CITRUS RESEARCH BOARD
# Citrograph
MAGAZINE
SPRING 2015


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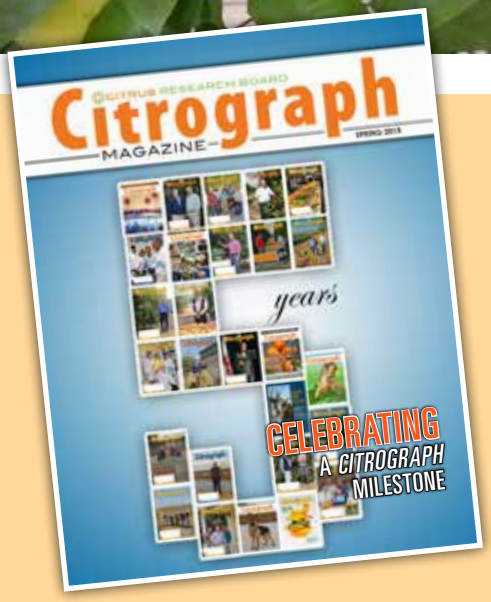
# IN THIS ISSUE

SPRING 2015 | VOLUME 6 • NUMBER 2 THE OFFICIAL PUBLICATION OF THE CITRUS RESEARCH BOARD


***About The Cover:***
*With this issue, the Citrus Research Board's quarterly magazine,* Citrograph, *is celebrating its fifth anniversary of service to the citrus industry (primarily in California, but also nationally and globally), as well as the centennial of the* Citrograph *brand. See story on page 16.*

# 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 2014-2015 (TERMS EXPIRE JULY 31)

### District 1 – Northern California

| Member | Expires | Member | Expires |
|---|---|---|---|
| Etienne Rabe | 2015 | Donald Roark | 2016 |
| John Konda | 2015 | Dan Dreyer | 2016 |
| John Richardson | 2015 | Jim Gorden | 2017 |
| Jeff Steen | 2015 | Greg Galloway | 2017 |
| Richard Bennett | 2015 | Joe Stewart | 2017 |
| Justin Brown | 2015 | Franco Bernardi | 2017 |
| Toby Maitland-Lewis | 2016 | Kevin Olsen | 2017 |
| Jack Williams | 2016 | | |

### District 2 – Southern California – Coastal

| Member | Expires | Member | Expires |
|---|---|---|---|
| Joe Barcinas | 2015 | John Gless III | 2017 |
| Alan Washburn | 2015 | Mike Perricone | 2017 |

### District 3 – California Desert

| Member | Expires | Member | Expires |
|---|---|---|---|
| Mark McBroom | 2016 | Craig Armstrong | 2016 |

### Public Member

| Member | Expires |
|---|---|
| Vacant | 2015 |

# CALENDAR OF EVENTS 2015

**March 5**
California Citrus Mutual Citrus Showcase, Visalia Convention Center, Visalia, California. