.D., from the U.S. Department of Agriculture-Agricultural Research Service-U.S. Horticultural Research Laboratory at Fort Pierce, Florida, the board conducted a full-day deliberation in January 2018 to prioritize those findings.

Those items with the highest priorities included:

**Strategies for Identifying Priority Projects**

- Explicitly and succinctly identify priority needs for the citrus industry and ensure that there are proposals meeting the highest priority needs.
- The current strategies in California should include emphasis on containment and limitation of HLB to prevent it from entering commercial groves.
- Develop committees or workshops (including several outside experts for the opportunity to identify potentially valuable technologies from other research areas) to assess opportunities for solicited proposals in specific areas and possibly from specific researchers.

**Citrus Research Board Commits to a Strategic Plan**

Gary Schulz
Reach for entirely new ideas through a “Grand Challenge and Exploration”-like procedure.

Recognize that the CRB research portfolio should include projects representing a range of low risk to high risk, short implementation time to long implementation time, etc.

Conduct workshops on identified priority project areas that will benefit especially from collective discussion. Emphasize the importance that participants focus on the big picture rather than serve solely as advocates for their own work.

**Strategies for Selecting Projects**

- When multiple projects focus on the same topic, or the same researcher has multiple funded projects, it may make sense to systematically review the justification for such a concentration of effort.

- When working with for-profit partners, assure that costs are shared fairly. Such projects should include a product development plan from the beginning, with sources of funding identified for downstream development and manufacturing costs. When a company submits a proposal, the CRB needs to be especially active in establishing intellectual property (IP) sharing.

- Early timeline deliverables should be met for sustained funding in most cases. Researchers must understand that they should deliver enough detail in progress to justify funding. Perhaps they can share in a closed-door session to sustain IP protection.

**Strategies for Managing Selected Projects**

- The CRB needs to be more actively involved in developing some research.

- Identify projects requiring grower cooperators for research projects and identify/designate cooperators when funded. A project slowed by failure to find experimental sites is unacceptable.

- Follow higher-risk projects closely, and be prepared to cut funding when they appear to be dead-ends. Such projects should be designed with firm go/no-go stage gates.

Conduct a review of the regulatory hurdles that will delay implementation in a project if successful. Consider outside consultant to review? Maybe could be potential partner for commercialization and, therefore, low or no cost? Consider developing Cooperative Research and Development Agreement-like mechanisms to achieve?

These various strategies are in the process of implementation as we continually work with our researchers, develop research priorities for upcoming deliberation cycles and work to find solutions to citrus pests and diseases.

Thank you to the members of the Citrus Research Board, staff and Ed Stover, Ph.D., for these meaningful strategies. 😊

Gary Schulz is the president of the Citrus Research Board, based in Visalia, California. For more information, contact gary@citrusresearch.org
The 39th Annual Post-harvest Pest Control Conference was held April 17-18, 2018, at the Embassy Suites Mandalay Beach Hotel and Resort in Oxnard, California. Its technical agenda focused on updates and the most recent developments in post-harvest disease control, food safety and post-harvest pest control tailored toward an audience of researchers, service industry personnel and packinghouse post-harvest experts. Co-organized by the Citrus Research Board (CRB) and the University of California, this year’s meeting was well attended with more than 80 registrants, including an increased number of international visitors from Belgium, Israel, Mexico and South Africa.

Wilma du Plooy, Ph.D., post-harvest disease research and program director of Citrus Research International in Nelspruit, South Africa, served as the keynote speaker. In her keynote address, she spoke on challenges facing the South African citrus industry and the parallels with those challenges faced in the U.S. A second presentation addressed post-harvest disease and decay management, highlighting recent research in optimizing the use of imazalil, the current key fungicide for control of *Pencillium* decay (blue and green mold). In the same presentation, du Plooy also covered on-going work on sanitizers, which nicely complemented other talks given by speakers at this meeting. Additionally, she provided a research update on work on citrus black spot, specifically the differences between susceptibility for different citrus types.

A third of the program was dedicated to the topic of food safety. First, Trevor Suslow, Ph.D., discussed how the Food Safety...
Modernization Act (FSMA) and Market-Access Standards are shifting produce supply-chain practices. He explained that survival of human pathogens is likely under many conditions and that a low infectious dose means growth is not essential for harm. Suslow also spoke about the key challenge of _Listeria_ management. He said that environmental monitoring programs (EMPs) are a prudent approach that may be required and stressed that prevention is the best tool.

Next Linda Harris, Ph.D., presented a summary of her recent research, which focused on examining the potential for _Salmonella_ species and _Listeria monocytogenes_ survival in float tanks, drench systems and other packline systems where sodium bicarbonate, imazalil (with or without peracetic acid [PAA]) and soda ash are recirculated or reused.

Daniel Chen, a Ph.D. student working with Jim Adaskaveg, Ph.D., presented the results of his research on alternative sanitizers, which can be used both for citrus disease management and to address FSMA requirements. Their focus has been on organic acids as replacements for commonly used sanitizers such as sodium hypochlorite, PAA or chlorine dioxide.

Attendees also heard from Brian Leahy, director of the California Department of Pesticide Regulation, who discussed his department’s functions, along with specific areas of concern. Active audience participation was a highlight of this session.

Jim Cranney, president of the California Citrus Quality Council, discussed the role of California post-harvest issues in international trade, including how these issues relate to maximum residue levels (MRLs) and the current trade barriers facing the citrus industry. Highlighted in his presentation was the increasing focus on systems-based approaches to post-harvest handling, especially for pests of quarantine significance, as methyl bromide use continues to be phased out.

Alternatives to methyl bromide, the traditional treatment method of choice for eliminating quarantine pests, were discussed during the conference, including:

- **Phosphine** – Spencer Walse, Ph.D., and his research group completed their work on this compound, which has been officially approved for use as a quarantine pre-shipment treatment this fall for the control of bean thrips on navels to Australia. This is the first time any U.S. fresh fruit industry has used phosphine in this capacity and will be setting regulatory precedence for others.

- **Ethyl Formate** – Studies are continuing with registration expected in approximately two years. Elizabeth Mitcham, Ph.D., at the University of California, Davis, has been spearheading this effort, and the latest results from her research group were presented by Veronique Bikoba, Ph.D.
**Malic Acid** – This compound is already approved for a number of non-food uses, and work in the Walse lab is studying the potential of this compound as an early stage packinghouse fruit dip treatment for Fuller rose beetle that would not leave residues on packed fruit.

**Cold Treatments** – This treatment has been an historic alternative to fumigation, but one not embraced to-date by the California citrus industry. Research was presented by Walse and Sandipa Gautam, Ph.D., on the effects of combining cold treatments with lower dose fumigation treatments to provide pest control while minimizing phytotoxic effects.

Carolina Evangelo is the director of communications for the Citrus Research Board in Visalia, California, where she also serves as co-publisher and project manager of Citrograph. For additional information, contact carolina@citrusresearch.org
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Economic Impact Of California’s Citrus Industry

Bruce Babcock

Project Summary

The objective of this research project is to provide policymakers and industry observers estimates of the economic impact of the California citrus. The value of California citrus as measured at the point of shipment was $3.389 billion in the 2016-17 marketing year. The total value created by California citrus in the 2016-17 season was $7.1 billion after including the indirect value generated by supporting industries and the induced value from households spending income paid by the industry. The industry directly supports more than 21,000 full-time equivalent jobs paying $452 million in wages and added $1.695 billion to the state's Gross Domestic Product (GDP) in 2016.

An important contributor to California's economic success is agriculture. California's farmers produced $49 billion worth of agricultural commodities in 2016, which, in turn, supported California's large food and beverage processing industry. A major part of California agriculture is citrus, which generated $2.2 billion in farm revenue in 2016. Estimating the total economic contribution of California's citrus industry requires measurement of direct sales revenue, as well as estimates of the dependence of other California industries on citrus production and processing. Analysts typically use various economic multipliers to capture the broad economic impact of an industry on a state. The total economic impact of the
California citrus industry includes the direct effect from sales, the indirect effects\(^1\) from dependent industries and the induced effects\(^2\) caused by Californians spending income earned in citrus and dependent industries.

**California Citrus Data**

The value of citrus grown in California increases as fruit moves along its supply chain. Value is added when fruit on the tree is picked and then again when it is transported to the packinghouse. Once at the packinghouse, fruit is either packed for the fresh market or aggregated and sent to the processing market. The proportion of California citrus packed for the fresh market is shown in Figure 1 for the three most recent years for which data are available. Across the three years, an average of 80 percent of oranges and grapefruit, 75 percent of lemons and 70 percent of mandarins have gone to the fresh market.

A large portion of California citrus is consumed in other states and other countries. Thus, the logical point on the supply chain to calculate the impact of citrus on California’s economy is just before this fruit is shipped to buyers, which is after it has been packed and sold. Ideally, the value of California citrus destined for the processing market should be the value of fruit contained in processed products to capture the value added by processors. However, no data are available that break out the value of processed citrus, so instead I used the price of fruit delivered to processing plants. Any costs incurred at the packinghouse and transportation costs from the packinghouse to the processing plant would be included in this price.

Although the U.S. Department of Agriculture National Agricultural Statistics Service (NASS) reports quantities of each type of citrus that is destined for fresh and processing markets, it does not report average prices received for California fresh citrus vs. California processed citrus due to confidentiality concerns. NASS does not even report average prices received or total state value for California lemons and mandarins.

One method for estimating packinghouse value can be inferred from citrus price data contained in NASS reports. NASS reports monthly prices for freight on board (FOB) packed fresh citrus for California oranges and lemons and for all U.S. grapefruit. NASS bases their estimates of monthly prices on packinghouse surveys.

The difference between the FOB price and the NASS price for fruit delivered to the packinghouse door is a measure of the value added by packinghouses. The average price for California navel oranges in 2016-17 as reported by NASS was $9.73 per box or 12.16¢ per pound. The average difference for California lemons was $9.83 per box or 12.26¢ per pound, while the average difference for U.S. grapefruit across the 2015-16 and 2016-17 marketing years was $9.70 per box or 12.13¢ per pound. No prices for California mandarins were reported by NASS. Given that California produced about 95 percent of U.S. mandarins in 2016-17, use of the Florida NASS value is not justified.

Rabobank (2015) released a market outlook for U.S. citrus and estimated the cost of moving delivered citrus through the packinghouse. This report offered an independent check on the NASS price differences. The reported cost was 12.8¢, 30.6¢ and 16.1¢ per pound for oranges, mandarins and lemons, respectively. Applying these per-pound values to an 80-pound box yields values of $10.24, $24.48 and $12.84 for oranges, mandarins and lemons, respectively, which are generally consistent with the values inferred from NASS data. Given the lack of a NASS estimate for mandarins, the Rabobank estimate was used in its place.

First, the values were adjusted to account for the proportion of fruit not packed for the fresh market. Fruit that goes to the processing market is washed, sorted and then sent to processors. Packinghouse value added to processing fruit is 40 percent of the value added to fresh fruit. To calculate the packinghouse value of California citrus, the resulting per-box estimates of packinghouse value for oranges, lemons, grapefruit and mandarins were multiplied by the total number of boxes produced, as reported by NASS. Table 1 presents the final estimates of California citrus value produced in 2016-17.
The direct, indirect and induced economic contributions of California’s citrus industry are presented in Table 2. Total direct value of California citrus production was $3.389 billion in 2016-17 from Table 1. This value generated an additional $1.263 billion in economic activity from related businesses supplying material and services to the California citrus industry. An additional $2.464 billion in economic activity was generated by households spending income they received from California’s citrus industry, making the total economic impact equal to $7.117 billion.

California Citrus Production Employment

No citrus employment data are publicly available, so employment was estimated based on available estimates of labor required to produce, harvest and pack citrus. University of California Extension budgets estimate that production of oranges and lemons requires 23 and 20 hours of labor per acre, respectively. Harvest costs for the two crops are estimated to be $926 per acre and $2,699 per acre, respectively. Adjusting these harvest costs to reflect actual yields in 2016-17 increases orange costs to $1,095 per acre and decreases lemon costs to $2,205 per acre. Lacking data on harvest costs for grapefruit and mandarins, per-acre grapefruit harvest costs were set equal to oranges, and mandarin harvest costs equal to lemons.

The estimated numbers of hours to pick and haul fruit and the estimated number of hours per acre for each citrus fruit type are presented in Table 3.

The total number of hours to grow, harvest and haul California citrus is calculated by multiplying the Table 3 results by the number of acres for each type of fruit. Dividing the result by the number of work hours per year (2,087 in an average year) gives the number of full-time equivalent jobs involved in producing California citrus and delivering it to the packinghouse. Here, the estimated employment in producing, picking and hauling fruit is 14,702 full-time equivalents (Table 4).
There are no published data showing employment in citrus packinghouses, so the estimate in this report is based on published data from the Hodges et al. (2014) study of the Florida citrus industry, which estimated that fresh citrus packinghouses employed 831 people in 2012-13. NASS reports that 6,031,000 80-pound boxes of fresh oranges were packed in that year. If California packinghouses have the same ratio of jobs per thousand boxes packed as assumed by Hodges et al., then this ratio - 137.8 jobs per million boxes packed - can be used to estimate the number of California citrus packinghouse jobs (2014). According to USDA-NASS (2017), California packinghouses packed 77,980,000 80-pound boxes of citrus in 2016-17. Applying the Florida results equals 10,746 California citrus packinghouse jobs.

Due to the seasonal nature of citrus production, most jobs in the packinghouse industry are probably less than full time. If two-thirds of the jobs are half-time and one-third of the jobs are full-time, this would translate into 7,163 full-time equivalent jobs. Adding 7,163 jobs in the packinghouse industry to the 14,702 jobs needed to produce fruit and get it to the packinghouse results in a total of 21,865 full-time equivalent California citrus industry jobs. If the average wage in the citrus industry was $10 per hour, then this amounts to total wage income of $455 million.

Conclusions

California citrus generated $3.4 billion in sales in the 2016-17 marketing year. These industry sales, in turn, directly generated more than 21,000 jobs and an additional $3.8 billion in economic activity in supporting industries, as well as industries boosted by households employed by the citrus industry spending their earned income. The total economic impact of the California citrus industry was $7.1 billion. Maintenance of these important contributions to the California economy will be difficult due to current and future challenges facing the industry. Foremost among the challenges are future production declines if huanglongbing spreads into commercial groves and escalating labor costs due to changes in California labor law and Federal immigration enforcement.

References


Glossary

1. Indirect effects: The impact of purchases by the citrus industry that cycle through supporting industries in the state.

2. Induced effects: The response of an economy to an initial change in value of citrus production resulting from households spending income generated by the initial change.

Bruce Babcock, Ph.D., is an agricultural economist and professor in the School of Public Policy at the University of California, Riverside. For additional information, contact bruce.babcock@ucr.edu
With more than 838 huanglongbing (HLB) detections in residential citrus trees during 2018 alone, the fatal plant disease is posing its greatest threat yet to California’s commercial citrus industry at a time when the industry is booming.

According to a recent study by the University of California, Riverside (see “Economic Impact of California’s Citrus Industry,” page 36), the California citrus industry had a $7.1 billion economic impact on the state’s economy and supported more than 21,600 jobs in 2016-17. It has never been more important for all of us to work together to prevent the spread of the disease and save California’s citrus industry.

The Citrus Pest and Disease Prevention Program (CPDPP) has been working diligently to address the alarming increase in HLB detections over the past year and recently created a strategic plan to fight the disease. A key factor is outreach to California homeowners, elected officials, city governments and the industry about Asian citrus psyllid (ACP) and spread of the HLB-associated bacterium ‘Candidatus Liberibacter asiaticus.’
Given the alarming increase in HLB detections in southern California residential citrus trees during recent months, the outreach team intensified efforts during the summer travel season and worked closely with the California Department of Food and Agriculture (CDFA) to educate key audiences.

**Don’t Move Citrus Campaign**

To capitalize on the busy travel season during summer months, the CPDPP launched a Don’t Move Citrus campaign, emphasizing the importance of not transporting citrus trees and plant material, including fruit, through the state when traveling. The campaign included outdoor billboards placed strategically on major transportation corridors, outreach to daily newspapers and local broadcast stations across the state and digital content shared on web sites and social media platforms.

From May through July, billboards at the U.S.-Mexico border, on I-5 near the Grapevine at the southern end of the San Joaquin Valley, and off Highway 99 in McFarland read “Protect Backyard Citrus From Plant Disease. Don’t Move Citrus.” or “Protect Local Ag From Plant Disease. Don’t Move Citrus.” Combined, these billboards were seen more than 4.7 million times.

During the same time period, a press release cautioning residents against moving citrus trees, leaves and plant material during summer travel was issued to media outlets across the state. The announcement included details on the harm HLB can have on residential and commercial citrus trees. As a result, 23 stories from various news sources throughout the state were published, including the following:

- An article specific to southern California residents was published by ten daily newspapers serving areas with or very near HLB detections. The report included comments from county agricultural commissioners and Beth Grafton-Cardwell, Ph.D., about the threat of HLB and how residents should take action to protect California citrus.
- Broadcast coverage during local morning and evening news programs in citrus-producing regions like Bakersfield and Palm Springs.
- Articles in five Asian-language media outlets serving multicultural California residents.

Lastly, to reach audiences who spend a majority of their time online, an informational video and an infographic about the importance of not moving citrus material were developed and shared on the CPDPP’s social media profiles before holidays known for travel, like Memorial Day Weekend and the Fourth of July. This content was shared more than 80 times by Facebook and Twitter users with their network of friends and family.
The Don’t Move Citrus campaign reached millions of Californians with an important message at a relevant and critical time.

**Encouraging Participation with Agriculture Crews**

CDFA field crews serve as “boots on the ground” in residential areas for the CPDPP and have a strong presence in southern California cities where ACP and HLB have been found. These crews are responsible for surveying and sampling backyard citrus trees, treating for ACP and removing HLB-positive trees. For the program to detect and eradicate diseased trees, it is critical that homeowners allow CDFA staff onto their properties.

The outreach team works hand-in-hand with the CDFA to identify local communities with higher refusal rates or homeowners who do not allow the CDFA on their property to inspect their citrus tree(s). The team then distributes door hangers, runs social media ads, attends community events and refers California Citrus Mutual’s free tree removal program in specific zip codes and neighborhoods with higher refusal rates. Communication emphasizes the threat of HLB, the importance of working with agriculture crews and what to expect when crews visit one’s home.

While many in the citrus industry are aware of the dangers HLB poses and how the disease spreads, continuous communication is needed for residents with citrus trees. Every commercial citrus farmer, grower, packer, hauler and supporter can help spread the word to others about the danger of HLB. Consider sharing content from the program’s Facebook or Twitter accounts – like the infographic and video mentioned above – on your personal social media profiles or with your network. Hand out informational materials about HLB at community events. If we sit idle – hoping others will take action for our benefit – we are welcoming this devastating disease into our groves. But by working together to fight HLB, we can help ensure our industry will be profitable for generations to come.

*Mark McBroom is the outreach subcommittee chair of the Citrus Pest & Disease Prevention Program and a CRB Board Member. For more information, contact desertcitrus@aol.com*
It won’t be long before Jack Frost is nipping at your citrus

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No Matter What You’re Growing, We Have You Covered!
Join us as we take a look back at 50 years of the Citrus Research Board as seen through the covers of Citrograph, the magazine of the California citrus industry.
The development of commercial citrus varieties with resistance to *Candidatus Liberibacter asiaticus* (CLas), the bacterium associated with huanglongbing (HLB), is considered by many within the citrus research community as the best long-term solution to this disease. Research projects currently underway to develop such varieties use a range of approaches, with different varieties in different laboratories funded by various government and citrus industry-associated funding agencies across the United States and abroad. The strength of such an approach is that it casts a wide net in identifying potential sources of CLas resistance and/or tolerance and reflects differing market and geographic needs across the U.S. citrus industry. The downside is that it creates duplicative efforts with overlapping goals, redundancy and a degree of disorganization that slows the overall pace of varietal development. For these reasons, there is concern that the U.S. citrus industry’s needs for timely development and implementation of CLas-resistant rootstock and scion varieties are not being adequately met. Accordingly, a comprehensive nationwide, collaborative citrus breeding effort has been proposed to better focus current breeding efforts and address current critical industry needs.

The benefits of a more centralized approach to new citrus variety development with CLas-resistance has been recognized by the Citrus Research Board (CRB) and in other external reviews of HLB research progress (National Academies of Sciences, Engineering, and Medicine 2018).
Last fall, with CRB board member involvement, a white paper was developed to highlight the shortcomings of current citrus breeding efforts. With financial support from the CRB, the U.S. Department of Agriculture (USDA)-Animal and Plant Health Inspection Service Huanglongbing Multi-Agency Coordinating Group and Sunkist, a two-day facilitated summit was organized earlier this year to identify and address current barriers limiting the development of new CLas-tolerant and/or resistant citrus varieties. The summit was planned and organized by a nationally-represented coordinating committee that included a meeting facilitator, and staff from the CRB, the Citrus Research & Development Foundation (CRDF) and USDA-National Institute of Food and Agriculture (NIFA). As described in the final report, the expected outcomes for this two-day meeting included:

- Identify the scientific, intellectual property, tech transfer, commercialization and other barriers to collaboration and new variety development.
- Outline structures or tools to overcome said barriers going forward.
- Create a mutually agreed-upon standard testing protocol to screen candidates for CLas/HLB resistance.
- Initiate data sharing agreements/programs across citrus breeding programs to limit duplication of efforts.

To prepare for the summit, attendees were provided relevant background reading material – the white paper highlighting industry concerns and background information on Collaborative Research Projects (http://ccnmtl.columbia.edu/projects/rcr/rcr_science/foundation/index.html) – and a survey to identify current perceptions regarding scientific and non-scientific barriers to the development of CLas-resistant or tolerant citrus rootstocks and scions. Questionnaire responses provided the coordinating committee a chance to identify the greatest barriers (perceived or real) within and between the different groups in attendance.

The summit was held February 27-28 in Denver, Colorado, and brought together 62 attendees from across the country, including current university and federal government citrus rootstock and scion breeders and molecular geneticists, relevant administrative representatives from host research institutions, relevant funding agency staff and industry members. The summit began with a review of the state of the citrus industry, especially as it has been impacted by HLB, followed by presentations highlighting successful scientific collaborative projects: Jeff Gwyn, Ph.D., representing the International Wheat Yield Program and Genaro Fazio, Ph.D., of the USDA-Agricultural Research Service spoke on their respective multi-agency, multi-location breeding programs for wheat and tree fruit rootstocks (NC-140 trial), respectively. The pre-meeting survey results were presented to the participants and provided food for thought as the attendees then broke into four separate breakout sessions and worked independently to address scientific, regulatory, intellectual property and technology transfer, and funding-related barriers. The groups came together at the end of the second day to highlight their findings to the larger group. Three priority action items highlighted at the end of this meeting and included in the final meeting report were the following:

1. Establish an organization similar to the International Wheat Yield Partnership and/or the NC-140-type program to conduct citrus germplasm evaluation in greenhouse and field trials, the coordination of data collection standards, and other service functions to achieve greater coordination and collaboration among citrus researchers to expedite the development of CLas resistant/tolerant citrus.
2. Hold a regulatory summit to address the issue of interstate movement of citrus plant materials for research purposes.
3. Set up a multi-agency project through USDA-NIFA to conduct better coordinated trials.

The full report including the survey and responses, a listing of participants, a description of each of the four types of potential barriers to new variety development and the potential means to address these barriers to CLas-resistant citrus rootstock and scion development can be found on the Citrus Research Board website at http://citrusresearch.org/wp-content/uploads/Final-NCBCM-Report.pdf. There already has been forward movement following this meeting with the development of draft guidelines for data collection for citrus HLB greenhouse and early-stage field trials. Funding has been secured, and planning is underway for the regulatory meeting highlighted in Action Item #2, as well.

The need for new CLas-resistant varieties is a universal priority across U.S. commercial citrus-producing regions, and this meeting was designed to facilitate a more coordinated national approach to long-term solutions for HLB. CRB staff and board members continue to work with other funding organizations and researchers to streamline this process – keep an eye out for more information in the coming months. ☮

References


Melinda Klein, Ph.D., is the chief research scientist for the Citrus Research Board in Visalia, California, where she also serves as the science editor of Citrograph. For additional information, contact melinda@citrusresearch.org
HLB Research At The UC Davis Contained Research Facility

Kris Godfrey, Elizabeth Foster, Tiffanie Simpson and Karla Araujo

Project Summary

Research is critically needed on many aspects of the huanglongbing (HLB) pathosystem¹ to predict the problems caused by the HLB-associated bacterium ‘Candidatus Liberibacter asiaticus’ (CLas) and its vectoring insect, the Asian citrus psyllid (ACP) in California. Research targeting detection and management of HLB and CLas has been conducted at the Contained Research Facility (CRF) at the University of California, Davis (UCD) for the past four years. Cultures of CLas and ACP colonies were maintained in the CRF and used by researchers from a variety of disciplines for research on detection of HLB and CLas prior to symptom appearance and evaluation of a variety of genetically modified citrus plants, citrus hybrids and citrus relatives for tolerance and/or resistance to CLas.
Since the invasion of ACP and the appearance of HLB and CLas in California, research has been needed on the detection and management of the pest and the disease. However, because of the invasive nature of ACP and its ability to transmit CLas, governmental regulations require research on this pathogen to be conducted in a venue that protects the environment from further spread of either the disease or pest. The CRF at UCD is such a venue because it is a Biosafety Level III-Plant quarantine (Godfrey et al. 2015). For the past four years, cultures of CLas (the bacteria thought to cause HLB) and colonies of ACP have been maintained at the CRF for use by researchers from a variety of scientific disciplines investigating a number of research topics. Two isolates of CLas are maintained in culture, Hacienda Heights isolate and the San Gabriel isolate (Figure 1). The research topics include detection of HLB and CLas in a plant before either symptoms appear or the bacteria can be detected by molecular means, and assisting in the evaluation of genetically modified citrus, citrus relatives and citrus hybrids for tolerance of CLas infection. This report summarizes the infrastructure that supports these research projects.

The research project that had the most need of CLas cultures and ACP colonies looked to detect the presence of CLas within a citrus plant prior to the appearance of symptoms using a variety of methods from several scientific disciplines (reviewed in Chin et al. 2014). These are referred to as “early detection” methods because they purport to detect the presence of CLas before actual detection of the bacteria using federal and state regulatory-approved quantitative polymerase chain reaction (qPCR) methods. Some early detection methods detect changes in the plant in response to infection by CLas while another one attempts to improve the detection ability of PCR methods such as digital droplet PCR to improve PCR sensitivity (McCollum et al. 2016).

The early detection methods studied included the metabolic response to infection by CLas as measured by changes in the plant’s metabolism in the leaves (Chin et al. 2015, 2018) and by measuring changes in the volatile organic compounds (VOCs) that infected plants emit (Thuesen et al. 2015). Another method capitalized on the fact that the microbial communities found on leaves shift with changes in the VOCs. By characterizing the changes in the microbial community or leaf microbiome, CLas-infected plants could be confirmed before qPCR detected the presence of CLas (Leveau and Rolshausen 2016). Proteomics-based early detection methods investigated the changes in the protein composition of the phloem caused by either the plant response to infection or secretion of bacteria proteins called “effectors” by the CLas bacterium (Franco et al. 2017). All of the early detection methods studied using plants in the CRF are now being field tested and/or being commercialized in an attempt to provide detection tools for California citrus growers.

To investigate the plant responses to CLas infection, three sets of experimental plants (Lisbon lemons, Washington navels and Tango mandarins grafted on Carrizo rootstock) were produced for use in the study (Figure 2). The first set of plants was assigned to the following treatments: control-control (no manipulation), graft-control (grafted with healthy scion) and grafted with CLas-infected plant material (Table 1). Grafted plants each received three grafts of the appropriate plant material, and the Hacienda Heights isolate of CLas was used for this set. After being subjected to the treatments, the plants were held in the greenhouse at 80°F with supplemental lighting (16 hours light, eight hours dark) and sampled monthly to determine the presence of CLas using qPCR (U.S. Department of Agriculture-Animal and Plant Health Inspection Service [USDA-APHIS] 2012). It was necessary to sample the plants and subject them to the same testing established by the USDA-APHIS protocol so that comparisons could be made among the times of first detection by the early detection methods and that of the USDA-APHIS protocol standard detection method.

The second set of experimental plants was insect-inoculated. Treatments included control-control (no manipulation), exposure to CLas-negative ACP adults and exposure to CLas-positive (Hacienda Heights isolate) ACP adults (Table
ACP adults were confined in sleeve cages on one branch of each treatment plant and allowed to feed for ten days (Figure 3). The ACP then were removed and assayed by qPCR to determine the presence of CLas within the insects. The percent of CLas-positive ACP used in the exposures was zero for the CLas-negative ACP exposures, a mean of 29.7 percent (range 4 – 69.6 percent) for insects used for the lemon exposure; a mean of 31.3 percent (range 4.2 - 68.8 percent) for insects used for the navel orange exposures; and a mean of 23.4 percent (range 4 – 72.2 percent) for insects used for mandarin exposure. Any flush growth on exposed plants was excised to remove eggs and nymphs, and then the plants were treated with foliar and systemic insecticides to kill any remaining ACP. These plants were held in the greenhouse at 80ºF with supplemental lighting (16 hours light, eight hours dark). All plants were sampled monthly to determine the presence of CLas using qPCR (USDA-APHIS 2012).

The third set of experimental plants also was insect-inoculated. The treatments were exposure to CLas-negative ACP adults and to CLas-positive (Hacienda Heights isolate) ACP adults (Table 1). Insects were confined to one branch using sleeve cages and allowed to feed for 14 days. The ACP then were removed and stored at -80ºC for later assay by qPCR to determine the presence of CLas within the insects. The results of these assays are pending. For this set, the flush growth was not removed; rather the flush tips were inspected for the presence of eggs and nymphs, and any found were destroyed. The plants also were treated with foliar and systemic insecticides before being moved to a greenhouse held at 80ºF with supplemental lighting (16 hours light, eight hours dark). All plants were sampled monthly to determine the presence of CLas using qPCR (USDA-APHIS 2012).

Table 1. The number of plants of each cultivar in each treatment for the three sets of experimental plants used in the early detection study and the outcome of each treatment in terms of infection by ‘Candidatus Liberibacter asiaticus’ using USDA-APHIS standard testing.

<table>
<thead>
<tr>
<th>Set 1</th>
<th>None</th>
<th>Graft Control</th>
<th>Graft Inoculation</th>
<th>Mean days ± std. error to first CLas positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lisbon Lemon</td>
<td>5/0</td>
<td>17/0</td>
<td>22/10</td>
<td>122.1 ± 17.2 days</td>
</tr>
<tr>
<td>Washington Navel</td>
<td>3/0</td>
<td>15/0</td>
<td>19/15</td>
<td>139.5 ± 14.6 days</td>
</tr>
<tr>
<td>Tango Mandarin</td>
<td>3/0</td>
<td>13/0</td>
<td>16/4</td>
<td>296.3 ± 31.6 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Set 2</th>
<th>None</th>
<th>ACP Exposure</th>
<th>CLas positive ACP</th>
<th>Mean days ± std. error to first CLas positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lisbon Lemon</td>
<td>6/0</td>
<td>13/0</td>
<td>16/4</td>
<td>185.8 ± 45.3 days</td>
</tr>
<tr>
<td>Washington Navel</td>
<td>9/0</td>
<td>9/0</td>
<td>10/0</td>
<td>--</td>
</tr>
<tr>
<td>Tango Mandarin</td>
<td>5/0</td>
<td>6/0</td>
<td>14/1</td>
<td>363 days</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Set 3</th>
<th>None</th>
<th>ACP Exposure</th>
<th>CLas positive ACP</th>
<th>Mean days ± std. error to first CLas positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lisbon Lemon</td>
<td>--</td>
<td>--</td>
<td>6/0</td>
<td>10/7</td>
</tr>
<tr>
<td>Washington Navel</td>
<td>--</td>
<td>--</td>
<td>6/0</td>
<td>10/1</td>
</tr>
<tr>
<td>Tango Mandarin</td>
<td>--</td>
<td>--</td>
<td>4/0</td>
<td>6/1</td>
</tr>
</tbody>
</table>

*Set II plants had flush growth removed after insect inoculation to remove eggs and nymphs. Set III plants did not have flush growth removed after insect inoculation.

1) ACP adults were confined in sleeve cages on one branch of each treatment plant and allowed to feed for ten days (Figure 3). The ACP then were removed and assayed by qPCR to determine the presence of CLas within the insects. The percent of CLas-positive ACP used in the exposures was zero for the CLas-negative ACP exposures, a mean of 29.7 percent (range 4 – 69.6 percent) for insects used for the lemon exposure; a mean of 31.3 percent (range 4.2 - 68.8 percent) for insects used for the navel orange exposures; and a mean of 23.4 percent (range 4 – 72.2 percent) for insects used for mandarin exposure. Any flush growth on exposed plants was excised to remove eggs and nymphs, and then the plants were treated with foliar and systemic insecticides to kill any remaining ACP. These plants were held in the greenhouse at 80ºF with supplemental lighting (16 hours light, eight hours dark) and sampled monthly to determine the presence of CLas using qPCR (USDA-APHIS 2012).

Figure 3. Sleeve cages on experimental plants, exposing the plants to ‘Candidatus Liberibacter asiaticus’ through Asian citrus psyllid feeding.
The production of CLas-positive plants in the three experimental sets is summarized in Table 1. Graft-inoculation produced proportionally more CLas-positive plants than insect inoculation. Comparison of the proportion of CLas-positive plants produced between Set II and Set III demonstrated the role of leaving the flush growth on a plant after insect inoculation. In Set II, flush growth was removed after insect feeding, and in Set III, the flush was left on the plant. More CLas-positive plants developed in Set III than in Set II.

The mean days until the first detection of CLas varied among cultivars and among the experimental plant sets (Table 1). This is not too surprising, considering that the amount or titer of CLas bacteria introduced into the experimental plants varied across time and cultivar. Since CLas cannot be cultured (i.e., grown on or in artificial media), it is very difficult to standardize or put the same amount of bacteria in each experimental plant. In general, Lisbon lemons appear to be more susceptible to infection (more positive plants and, at times, fewer days to first detection of CLas infection) than either Washington navels or Tango mandarins, although all three cultivars are susceptible to CLas infection (Table 1).

Infrastructure support also was provided to two projects that are investigating genetically-modified citrus plants, and citrus hybrids and relatives. Details and results of these projects have been reported in Louzada and Thomson (2017) for the genetically modified plants and in Ramadugu et al. (2016) for the citrus hybrids and relatives. The infrastructure project provided CLas-infected plant material for grafting or the CLas-positive ACP that were used to challenge the plants with CLas. When needed, the infrastructure project conducted the challenges, maintained the plants and collected samples for both projects.

Conducting research in the CRF allowed work to begin on this important pathosystem soon after initial introduction by researchers who are familiar with citrus production and pest management issues in California. While some of the production and pest management issues are shared with other citrus-producing states, many of the issues are unique to California citrus. In addition, the research was conducted using cultivars and rootstocks common to California, as well as ACP populations and CLas isolates found in California. Conducting California-centric research may provide data on this pathosystem that reflects conditions in the field more realistically than data from other states.

CRB Research Project #5300-161

References


Glossary

‘HLB pathosystem: A subset or type of ecosystem, in this case characterized by the host (citrus plants) and two “parasites,” the Asian citrus psyllid (ACP) and the ACP-spread (or vectored) bacterium ‘Candidatus Liberibacter asiaticus’.

Kris Godfrey, Ph.D., is a project scientist, Elizabeth Foster is a staff research associate, Tiffanie Simpson is a staff research associate and the manager of the Contained Research Facility and Karla Araujo is a junior specialist. All work at the Contained Research Facility, University of California, Davis. For more information, contact kegodfrey@ucdavis.edu
Correlating Citrus Tree Health with Microbes

Philippe Rolshausen, Tyler Dang, Sohrab Bodaghi, Nichole Ginnan, Paul Ruegger, Beth Peacock, Caroline Roper, James Borneman, Greg McCollum, Georgios Vidalakis and Gary K. England

Project Summary

This work provided a DNA database of the bacteria and fungi associated with Florida citrus tree budwood, leaves and roots impacted by huanglongbing (HLB). Bacterial and fungal communities (microbiome¹) profiling was performed for two consecutive years. In addition, HLB severity was recorded using a disease rating scale, and ‘Candidatus Liberibacter asiaticus’ (CLas) titer was measured by quantitative polymerase chain reaction (qPCR)² for all sampled trees. Some microbes (mycorrhizae, Bacillus, Streptomyces) surfaced as potential plant health promoters because they were more frequently found in association with trees exhibiting limited HLB symptoms. This research provides the basis for obtaining a greater understanding of the factors that shape the citrus microbiome, as well as identifying individual microorganisms or microbial consortia of microorganisms that potentially play a role in plant health and HLB suppression or exacerbation. This Citrus Research Board (CRB)-funded research has facilitated additional funding from state and federal agencies that will support long-term research and identification of microbial-based prophylactic and/or curative treatments for HLB management.

Figure 1. Huanglongbing (HLB)-affected trees in a commercial orchard in Florida. Left tree is showing no or minimal HLB symptoms in comparison to the tree on the right (symptomatic). Note the disease progression from 2016 to 2017.
**Introduction**

In recent years, resources have been invested in plant microbiome research to explore the biological functions of microbial communities with a larger goal to harness the power of beneficial microbiomes for enhanced crop productivity. One goal of this CRB-funded project was to identify microbes that are potentially beneficial to overall citrus health. Using DNA-based technologies, we profiled the microbial communities associated with leaf, root and budwood of apparently minimally-symptomatic and HLB-impacted Florida citrus trees. The resulting dataset will aid in identifying bacteria and/or fungi that support citrus health. This information could provide a scientific foundation for commercial bio-product development that could be incorporated into orchard management practices.

**Results and Discussion**

We visited more than ten Florida orchards, focused on five (Table 1) that displayed minimally-symptomatic trees despite being under high HLB pressure (Figure 1) and monitored those trees for two consecutive years. We refer to those trees as minimally symptomatic because they show few or no HLB-like symptoms and yielded marketable fruit. We hypothesized that those trees host beneficial microbes that protect them in some capacity against CLas. Three of those five orchards were planted before HLB was first confirmed in Florida in 2005, so their lives have spanned pre- and post-HLB exposure. Two orchards were planted after 2005 and, hence, have been exposed to HLB pressure since their inception.

**Table 1. Description of the five Florida orchards used in this study.**

<table>
<thead>
<tr>
<th>FL Orchard #</th>
<th>Year Planted</th>
<th>Rootstock</th>
<th>Scion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2013</td>
<td>US-897</td>
<td>Ruby Red</td>
</tr>
<tr>
<td>2</td>
<td>2006</td>
<td>Swingle Citrumelo</td>
<td>Valencia</td>
</tr>
<tr>
<td>3</td>
<td>1990</td>
<td>Swingle Citrumelo</td>
<td>Hamlin</td>
</tr>
<tr>
<td>4</td>
<td>1990</td>
<td>Sour Orange</td>
<td>Parson Brown</td>
</tr>
<tr>
<td>5</td>
<td>1990</td>
<td>Sour Orange</td>
<td>Parson Brown</td>
</tr>
</tbody>
</table>

Using a Disease Rating (DR) scale ranging from 0-5 (0=minimally-symptomatic and 5=dead or dying), we selected a range of trees with HLB symptoms within each orchard, including five minimally-symptomatic (DR=1-2) and five severely symptomatic (DR=3-4) trees (50 trees total - Figure 2). We collected root, budwood and leaf samples from 50 trees in each of two consecutive years. The identity and relative abundance of the microbes living on and within the plant samples was determined using DNA-based technologies and traditional culturing techniques. Because a large percentage of microorganisms do not grow in culture, the DNA-based approach provides a more comprehensive profile of the microbes present.

Culturable organisms were recovered from plant tissues on bacterial and fungal nutrient media and harvested in a buffer solution. The DNA of these cultures was extracted, and species identity was determined by DNA sequencing. Stock suspensions of our microbial collection were stored at -80°C for additional experiments to identify the culturable microbes that inhibit *Liberibacter crescens*, a bacterium closely related to CLas. CLas has yet to be cultured in vitro, which makes it challenging to screen for active molecules or biological control agents (BCAs). Thus, we are using *L. crescens* as a surrogate of CLas for in vitro screening (Figure 3). This is part of an ongoing project funded by the U.S. Department of Agriculture-National Institute of Food and Agriculture Citrus Disease Research and Extension (Project #2017-70016-20563) to characterize new anti-CLas molecules.

Bacterial and fungal DNA-based community analyses were performed on the budwood, leaf and root samples of trees. In addition, CLas titer was measured by qPCR in all plant samples collected (Li et al. 2006; McCollum et al. 2014). In the five orchards, HLB severity increased over the sampling period with the total number of visually minimally-symptomatic trees (DR=1-2) (Figure 2) decreasing from 50 percent in 2016 to 34 percent in 2017 (Figure 4). Interestingly, the minimally-symptomatic appearance of the trees was specifically maintained in the three oldest orchards that were planted

![Figure 2. Huanglongbing (HLB) disease rating severity scale. 0 = healthy tree (not shown). ‘Candidatus Liberibacter asiaticus’ (CLas) not detected by DNA detection methods. 1 = Minimally symptomatic (full canopy) tree, but CLas-positive at time of sampling; often shows a few signs of infection (e.g. yellow shoots, blotchy mottle on leaves). 2 = Tree showing canopy thinning on 50 percent or less of the canopy; CLas-positive. 3 = Extensive canopy thinning (see-through) on 100 percent of the tree canopy; CLas-positive. 4 = Extensive branch dieback and CLas-positive on 50 percent or less of the canopy. 5 = Tree is dead or dying and CLas-positive; extensive branch dieback on 50 percent or more of the canopy. (Ginnan et al. 2018)
before the onset of the HLB epidemic (#3-5 in Table 1), and those trees were further referred to as minimally-symptomatic in our analyses (see below).

In addition, the percent of samples that tested negative for CLas in budwood and leaves dropped from 56 and 58 percent in 2016 to 16 and 22 percent in 2017, respectively (Figure 5). Overall, there was a general trend between our HLB disease severity rating and CLas titer in leaves (Figure 6). The relatively high percentage of CLas-free budwood and leaf tissue in 2016 (more than 50 percent) was somewhat surprising for 2016 given that these trees had been exposed to CLas for an extended period of time.

The most distinct differences in both the bacterial and fungal communities among the three tissue types were between the roots and the other two tissues. Specifically, tree roots were associated with specific organisms that were not found in leaves and budwood and vice versa. For the bacteria, budwood and leaves had more Methylobacterium and Hymenobacter than the roots. Conversely, the roots had considerably more Streptomyces and Bacillus than either the budwood or leaves. All of these taxa3 have been previously identified from citrus (Gomba et al. 2017; Trivedi et al. 2010, 2011, 2012; Zhang et al. 2017).

For the fungi, budwood and leaves had higher abundance levels of the genera Cladosporium and Sporobolomyces, both of which have been identified previously on citrus leaves (Araújo et al. 2001; Furuya et al. 2012). Conversely, roots contained a larger amount of the Glomeromycota than budwood or leaves, a group of fungi which includes mycorrhizal fungi4. The two mycorrhizal genera we commonly identified, Rhizophagus and Glomus, were previously identified in citrus (Song et al. 2015; Wu et al. 2017). Computational analyses of those samples showed that mycorrhizal fungi, Streptomyces, Bacillus and Methylobacterium, were more abundant in minimally-symptomatic than in symptomatic trees. These specific organisms are known biological control agents and plant growth promoting agents in many agricultural systems. Their importance for protection against drought, soil salinity, pests and diseases in citrus has been previously demonstrated (Caicedo et al. 2016; Chen et al. 2016; Giassi et al. 2016; Shutsrirung et al. 2013; Wu et al. 2017; Yen et al. 2008).

It has been proposed that CLas infection alone is not the cause of tree death. The host's response to pathogen invasion (i.e., plugging the phloem sieve pore with callose), leads to a decrease of sugar transport from the leaf to the root, which weakens the plant (Kim et al. 2009). Because sugar (i.e., in the

Figure 3: Liberibacter crescens in vitro inhibition assay. This assay is used to screen for active molecules or biological control agents (BCAs) that inhibit growth of L. crescens. The disc in the center of the Petri plate contains the molecule or BCA to be tested on L. crescens. A: no inhibition (control); B: inhibition of L. crescens (50µg of Spectinomycin) as indicated by the halo around the disc (dotted circle).

Figure 4: Huanglongbing disease incidence progression in the five orchards (Table 1) studied between 2016 and 2017.
form of starch) is an energy reserve for trees, plant integrity is compromised to the point that it succumbs to biotic (e.g., secondary pathogens) or abiotic stresses (e.g., frost, drought). Florida orchards suffered an extended drought period between 2015-2017 that exposed trees to environmental stresses. We speculate that the organisms identified may protect minimally-symptomatic trees against drought conditions, allowing trees to respond better to CLas infection, minimizing HLB symptom development. Developing tools that allow an early interaction and/or symbiosis between beneficial microbes and citrus trees under field conditions may support tree health and extend tree longevity of HLB-affected trees.

**Conclusion**

The data generated by the DNA sequencing approach is useful for the discovery of microbes that correlate with tree health. Identifying specific microbes through this approach will also be useful for future research to evaluate the biological functions of those specific microorganisms. Several of the microorganisms identified in this study are known biological control agents and promoters of plant vigor, many of which are available commercially and used in crop production systems. Some of those microorganisms are among our collection of culturable organisms and could be incorporated in controlled experiments in future work. This approach provides opportunities for the patenting of novel technologies and for the development and commercialization of new science-based bioproducts.

**CRB Research Project #5300-164A

**References**


**Glossary**

1. **Microbiome**: The collection of all microbes (bacteria, fungi, viruses) associated with a particular environment.

2. **Quantitative polymerase chain reaction (qPCR)**: A laboratory technique developed to amplify a targeted DNA sequence and to quantify it in real-time.

3. **Taxa**: Groups into which biological organisms are classified.

4. **Mycorrhizal fungi**: Fungi that form a symbiotic association (via mycelium) with plant roots.

Philippe Rolshausen, Ph.D., is an extension specialist; Tyler Dang is a Ph.D. candidate; Sohrab Bodaghi, Ph.D., is a research scientist; Nichole Ginnan is a Ph.D. candidate; Paul Ruegger, Ph.D., is a research scientist; Beth Peacock is a Ph.D. candidate; Caroline Roper, Ph.D., is a professor in microbiology and plant pathology; and James Borneman, Ph.D., is a professor in microbiology and plant pathology. All are at the University of California, Riverside. Greg McCollum, Ph.D., is a research physiologist at the U.S. Department of Agriculture-Agricultural Research Service in Fort Pierce, Florida. Georgios Vidalakis, Ph.D., is director of the Citrus Clonal Protection Program and an extension specialist at the University of California, Riverside. Gary K. England is an extension agent at the Hastings Agricultural Extension Center, University of Florida. For additional information, contact philrols@ucr.edu
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A Microbiota-based Approach to Citrus Tree Health

In search of microbial biomarkers to pre-diagnose trees for HLB infection

Xiaochen Yin, Kaitlyn Kelly, Nilesh Maharaj, Philippe Rolshausen and Johan Leveau
Project Summary
The overall objective of this project was to describe, by DNA-based technology, the microbial communities (also known as microbiota⁴) that associate with healthy and huanglongbing (HLB)-impacted citrus trees. The composition of microbial communities associated with citrus samples (mostly leaf material, but also root, stem and soil) from different locations (California, Texas and Florida) and under different management regimes (greenhouses, experimental stations, commercial fields and residential areas) were characterized to determine potential correlations between microbiota composition and tree health information. Here, we detail a subset of the data generated representing a case study to highlight the promises, outcomes and challenges that underlie the identification of pre-diagnostic biomarkers for HLB status in commercial citrus trees.

Introduction
Numerous microorganisms (including bacteria, fungi and viruses) colonize the outside surfaces and inside tissues of plants and trees. These microbial communities are referred to as "microbiota". The composition of plant-associated microbiota can be measured using DNA-based tools and is a function of host factors, including species or cultivar, growth stage, plant compartment (root vs. leaf, inside vs. outside), plant health status and environment (e.g., soil type, nearby vegetation, climate, and application of fertilizers or fungicides). An unhealthy or diseased plant or tree often displays abnormal microbial patterns, a phenomenon referred to as dysbiosis⁵. It is not always clear whether these changes in the microbiota are a result of the disease, or whether differences in the microbiota lead to different disease outcomes. Citrus Research Board Project #5300-164 addressed this issue in the context of HLB. In #5300-164A (see report on page 52), Philippe Rolshausen, Ph.D., and his team explored the question of how variation in citrus microbiota (in particular, those living inside the tree) might contribute to the tolerance of trees to the HLB pathogen Candidatus Liberibacter asiaticus (CLas). In the other part of the project (#5300-164B, and subject of this Citrograph article), Johan Leveau, Ph.D. and his group have focused on the reverse, namely how CLas infection impacts the microbial community structure of citrus trees (in particular, those living on leaf and root surfaces).

HLB is one of the most devastating citrus diseases. It affects major citrus-producing areas worldwide and has been responsible for huge economic losses to the Florida citrus industry with the potential to do the same in California. The bacterium that is believed to cause this disease, CLas, is vectored by the Asian citrus psyllid (ACP), Diaphorina citri. Polymerase chain reaction (PCR) is the currently approved method for confirming CLas infection. However, due to the uneven distribution and low titer of CLas in an infected citrus tree, PCR is not an ideal CLas detection method. HLB diagnosis based on symptoms also is problematic, because the symptoms typically develop long after initial infection. Given that infected but asymptomatic trees represent a source of CLas, there is much interest in pre-diagnostic or early detection technologies (EDTs) to assess asymptomatic trees for CLas infection status. Early results showed that the leaf and root microbiota composition of CLas-infected citrus trees in the greenhouse were different from non-CLas infected citrus trees (Leveau et al. 2016), leading to the idea that CLas-infection status might be inferred from analysis of the microbial community composition. We focus here on a data set that captures the promise and challenges of this idea, in search of microbes that can serve as specific and sensitive early indicators of CLas infection in commercial orchard citrus trees.

Approaches
In collaboration with Rolshausen and researchers at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) in Fort Pierce, Florida, we sampled a total of 170 trees from 10 groves in central Florida in March 2016 (90 trees) and March 2017 (80 trees), representing various sweet orange and grapefruit cultivars. These groves were chosen because they showed variable degrees of HLB-like symptoms, as documented for each individual tree using a numeric scale ranging from 0 to 5 in 0.5 increments, with 0 being asymptomatic and 5 being severely symptomatic (Table 1). Disease ratings were based on assessment of yellowing, foliage loss, fruit production/drop and stunting. There was a general trend between disease rating and CLas titer as measured by qPCR (see report on page 52). Both leaf

<table>
<thead>
<tr>
<th>Year</th>
<th>Disease rating</th>
<th>Total # of trees</th>
</tr>
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<tbody>
<tr>
<td>2016</td>
<td>0.5 1 1.5 2 2.5 3 3.5 4 4.5 5</td>
<td>90</td>
</tr>
<tr>
<td>2017</td>
<td>0 9 5 5 3 24 10 6 6 12</td>
<td>80</td>
</tr>
</tbody>
</table>
swabs and root samples were taken from citrus trees for microbial profiling following an established protocol in the Leveau lab (Figure 1A). Microbial DNA was extracted from samples, amplified using bacterial 16S ribosomal RNA gene3 and fungal Internal Transcribed Spacer (ITS⁴) primers and sequenced using a high-throughput sequencer (Illumina MiSeq) at the University of California, Davis.

**Results**

Figure 1B is a graphic representation of the similarities and differences in microbial community composition in samples collected from the Florida citrus groves in 2016 and 2017. We found that the fungal communities associated with
leaves (green circles) were very different from those associated with roots (red squares) and soil (brown triangles). Root and soil samples overlapped in community structure (Figure 1B), which is consistent with the root microbiota being a subset of the soil microbiota. The tree compartment (leaf, root, soil) explained 25 percent of the fungal community differences, and we were able to predict with greater than 90 percent accuracy whether a sample was taken from leaves, roots or soil based on the fungal community information. Sampling year also correlated with fungal community composition (Figure 1B), indicating that citrus-associated microbiota are dynamic through time.

Location and citrus cultivar also impact leaf microbiota. Focusing on leaf microbiota only, we found that there was a strong location effect (Figure 2): samples from the same grove were more similar in terms of microbiota than they were to samples from other fields. We also observed a sampling year effect in that trees from one field showed similar fungal composition to other fields sampled in the same year, rather than to the same field sampled in a different year. This means that location and time (where and when a tree is growing) are important in terms of fungi that are available to colonize foliage. One of the locations sampled was the USDA-ARS facility in Fort Pierce, Florida, where different citrus cultivars are grown. When labeled by cultivar, samples from this facility (same location, same sampling year, i.e. 2016) separated roughly in two clusters: grapefruit (Jackson, Marsh and Triumph) and sweet orange (Valencia) (Figure 3). This is

![Figure 3. Cultivar impacts leaf fungal community structure. Leaf swabs were collected at the same location (USDA site at Fort Pierce, Florida) and in the same year (2016). PCoA was performed based on the Bray-Curtis distance matrix. Cultivars are labeled in different colors and shapes. Each data point represents a sample. The closer two points are, the more similar the fungal communities are between these two samples. The dashed line roughly separates orange samples from grapefruit samples.](image)

Figure 3. Cultivar impacts leaf fungal community structure. Leaf swabs were collected at the same location (USDA site at Fort Pierce, Florida) and in the same year (2016). PCoA was performed based on the Bray-Curtis distance matrix. Cultivars are labeled in different colors and shapes. Each data point represents a sample. The closer two points are, the more similar the fungal communities are between these two samples. The dashed line roughly separates orange samples from grapefruit samples.

![Figure 4. Fungal taxa that showed positive correlation with HLB disease rating. The relative abundance of the fungal taxa on citrus leaves is shown on the y-axis (per 3,000 counts) with disease rating on the x-axis.](image)

Figure 4. Fungal taxa that showed positive correlation with HLB disease rating. The relative abundance of the fungal taxa on citrus leaves is shown on the y-axis (per 3,000 counts) with disease rating on the x-axis.
consistent with the notion that host genotype also can be a driver of leaf microbial community structure (Whipps et al. 2008).

3 **HLB-related fungal signatures.** We mined the 2016 and 2017 leaf microbiota data for fungal taxa that were positively correlated with the level of HLB symptoms – i.e. whose relative abundance on leaf surfaces was significantly greater on trees with higher disease ratings than on trees with lower ratings. Six fungal taxa, including *Phoma calidophila*, showed strong and significant responses as seen in Figure 4. Some of these fungal taxa include foliar plant pathogens such as *Capnodium* (sooty molds, an indicator for insect infestation) (Klotz, 1978) and *Corynespora cassiicola* (causes leaf spot in cucumber, papaya, tomato) (Dixon et al. 2009). These fungal taxa could be explored further as potential biomarkers of CLas infection. Similarly, we identified several taxa that negatively correlated with HLB symptom-based rating (Figure 5). These taxa included non-pathogenic fungal species, such as representatives of the yeast genus *Sporobolomyces* (Last et al. 1965).

**Conclusion and outlook**

Project #5300-164B gave the Leveau lab an opportunity to educate many stakeholders, including growers, about the importance, complexity and potential use of plant-associated microbial communities in relation to citrus tree health. It laid the basis for a potential microbiota-based EDT for HLB. The need for EDTs remains urgent. With this project, we informed growers about the advantages and limitations of a microbiota-based EDT. This Florida project exposes the challenges of confounding factors such as location, time and cultivar in identifying HLB-specific biomarkers. In doing so, it highlights the need to establish a baseline understanding of the variation in microbial community composition across different locations, seasons and cultivars, to facilitate the identification of HLB-related microbial biomarkers specific for California conditions. 

**CRB Research Project #5300-164B**

**References**


Glossary

1Microbiota: The collection of all microbes including bacteria, fungi and viruses of a particular site or habitat.

2Dysbiosis: A microbial imbalance or maladaptation on or inside the host.

316S ribosomal gene: Gene common to all bacteria, useful for assigning taxonomic classification (species, genus, etc.).

4ITS: Abbreviation for internal transcribed spacer, which is the DNA sequence located between the small sub-unit ribosomal RNA (rRNA) gene and large sub-unit rRNA gene. This region is commonly used for fungal taxonomy assignment.

5Principal coordinate analysis: Method to explore and visualize similarities or dissimilarities within complex data sets.

6Bray-Curtis distance: Distance calculation commonly used in biology and ecology to quantify the compositional dissimilarity between two samples, named after J. Roger Bray and John T. Curtis.

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Reducing Breeding Time in Citrus Through Biotechnology

Gloria A. Moore, Katie L. Rogers, Qingchun Shi, Vicente J. Febres, José X. Chaparro and Ed Stover

Project Summary
Economically important traits for the improvement of citrus such as resistance or tolerance to huanglongbing (HLB) have been identified in several citrus types and relatives, and markers for these traits are being identified. In addition, early flowering citrus types for use in citrus variety breeding are urgently needed. Early flowering has been achieved in many plant species; however, this trait has been unexpectedly difficult to reproduce in citrus. This project is exploring alternative strategies for producing early flowering citrus types.
Citrus variety improvement using conventional methods (i.e., hybridizing two citrus types with desirable traits, growing out the resultant hybrid seedlings, evaluating the hybrids for improved tree and fruit characteristics and using selected hybrids in further crosses) is very time consuming. Despite several biological constraints, it is possible to produce many hybrids; both through the crossing of closely related citrus types and by crossing citrus varieties with more distantly related relatives (i.e. wide hybridization). However, many diverse traits in the citrus gene pool will take decades to exploit using only conventional breeding. The major constraint in citrus breeding is the lengthy period of juvenility (the time between planting hybrid seed and their development into flowering, fruiting trees), which can be eight years or longer.

The overall goal of our research is to develop and use early flowering citrus types to speed up the breeding process, making it possible to rapidly introduce valuable traits from citrus relatives or non-commercial citrus types into high-quality commercial varieties. Our original goal was to use transgenic methods to produce transgenic hybrids that flowered in one year or less. This method has been developed in annual plants and is being implemented in other perennial plants with long juvenile periods, including plums, apple and poplar (Tränkner et al. 2010). A rapid cycling method would make it possible to incorporate priority traits (such as HLB resistance) and should allow breeding to occur much more rapidly than is possible with conventional breeding alone.

We have conducted many experiments with citrus involving the use of molecular methods and genetic transformation. Most of the experiments were conducted with two genes that facilitate the identification of transgenic plants during the transformation process: the selectable kanamycin-resistance gene (nptII) which allows selection of transgenic plants since only they survive in the presence of the kanamycin antibiotic; and the β-glucuronidase (GUS) gene as a scorable marker, which produces a visible blue color in transgenic tissue when the appropriate substrate is present. In addition, a gene promoter that was expected to induce constitutive (always on) expression was used to control the flower-inducing transgene. The flowering gene that we have tested most extensively is called FLOWERING LOCUS T (FT). This gene is believed to encode the “florigen” flower inducer (the hypothesized hormone-like molecule responsible for controlling and/or triggering flowering in plants). Over-expression (expression of a transgene copy of an already present gene) of FTs in many different species has led to early flowering. There are several slightly different copies of this gene in citrus.

We cloned three citrus genomic sequences and characterized them. They have been named FT1, FT2 and FT3. FT1 and FT2 may be alleles (two forms of the same gene) at a single locus (location in the DNA). FT3 is clearly different and appears to be the FT gene whose expression is most closely associated with flowering. We have used all three of these to transform both tobacco, as a model species, with which we can get rapid results, and also some citrus types. In tobacco, transformation with any of the sequences leads to early flowering. When the citrus FT3 gene

Figure 1: Alemow grafts infected with CTV-FT3 flowering in March 2017 in Fort Pierce, Florida (A and detail in B), and in June 2017 in Gainesville, Florida (C-D). Alemow grafted on finger lime (Eremocitrus) is shown in panel D.
was overexpressed in citrus (‘Carrizo’ citrange, ‘Hamlin’ sweet orange and ‘Duncan’ grapefruit) with a constitutive promoter, we obtained flowering too soon, and transgenic plants were difficult to regenerate.

We are conducting a number of experiments to overcome our problem of too much FT gene expression in citrus, by concentrating on FT3. One general strategy is to try to "dial down" the expression of the transgene. We are testing other, non-constitutive (less intense) promoters. Some of these promoters limit gene expression to specific plant tissues – for example, only in the phloem (part of the plant vascular system), where FT is thought to be naturally expressed. Such localized expression may avoid production of FT that affects important plant processes other than flowering. For example, we have 13 transgenic ‘Carrizo’ plants with the phloem-specific citrus sucrose synthase (CitSS) promoter driving FT3 expression (CitSS::FT3) and 43 ‘Carrizo’ plants with 396SS::FT3 (another citrus phloem-specific promoter) that will be validated soon.

In addition, we are testing inducible promoters. We have 20 transgenic ‘Carrizo’ plants that have a PR1::CiFT3 (pathogenesis-related protein 1 gene promoter) that can be induced by application of salicylic acid. Finally, we have made a construct wherein FT3 is controlled by a promoter that should be induced only when the transgenic plants are exposed to a specific inducing agent, in this case, a pesticide that is already registered for use in citrus. The construct has been tested carefully to determine that it is correctly cloned. Both tobacco and citrus explants have been transformed with this construct, and we are awaiting further results. These were described previously (Moore et al. 2016).

A different approach uses a citrus tristeza virus (CTV) vector (which was partially constructed by others and generously provided to us) containing the FT3 gene for in planta expression. The virus can be delivered to already established plants, and the FT gene sequence is expressed in the citrus plant, but not incorporated into the genome; hence, this method does not require production and regeneration of transgenic plants via tissue culture. CTV inhabits the phloem, which is also the site of FT action. One CTV-FT3 vector was produced by Siddarama Gowda, Ph.D. (University of Florida, Citrus Research and Education Center) in collaboration with Ed Stover, Ph.D., and used to inoculate an alemow plant (Citrus macrophylla) (Folimonova et al. 2007). This plant was confirmed to have CTV-FT3 in all newly grown branches. Using budwood from this plant, a group of 90 seedlings (largely pomelo hybrids) were graft-inoculated from July to August 2016 in Fort Pierce, Florida (Figure 1 A-B). From December 2016 to January 2017, two plants showed flowering at the grafting site. Thirty additional trees, mainly ‘Pineapple’ sweet
orange, 'Duncan' grapefruit, sour orange and ‘Kuharski’ rootstock have been graft-inoculated in Gainesville, Florida, using a different CTV vector construct (provided by Svetlana Folimonova, Ph.D., University of Florida, Plant Pathology Department) and confirmed to be CTV-FT3 positive (Figure 1 C-D). So far, early flowering has not been observed on varieties other than alemow.

FT is only one gene of many involved in the process. There are other naturally occurring genes that also promote flowering and some genes whose expression delays flowering. Therefore, we are testing methods to reduce the production of an important naturally occurring citrus "flower delaying" gene using RNA interference technology, where the RNA product of a transgene reduces the expression of a target gene by inducing degradation of its RNA. Two hair-pin RNA3 constructs of different lengths [156 base pairs (bp) and 313 bp] were used to silence the terminal flower gene (TFL1), which delays flowering. Transformation with these constructs has been completed, and 46 ‘Carrizo’ transgenic plants were obtained. These plants are being tested for early flowering. In addition, we are using a non-transgenic approach through the direct application of chemically synthesized double-stranded RNA (dsRNA) to induce the degradation and inhibit the expression of citrus genes. In one test, as proof of concept, application of a dsRNA designed to target CsPDS (involved in chlorophyll synthesis) resulted in a photo-bleached appearance (Figure 2 A-B), along with reduced CsPDS expression and chlorophyll content (Figure 2 C-D). This suggests that dsRNA could be applied to plants to effectively regulate citrus genes. To test this, a 150-bp dsRNA targeting TFL1 was applied to Persian lime plants as a soil drench (200 µg dsRNA per pot weekly). The lime plants, under greenhouse conditions, flowered after 11 treatments (Figure 2 E-F).

**Future Directions**

In future experiments, we will explore two additional approaches. One is the use of a genome editing method called CRISPR/Cas9. This technology allows very precise editing or addition of citrus genes without needing to insert sequences from other organisms (e.g., bacterial or viral DNA), a step

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Figure 3: Early flowering individuals were identified from a two-month-old F2 mapping population derived from crosses between 'Pineapple' sweet orange and Eremocitrus glauca planted from seed in an insect proof greenhouse (A). Photographs were taken in November 2015, with a greenhouse temperature setting of 80°F. Two-month-old seedling flowering in seedling tray (B). Appearance of early flowering seedlings (C and D). Fruit development (E).
necessary with Agrobacterium-mediated transformation. This is not only more precise, but also avoids the presence of foreign DNA that is subject to federal regulation. The other approach is to hybridize valuable varieties with non-transgenic early flowering mutants that we have identified. Again, such hybrids would not be regulated for field planting.

We hope with these additional strategies, we will be able to rapidly produce and test genes of interest that may induce the early flowering characteristic that is needed for shorter breeding times. Then we can concentrate on the rapid production of improved citrus types, using crosses between citrus varieties and with citrus relatives, to provide new alternatives to citrus breeders and producers, especially HLB tolerance.

CRB Research Project #5200-146

References


Glossary

1Transgenic Methods: The process of introducing foreign DNA into a host organism's genome (its complete set of genetic material). The DNA that is transferred to the recipient can be from other individuals of the same species or even from unrelated species.

2Gene Promoter: a DNA sequence that determines how much, where and under what conditions a gene product is made.

3Hairpin RNA: A short fragment of RNA that can bind to itself due to a complementary sequence making a “hairpin” type of structure; this type of RNA fragment can be used to silence target gene expression via RNA interference mechanisms.

Gloria A. Moore, Ph.D., is an emeritus professor in the University of Florida (UF) Horticultural Sciences Department (HOS) and the Plant Molecular and Cellular Biology Program, Katie Rogers is a Ph.D. student in UF HOS, Vicente Febres, Ph.D., is a research assistant scientist at UF HOS and José X. Chaparro, Ph.D., is an associate professor at UF HOS. Qingchun Shi, Ph.D., is a research biologist and Ed Stover, Ph.D., is a horticulturist and geneticist at the U.S. Horticultural Research Laboratory (USDA-ARS) in Ft. Pierce, Florida. For additional information, contact gamoore@ufl.edu
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The Quest for a Non-vector Psyllid

A new management strategy based on variation in pathogen transmission by Diaphorina citri

Michelle Heck, El-Desouky Ammar, Kathy Moulton and David Hall
Project Summary

Huanglongbing (HLB) is a citrus disease involving citrus host trees, an insect vector¹ called Diaphorina citri, known as the Asian citrus psyllid (ACP), and a bacterial pathogen known as ‘Candidatus Liberibacter asiaticus’ (CLas). HLB, also known as citrus greening disease, is considered the most devastating of all citrus diseases, and there is currently no adequate control strategy. In this work, a team of Agricultural Research Service (ARS) scientists showed that different psyllid populations vary in their ability to acquire and transmit CLas. Some populations transmit the pathogen very efficiently and others not at all. The ability or inability to transmit the pathogen is passed on from parents to offspring for several generations, proving that psyllid genes regulate the spread of the CLas. These populations varying in transmission competency will hopefully enable scientists to develop new ways of halting the spread of CLas to new citrus trees in Florida, Texas, California and other citrus growing regions of the world.

In the U.S., the bacterium associated with HLB is CLas. ACP (Figure 1), is the primary insect vector for CLas in North America. HLB management has eluded citrus growers in Florida for more than ten years, and the disease has significantly affected commercial citrus production across the state. A team of ARS scientists is focusing on research that could prevent new plantings from becoming infected.

CLas is transmitted by ACP in a circulative, propagative manner. The bacterium must be acquired through feeding of the psyllids on infected citrus, followed by infection of the insect gut. Bacteria then replicate within the insect prior to infecting a new host tree (Inoue et al. 2009; Ammar et al. 2016). Thus, the CLas bacterium infects both the plant and the insect vector (Figure 2). Our team has shown that CLas infection levels in the insect correlate with the ability of the insect to transmit the bacterium to new citrus plants (Ammar et al. 2018).

Interestingly, despite the high disease pressure in Florida, two independent field surveys have shown that the percent of CLas infection in ACP varies widely across commercial groves (Coy and Stelinski 2015; Hall 2017). Our team hypothesized that the variation in CLas infection rates in the psyllids is genetically controlled; ACP genes² regulate whether the psyllid will be a competent vector, capable of efficiently transmitting CLas, or a poor or non-vector³, incapable of efficient transmission. Proving that there is a genetic basis for variation in CLas transmission by ACP is critical to develop new HLB management strategies that can block tree-to-tree transmission by ACP. Of special interest to us was the identification of a non-vector psyllid, one incapable of becoming infected with or transmitting CLas.

To test for a genetic basis underlying CLas transmission, we established ACP isofemale lines⁴ from wild psyllids collected throughout Florida. Each isofemale line descended from a single female and male pair. Isofemale lines are important for these studies so we can test whether any traits⁵ relating to CLas interactions are passed on from one generation to the next. We collected live ACP adults in Fort Pierce, Port St. Lucie, Homestead and Fort Myers between the summer of 2014 and the fall of 2015. The isofemale lines

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Figure 1. Developmental stages of Asian citrus psyllid: A: Adult in feeding position. B: Tiny eggs (and a newly-hatched nymph) laid by adult females on a very small semi-folded citrus leaf. C: Young nymphs feeding and excreting waxy honeydew on the lower surface of a citrus leaf.
we established were all CLas-free, which enabled us to test for various parameters of CLas transmission (Ammar et al. 2018). A total of 15 isofemale lines were established for the experiments (Figure 3).

The first step of CLas transmission is the acquisition⁶ of the CLas bacterium by the psyllid. To test for CLas acquisition, 50-75 adult psyllids from each isofemale line were caged onto either healthy or CLas-infected, flushing citrus plants. The females were allowed to lay eggs on the flushing plants, and the emerging nymphs were permitted to develop to adulthood on these plants. An average of 100 insects from each isofemale line were tested for the presence of CLas over the course of several generations, and insects from the healthy plants were sampled as a control for every line. We found that the isofemale lines varied in their ability to acquire CLas from infected trees (Figure 4). Some lines acquired CLas more than 30 percent of the time, and other lines acquired it less than five percent of the time. Remarkably, this variation was passed on across generations, proving that ACP genes regulate the rate of CLas acquisition.

Next, we tested whether variation existed in the ability of ACP to inoculate⁷ new plants with CLas. To do this, we used the excised leaf transmission assay (Ammar et al. 2013), which streamlines the time needed to study CLas acquisition by ACP from six months to two weeks. In this test, we placed 6-10 individual psyllids from each isofemale line onto excised citrus leaves and placed these leaves into individual tubes to contain the insects. The psyllids are allowed a one-week feeding period, after which the midrib, or center vein, of the leaf is removed and tested for CLas. Our results show that the isofemale lines varied in their ability to transmit CLas into citrus leaves and that there was an excellent correlation between the acquisition rate and the transmission rate for each line. Moreover, we obtained evidence in support of our hypothesis that ACP genes regulate CLas transmission – CLas inoculation rates for each isofemale line were passed on through several generations.

**Conclusion**

With the knowledge that CLas acquisition and transmission rates in ACP are variable and heritable, where does the research go from here? What are the next steps? What are the benefits of this new knowledge for citrus growers in California and elsewhere?

Generating a non-vector psyllid using transgenic⁸ technology was a major goal of the U.S. Department of Agriculture and the Florida Citrus Research and Development Foundation nu-Psyllid project. Although the project produced a great deal of new information about the psyllid-HLB pathogen interaction, the project team was not able to generate the transgenic psyllid that did not transmit CLas. Moreover, any insect that is genetically modified would face regulatory hurdles for eventual deployment into the field. A gene drive⁹ system would be needed to ensure success of spread of the transgene10 in the population. There are other challenges to this potential achievement, although none would be unsurmountable. In fact, transgenic technology is gaining traction to control insect vectors of animal viruses. A small Florida company called Oxitec (www.oxitec.com) has developed technology to produce a transgenic mosquito that would help to eliminate *Aedes aegypti*, the insect vector of the Zika virus and other serious animal-infecting viruses. One limiting factor for development of transgenic ACP to control HLB is the length of time necessary for the development and regulatory approval of a transgenic ACP technology to block CLas transmission.
Our work shows a clear path to a non-vector psyllid is possible without transgenic technology because non-vector psyllids exist in nature. Future work should focus on identifying the ACP genes that regulate CLas acquisition and transmission. Additional research is needed to test whether the isofemale lines we established show similar transmission for other isolates of CLas and when the psyllids are reared on other citrus host plants. Breeding non-vector psyllids for mass release should be the goal of future work in this area. Flooding the gene pool with insects that are resistant to CLas infection may be one way to tip the balance in favor of blocking transmission into new plantings. Some studies have shown that in the lab, CLas infection may have positive fitness benefits for the psyllid: in other words, it benefits the psyllid in some way to carry CLas (Pelz-Stelinski and Killiny 2016). However, very little is known about the ecology of the interaction between CLas and ACP in field populations, and the fact that many psyllids collected from the field are not infected suggests that carrying CLas may not be beneficial to all psyllids under all conditions.

Nothing is known about the acquisition and transmission rates of California ACP. Our labs are beginning these tests with ACP from California. Information about the potential of California psyllids to spread CLas may help limit the spread of HLB in the state. In the long-term
development of management strategies to control HLB, we hypothesize that research on strategies to block CLas transmission into new citrus plantings will be paramount to the U.S. citrus industry.

CRB Research Project #5300-163

References


Glossary

1. **Vector**: An insect capable of spreading a pathogen.
2. **Genes**: Units of heredity that are passed from parent to offspring which encode information about traits of the organism.
3. **Non-vector**: An insect incapable of spreading a pathogen.
4. **Isofemale lines**: Insect lines developed from single fertile females collected from the wild and maintained in the lab.
5. **Traits**: Genetically determined characteristics.
6. **Acquisition**: The process of an insect vector taking up a pathogen by feeding on infected materials.
7. **Inoculate**: The process during which an insect vector infects a new plant with a pathogen.
8. **Transgenic**: An organism with DNA from a different organism.
9. **Gene drive**: A genetic engineering approach that causes a gene to spread in a population.
10. **Transgene**: A piece of DNA inserted from one organism into another.

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"For apple growers like myself, a successful season is directly related to the quality of apples. Not only a blemish free, gleaming vibrant apples are appealing but they also can be sold at a higher price. Now with the PAL-HIKARI series, I can take Brix measurements while the fruits are on the tree, without cutting and crushing the fruits. I can keep the apples on the tree as long as needed, until it is exactly at the peak of its maturity. These precious fruits no longer have to be wasted"
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Update On Disease Forecasting and Management Of Septoria Spot Of Citrus

James E. Adaskaveg and Helga Förster

Septoria spot of citrus caused by the fungus Septoria citri has been of economic concern for California citrus exporters since 2003, when diseased fruit was detected in shipments to Korea where the fungus is a quarantine pathogen.

Previously, Septoria spot had been considered a minor disease. Little new information had become available since the 1940s-60s when the last California outbreaks occurred. Import restrictions for California oranges shipped into Korea initially required mandatory copper applications in the field, special packinghouse guidelines and the establishment of a fruit certification program (the Navel and Valencia Export to Korea program – NAVEK) (Adaskaveg and Cranney 2011, Adaskaveg and Förster 2011). Under a new 2010 work plan, NAVEK is now a voluntary program, but the Good Agricultural Practices (GAPs) that were developed are still strongly recommended to ensure that no citrus with S. citri fruiting structures arrive in Korea.
Closure of the Korean export market was an emergency situation that initiated research efforts to better understand, detect and manage the disease. Achievements include a quantitative polymerase chain reaction (qPCR)²-based system to detect the pathogen in infected fruit and properly diagnose disease when no signs of the pathogen are present and culturing the fungus is often difficult. We also identified and helped register five new pre-harvest fungicides (Adaskaveg and Förster 2016) for the management of the disease that are best used following a copper application at the beginning of the harvest season. In post-harvest research, we identified fungicides that are very effective in preventing fungi from producing spores and help to meet export requirements (Adaskaveg and Förster 2016). To help growers improve the timing of fungicide applications and as part of the GAPs, an environmental monitoring and disease forecasting program was developed.

A major outcome of our Septoria spot research was an improved understanding of the environmental conditions affecting fruit infection. This led to the development of an epidemiological model² for predicting disease risk by assessing favorable periods for infection and, subsequently, the most effective timing of fungicide applications in the field. It already was established in the literature that low temperatures are a critical determinant for the occurrence of Septoria spot; (Klotz and DeWolfe 1955); however, these environmental conditions had to be more exactly defined. For our modeling, environmental data to forecast disease were evaluated over several years and then applied and correlated with actual disease levels to validate the model (Adaskaveg and Förster 2011). Thus, much of the data used to develop the model were collected from the beginning of the project in 2003-2009 with the closure of the Korean export market. Use of the model has continued through 2017-18, allowing forecasts to be made each winter harvest season.

The first steps to model conditions for disease development were laboratory studies where oranges were subjected to selected temperatures between -2⁰C and -20⁰C for different time periods between four hours and 30 hours. Fruit then were incubated and observed for the development of cold-temperature injuries (“ice marks”) after seven days. Data graphing indicated that, as expected, cold damage increased with decreasing temperature and increasing time exposure (Figure 1). But more importantly, exposure of fruit for ten or more hours to moderate freezing temperatures of -2⁰C to -4⁰C that commonly occur in most winters in inland California citrus growing areas resulted in a definite increase in “ice mark” development. Therefore, this temperature range and time duration became the threshold for risk assessment.

Furthermore, we demonstrated that cold damage to fruit creates entryways for S. citri. After inoculation with conidia³, only fruit with “ice mark” developed symptoms of Septoria spot, but not fruit without such injuries (Mila et al. 2005). Additionally, when environmental conditions for low- and high-disease years were compared, high-disease years had significantly longer cold-temperature periods than...
low-disease years (Figure 2). Disease data in these comparisons were based on the incidence of Septoria spot detections in fruit submitted to the NAVEK fruit certification program.

The amount of precipitation occurring after the first low-temperature period was established as another important determinant for disease development. Rainfall and subsequent wetness in an orchard promotes fungal growth and sporulation and facilitates splash-dispersal of conidia to ripening fruit from dying or dead plant material on the tree where the fungus survives. There were clear positive statistical correlations⁴ between disease progress over the harvest season (Figure 3A), as well as the average amount of precipitation and the incidence of disease in submitted NAVEK samples (Figure 3B) for three winter seasons when disease progress was offset by 30-45 days from initial precipitation events. Disease incidence (natural log⁵ of incidence was used to linearize the graph) was plotted against accumulated precipitation after fruit were exposed to temperatures of less than -1°C.

This information ultimately was assembled into a simple numerical compartmental model⁶ based on the accumulation of both average number of hours less than or equal to -1°C and average daily total precipitation from several environmental monitoring stations in each county to predict disease risk. The California Irrigation Management Information System (CIMIS), as well as municipal and private weather monitoring systems, were used to obtain this information. Initially, this was done for counties in the San Joaquin Valley (Madera, Fresno, Tulare and Kern) and has been since expanded to coastal (District 2) and desert (District 3) counties with one to five monitoring stations per county.

The model is initiated at the beginning of the Navel orange season in mid-October and continues through mid-March. Environmental data are summarized daily for each county using a spreadsheet; and color-coded, numerical risk values are assigned using the compartmental model developed for forecasting Septoria spot risk (Figure 4). As more hours of low temperatures (i.e., less than or equal to -1°C) accumulate, the risk of disease increases even with low amounts of accumulated precipitation. This is indicated by an increasing color-coded numerical risk that goes from white (0), green (1), yellow (2) and red (3) to dark gray (4). Similarly, with less than ten hours of accumulated temperatures below -1°C, higher amounts of precipitation – 31-60, 61-90, 91-120, 121-150 or more than 151 millimeters (mm) – also increase the numerical risk to 0, 1, 2, 3 or 4, respectively. Thus, the interaction of accumulated hours of temperatures below -1°C and increasing precipitation increases the risk of disease (Figure 4).

Application of this model has been continuous throughout this project and the NAVEK program operation. The model has allowed forecasts to be made to help growers optimize timing...
Table 1. Summary of risk assessment model for Septoria spot of citrus in California - 2016-17 season.

<table>
<thead>
<tr>
<th>County (No. of stations)</th>
<th>Date</th>
<th>Total Hrs &lt; -1°C</th>
<th>Average accumulated precipitation (mm)</th>
<th>Risk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresno (3)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>46.7</td>
<td>0</td>
</tr>
<tr>
<td>Tulare (1)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>42.8</td>
<td>0</td>
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<tr>
<td>Kern (2)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>13.1</td>
<td>0</td>
</tr>
<tr>
<td>W. Riverside (2)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>26.0</td>
<td>0</td>
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<tr>
<td>Ventura (3)</td>
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<td>0.0</td>
<td>47.0</td>
<td>0</td>
</tr>
<tr>
<td>Santa Barbara (4)</td>
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<td>53.5</td>
<td>0</td>
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<tr>
<td>San Luis Obispo (3)</td>
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<td>0.0</td>
<td>98.4</td>
<td>1</td>
</tr>
<tr>
<td>Imperial (3)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>0.4</td>
<td>0</td>
</tr>
<tr>
<td>San Diego (5)</td>
<td>12-1-16</td>
<td>0.0</td>
<td>34.9</td>
<td>0</td>
</tr>
<tr>
<td>Fresno (3)</td>
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<td>120.4</td>
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</tr>
<tr>
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<td>44.9</td>
<td>1</td>
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<td>W. Riverside (2)</td>
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<td>90.3</td>
<td>1</td>
</tr>
<tr>
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<td>120.2</td>
<td>2</td>
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<tr>
<td>Santa Barbara (4)</td>
<td>12-28-16</td>
<td>22.0</td>
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<tr>
<td>San Luis Obispo (3)</td>
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<td>Imperial (3)</td>
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<td>0</td>
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<tr>
<td>San Diego (5)</td>
<td>12-28-16</td>
<td>2.0</td>
<td>111.0</td>
<td>2</td>
</tr>
</tbody>
</table>

* Risk is defined as the potential for disease following the numerical risk assessment, environmental accumulation model (shown in Figure 3).

Table 2. Summary of risk assessment model for Septoria spot of citrus in California - 2017-18 season.

<table>
<thead>
<tr>
<th>County (No. of stations)</th>
<th>Date</th>
<th>Total Hrs &lt; -1°C</th>
<th>Average accumulated precipitation (mm)</th>
<th>Risk*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresno (3)</td>
<td>1-3-18</td>
<td>14.0</td>
<td>21.0</td>
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<td>Tulare (2)</td>
<td>1-3-18</td>
<td>56.5</td>
<td>11.5</td>
<td>0</td>
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<tr>
<td>Kern (2)</td>
<td>1-3-18</td>
<td>35.0</td>
<td>3.6</td>
<td>0</td>
</tr>
<tr>
<td>Fresno (3)</td>
<td>1-10-18</td>
<td>14.0</td>
<td>43.5</td>
<td>1</td>
</tr>
<tr>
<td>Tulare (2)</td>
<td>1-10-18</td>
<td>56.5</td>
<td>29.6</td>
<td>3</td>
</tr>
<tr>
<td>Kern (2)</td>
<td>1-10-18</td>
<td>35.0</td>
<td>21.9</td>
<td>2</td>
</tr>
<tr>
<td>W. Riverside (2)</td>
<td>1-10-18</td>
<td>0.0</td>
<td>62.6</td>
<td>1</td>
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<tr>
<td>Ventura (3)</td>
<td>1-10-18</td>
<td>0.0</td>
<td>64.3</td>
<td>1</td>
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<tr>
<td>Santa Barbara (3)</td>
<td>1-10-18</td>
<td>45.0</td>
<td>69.4</td>
<td>3</td>
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<tr>
<td>San Luis Obispo (2)</td>
<td>1-10-18</td>
<td>0.0</td>
<td>93.6</td>
<td>2</td>
</tr>
<tr>
<td>Imperial (3)</td>
<td>1-10-18</td>
<td>0.0</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td>San Diego (5)</td>
<td>1-10-18</td>
<td>0.0</td>
<td>54.9</td>
<td>0</td>
</tr>
</tbody>
</table>

* Risk is defined as the potential for disease following the numerical risk assessment, environmental accumulation model (shown in Figure 3).

Figure 4. Numerical risk model for forecasting Septoria spot based on accumulated hours of fruit exposure to temperatures of less than -1°C and total amount of precipitation. Using the model a risk alert is posted when a risk factor reaches two or higher. Fungicide applications are recommended within 30 days after a risk factor of three is reached or after a risk factor of two is reached and high rainfall is forecasted for a county.
In summary, forecasting Septoria spot is an integral part of the NAVEK program that is a showcase of cooperation between regulatory agencies, citrus industry organizations and the University of California.

CRB Research Project #5400-119

References


Glossary
1Quantitative polymerase chain reaction (qPCR): A laboratory technique of molecular biology based on the polymerase chain reaction that can be used to detect and quantify DNA (genetic blueprint) of organisms.

2Epidemiological modeling in plant pathology: Mathematical method for assessing the potential damage to an agricultural crop from a plant disease.

3Conidia: Asexual spores or propagules produced by certain fungi.

4Statistical correlation: A measure that indicates the extent to which two or more variables are closely or distantly related.

5Natural log: Logarithm to the base e (2.71828…). A mathematical way to describe the dynamics of natural phenomena, like population growth or disease development, during an epidemic.

6Compartmental model: Technique used to simplify the mathematical modelling of infectious diseases. Compartmental models may be used to predict properties of how a disease increases or spreads in a geographical region.

James E. Adaskaveg, Ph.D., is a professor of plant pathology and Helga Förster, Ph.D., is a project scientist in the Department of Microbiology and Plant Pathology at the University of California, Riverside. For additional information, contact jim.adaskaveg@ucr.edu
UNTIL THEY FIND A CURE...
WE CAN PROVIDE A CONTROL

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

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

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

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

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

Development of Blood Orange and Cara Cara-like Cultivars

Kasturi Dasgupta, Min Shao and James Thomson

Project Summary

Blood orange and Cara Cara-like citrus cultivars with purple or red fruit color, increased antioxidants and modified flavor potentially can be the next generation of cultivars produced via genetic engineering (Dasgupta et al., 2015, 2016). The colored fruit are superior to other navel orange varieties in health benefits and are often in high demand when in season (Khoo et al., 2017). Despite increasing consumer interest, production of these citrus varieties remains unreliable due largely to a dependency on cold temperature during fruit maturation for full color formation. We proposed to develop these varieties using citrus genetic elements by targeting anthocyanin (Blood orange) and lycopene (Cara Cara) metabolic pathways and using modern molecular biology tools and methods. Citrus-specific promoters and genes were used to activate anthocyanin and lycopene pathways in different varieties of citrus such as Carrizo and Mexican lime. Anthocyanin accumulating lines are currently in process for field trial evaluation.

Plant genetic engineering requires precise transgene expression for various biotech applications. Constitutive promoters that activate gene expression throughout the entire plant are useful for initial expression studies, but they may not be the best choice for modifying fruit quality characteristics in a commercial variety due to the energy drain on the plant leading to stunted growth and/or low fruit production. Fruit-specific promoters are important tools for improving fruit quality traits by expressing genes associated with traits of interest only in fruit. These promoters can be identified and selected based on ability to express genes at the time of fruit development, when needed for coloring, which requires much less energy from the plant. Five candidate fruit-specific promoters were identified and tested in this project. Isolated
promoter regions were fused with the GUS reporter gene and were introduced into tomato for evaluation of promoter activity in their fruit. These promoters showed different levels of expression and varying degrees of specificity.

### Summary of Past Research

**Accomplishments**

Anthocyanin genes (necessary for the purple coloring in blood oranges), known as MybA, were identified and confirmed to be functional in plants. The activity of MybA genes obtained from four plant species (citrus, plum, grape and Arabidopsis) were examined in transgenic tobacco and citrus, (Collier et al. 2017; Dasgupta et al. 2017).

This work showed that the citrus (Moro blood orange) MybA gene clearly activated the anthocyanin biosynthetic pathway in transgenic tobacco and Mexican lime fruit. Therefore, the MoroMybA has been used for the rest of the project to develop a blood orange-like trait in other citrus cultivars. Also identified were the genes necessary for production of lycopene pigment, the compound responsible for the red coloring in Cara Cara oranges. However, due to a more complex metabolic pathway, results could not be visualized in tomato.

### Summary of Current Research

**Accomplishments**

1. Five fruit-specific citrus promoters were identified, with their genomic sequences isolated and assembled for testing. Candidate fruit-specific promoters were identified and selected from a known citrus gene expression database that show fruit-specific expression. All the candidate genes are highly expressed in fruit and flower with some degree of expression in peel, but not in leaves. The conclusion was that the promoter sequence for the corresponding genes was predicted and a 2,000-base pair sequence upstream of the gene start was used for promoter:GUS testing.

2. The functionality of the candidate fruit-specific promoters in tomato plants was tested. Results showed that candidate promoter sequences were active and capable of producing GUS expression (blue staining) specifically in tomato fruit.

3. Functional citrus fruit-specific promoters and citrus-derived MoroMybA gene were combined to generate multiple independent transgenic citrus plants for enhanced accumulation of anthocyanin.

Most citrus types need 5–15 years to begin flowering and fruiting. The long juvenile phase delays regular fruit production for years. Alternatively, early flowering has been achieved in transgenic trees, including citrus plants, by turning on a flower gene. The FLOWERING LOCUS T (FT) gene is a key regulator of flower timing. Therefore, to speed up study time, the FT gene was introduced into our system to reduce the long juvenile phase and more quickly assess citrus fruit accumulating anthocyanin (Figure 1).

Transgenic Mexican limes with early flowering phenotype and anthocyanin accumulation in fruit were produced (Figure 2). The pattern, distribution and level of anthocyanin accumulation in young immature transgenic Mexican lime varied among the different lines and developmental stages. The transgenic lines showed rapid time to first flowering, approximately 72 weeks after planting in soil.

### Conclusion

Native citrus genes and promoters can be used to successfully modify fruit for enhanced heritable trait profiles. Use of native citrus DNA to modify citrus traits may increase consumer acceptance of biotech citrus fruit.

1. Selected candidate promoters (CitWAXp, CitJuSacp and CitUNKp) were combined with native citrus genes, MoroMybA to develop blood orange-like cultivars.
2. Single copy Mexican lime lines identified in this project will help with federal deregulation procedures.

3. Higher FT expression exhibited early flowering and fruiting phenotype in transgenic Mexican lime lines.

**Ongoing work**

Production of transgenic citrus (Mexican Lime and sweet orange) containing the lycopene biosynthetic genes using candidate promoters (CitWAXp, CitJuSacp and CitUNKp) is completed and under evaluation.

**Figure 2.** Comparison of the visible phenotypes in 70-80 week old fruit from transgenic Mexican limes. MoroMybA gene is abbreviated as MM.

**Future Directions**

New Mexican lime and Carrizo citrus cultivars were generated to produce blood orange and Cara Cara-colored fruit using native citrus genes and promoters. These cultivars are now being used to help standardize regulatory procedures and, hopefully, increase consumer acceptance as the global impact of this nutritionally enhanced staple fruit is realized.

» **Field trial.** Selected characterized single copy number transgenic citrus lines expressing fruit-specific promoter-driving MybA are ready for field trials as is the regulatory paperwork needed. Clones of transgenic single copy
citrus lines currently are being generated in greenhouses at the U.S. Department of Agriculture (USDA)-Agricultural Research Service Laboratory in Albany, California, and will be transferred to an as yet undecided location for field evaluation.

**Permits.** Determining the exact procedure for getting approval is part of the learning process we hope to pass on to the Citrus Research Board (CRB) for potential future transgenic citrus projects. Sequence selected transgenic citrus line genomes generated in this project for APHIS deregulation.

**Intellectual Property (IP) rights and commercialization.** Based on field trial results, transgenic citrus lines will require IP protection for commercialization. A contract established between the CRB and USDA-ARS will help in determining these IP rights.

CRB Research Project #5200-141

**References**


Kasturi Dasgupta, Ph.D., was an associate scientist with the Citrus Research Board, housed on the campus of USDA-ARS, Albany, California. Min Shao, Ph.D., is a post-doctoral research fellow and James Thomson, Ph.D., is a research geneticist with the USDA-ARS Crop Improvement and Genetics Research Unit in Albany, California. For more information, contact james.thomson@ars.usda.gov

---

**Glossary**

**1Transgene:** Gene that has been transferred from the genome of one species into that of another.

**2Promoters and genetic elements:** Controls when, where and how much genes will be expressed

**3GUS:** Reporter gene that stains the tissue blue and allows one to see where, when and how much the promoter turns on.

**4Tomato:** Used as a model fruit testing crop due to its rapid fruit production and short life cycle.

**5Arabidopsis:** A small flowering plant related to cabbage and mustard; it is a model organism for plant biology studies because of its small genome size and rapid growth cycle.

**6Transgenic:** An organism containing genes or genetic material introduced from a different organism into its own genome (by artificial means).
MicroRNAs (miRNAs)¹ are small (about 22 nucleotides²) single-stranded RNAs that are common in all higher, complex organisms (e.g., plants and animals). They help to regulate gene expression in higher organisms by targeting and interfering with the activity of specific RNAs, but in recent years, they also have been used for RNA interference (RNAi³) applications to affect natural gene regulation and, thereby, induce desired traits in plants and animals. Our approach is to target specific gene expression in the Asian citrus psyllid (ACP or Diaphorina citri), the insect vector of ‘Candidatus Liberibacter asiaticus’ (CLas), which is the causal agent of huanglongbing (HLB), and then induce desired negative phenotypes⁴ or mortality. We are using two approaches – expressing artificial miRNAs (amiRNAs)⁵ to affect natural D. citri RNAs, and expressing antagomirs⁶ to target specific miRNAs in D. citri. Some of the antagomirs that we have tested so far show promise and significantly lowered the accumulation level of targeted D. citri miRNAs. Some also caused mortality of D. citri.

Figure 1. Experimental steps. The predicted miRNA and their target sequences identified from deep sequencing results were next verified in Drosophila S2 cells. The confirmed miRNAs were then used to design and synthesize specific antagomirs for micro-injection experiments in whole Diaphorina citri insects. The injected D. citri were used for further analysis including gene expression and screening for desired phenotypes including mortality. The selected miRNAs can be applied to produce transgenic citrus plants in the future. The illustration of deep sequencing was acquired from Lin et al. (2016).
Artificial microRNAs and Expression Vectors

MicroRNAs are small RNAs generated by one of the natural cellular RNA interference pathways. They help to regulate gene expression in higher organisms by targeting and interfering with the activity of specific RNAs. Several recent efforts have shown that amiRNAs can be used to intentionally manipulate gene expression and induce desirable phenotypes in plants and animals. Various tools have been developed and optimized for expressing miRNAs, both for fundamental research and translational applications (Liu and Berkhout 2011); however, all are not equal. Transgenic approaches primarily have been used for amiRNA expression in plants, but these can be time-consuming and expensive. Plant viruses could offer several advantages for expressing amiRNAs in whole plants, including faster and more economical means of application.

We tested three gene expression systems – a plant DNA virus, a plant RNA virus and a plasmid used for transgenic plants – for their abilities to express D. citri-specific amiRNAs in plants. Our results showed that the nuclear-replicating DNA plant virus expressed the highest quality and quantity of the desired amiRNAs. With the success of developing this tool for plant-based expression of amiRNAs, we are now attempting to express specific amiRNAs or anti-miRNA sequences to disrupt gene regulation in D. citri, and induce desired, negative phenotypes.

Deep sequencing analysis for miRNAs and target genes in D. citri

To ensure that our selection of amiRNAs and RNA targets was correct, we next focused on identifying authentic miRNAs and their RNA targets in D. citri. We took advantage of the recent breakthroughs in next-generation sequencing (NGS) technologies to analyze D. citri. We have so far identified eight different miRNAs and their target RNAs by bioinformatics analyses of the sequence data. We then confirmed the presence of the miRNAs and directly detected them in D. citri. To confirm that the miRNAs affected gene expression as predicted, we expressed the miRNAs and corresponding target RNAs in S2 cells of the fruit fly Drosophila melanogaster, which is a model insect species. This combination of approaches provided valuable information about miRNA profiles and RNA expression patterns in D. citri, and validated our approach to identify important D. citri targets for further studies. (Figure 1).

Antagomirs

Previous research in animals, including insects, has shown that directly interfering with the miRNA regulators of gene expression also can provide an efficient and straightforward way to block small RNA functions (Hutvágner et al. 2004) and cause specific desired phenotypic changes (Xiong et al. 2016; Zhang et al. 2016). For example, Zhang et al. (2016) showed that by targeting miR309 in the mosquito Aedes aegypti, ovarian development was interrupted and affected mosquito reproduction. Thus, our idea is that in addition to using amiRNAs to target specific D. citri RNAs, if a given miRNA is critical for normal D. citri gene regulation, it might be possible to directly target and interfere with the miRNA. If so, we also could use this approach to achieve desired phenotypic effects. Thus, we evaluated this as a second strategy to alter D. citri gene expression. Chemically-modified small RNAs were designed to be complementary and bind to the naturally occurring target miRNA in D. citri. These small RNAs are referred to as antagomirs (Krützfeldt et al. 2005) (Figure 2).

We designed three specific antagomirs – anti-miR317, anti-miR277 and anti-miR34 – based on our D. citri miRNA identification above. We separately injected these antagomirs and a negative control with a random sequence into D. citri whole insects and used RT-qPCR, which first reverse transcribes RNA into DNA, and then the DNA was used for quantitative real-time polymerase chain reaction, to analyze the accumulation level of each targeted miRNA (Figure 3). We also assessed phenotypic responses in the injected D. citri over time. The D. citri injected with the antagomirs showed much lower levels of the targeted miRNAs, but...
reductions were not seen for the random sequence negative control. This validated our idea and suggested we could use this second approach for modifying \textit{D. citri} gene expression. Most importantly, the anti-miR317 caused an average of 91.6 percent \textit{D. citri} mortality and the anti-miR34 caused an average of 74.6 percent mortality out of three biological repeats (data not shown). By contrast, the anti-miR277 caused an average of 56.3 percent while the negative control using random sequence for the all three treatments showed average 61.9 percent mortality (data not shown). The latter mortality is likely due to stress from injection, but because we replicated these experiments, we can conclude that anti-miR317 and anti-miR34 have good potential to induce negative phenotypes in \textit{D. citri}.

**Future Directions**

With our success in expressing specific amiRNAs in plants, identifying authentic \textit{D. citri} miRNAs and interfering with some of these via antagonirs, we are now well positioned to continue toward translational opportunities of using these RNAi approaches for efforts to target \textit{D. citri}. For example, we know that wild populations of \textit{D. citri} are infected by a number of different viruses (Nouri et al. 2015), and CLas-positive \textit{D. citri} are infected by CLas. Thus, in all of these, it is likely that \textit{D. citri} are responding to these infections via activation of natural \textit{D. citri} immune response pathways. We presently are identifying which miRNAs are involved in the \textit{D. citri} innate immune pathways. We plan to use specific xantagomirs to target some of these immunity-related miRNAs to identify those that are critical. We can then over-express or target these specific miRNAs to interfere with \textit{D. citri} immune responses to CLas and/or virus infections as one approach to help manage \textit{D. citri}.

**CRB Research Project #5300-169**

**References**


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**Figure 3.** \textit{Diaphorina citri} insects were injected with different antagonirs targeting specific miRNAs. (A) A psyllid is placed on a microscope slide to be injected by using a fine micro-injection needle. (B) The field image under microscope. (C) The injected psyllids were placed onto a healthy citrus plant. (D) Each plant with different antagonir injection is placed in an individual small cage for further analysis.


Glossary


2Nucleotides: nucleotides are the basic structural units or building blocks of nucleic acids, such as ribonucleic acid (RNA) and deoxyribonucleic acid (DNA).

3RNA Interference (RNAi): Biological processes that target specific RNA molecules to regulate gene expression and provide an anti-viral defense.

4Phenotypes: the heritable observable characteristics or traits of an organism.

5Artificial microRNA (amiRNA): Small RNA molecule artificially designed to target a gene of interest.

6Antagomir: A class of small synthetic RNA molecules that bind to complementary miRNAs preventing them from binding to target mRNAs resulting in gene silencing.

7Transgenic: Relating to the movement of genetic material from one organism to a second organism usually to modify a specific trait of interest, generally done using recombinant DNA.

8Next-generation Sequencing (NGS): A collective term describing DNA sequencing technologies that revolutionized genomic research.

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When Toni Siebert Wooldridge taught a University of California, Riverside (UCR) extension class on “Citrus in the Home Garden” in 2008, she had no idea that she was about to embark on a multi-year journey that could result in the public release of a promising new navel orange. In her class, she met Frank Shahani, who brought her a fruit from his navel orange tree. He was concerned because the navel orange had regions of red-pigmented flesh. Wooldridge explained that the flesh color was likely due to a bud sport that generated the anthocyanin red pigmentation. Wooldridge, David Karp and Tracy Kahn visited Shahani’s home a few weeks later in March 2008 to see the tree (Figure 1). All fruit observed had a smooth orange rind with varying amounts of red rind blush, red internal flesh pigmentation, a navel and few or no seeds (Figures 2 and 3). Shahani allowed the group to collect budwood for propagation and agreed to donate the material to UCR. After realizing the selection was stable, we offered to name it the Shahani Red navel in honor of his family.

Preliminary tree and fruit quality evaluations of three trees of this selection began at UCR in January 2013. The tree size of the Shahani Red navel orange is larger than standard navel orange trees at the same age and on the same rootstock (Carrizo and C-35 Citrange), but with more open growth habit than typically true of navel oranges. Ten fruit samples of Shahani Red navel fruit from each of three trees were evaluated and compared with ten fruit samples of Moro and Sanguinelli from each of two trees. These commercially significant standards were chosen for comparison as they both have anthocyanin pigmentation, but differ in timing of maturation.

Based on this small data set at a single Riverside location over five years, Shahani fruit had slightly higher soluble solid content (SSC), but the lowest percentage acidity based on citric acid or titratable acidity (TA) relative to Moro or Sanguinelli fruit at each of the sample dates tested. The ratio between these two characteristics (SSC/TA), used as a basis of legal maturity, reflects the consistently high SSC of Shahani. Although affected by fruit load and tree age, the average fruit weight of Shahani was consistently heavier than that of Moro or Sanguinelli. Shahani also had finer rind texture than either Moro or Sanguinelli for each of the sample dates, adding to the fruit’s attractiveness. Shahani was consistently less seedy than either Moro or Sanguinelli, which is typical of navel oranges relative to blood oranges.

At UCR Citrus Day in February 2018, growers had the opportunity to see and taste new introductions and hybrids with potential commercial promise, and were asked to rank them based on several characteristics. For overall flavor, more than 80 percent of participating growers ranked Shahani as having “very good” to “exceptional” flavor, and 92 percent rated Shahani as having a “sweet” to “perfectly balanced” flavor. For commercial viability, 75 percent expressed moderate to high market interest in this cultivar. Budwood of Shahani Red navel orange is currently in clean-up at the Citrus Clonal Protection Program. The cultivar should be large enough to be considered for the VI Index imminently, so the earliest Shahani could be publicly released for propagation is fall 2019.

A data summary for these three cultivars sampled five times per season in 2014-2018 is posted on the Citrus Variety Collection website: http://www.citrusvariety.ucr.edu/citrus/shahani.html

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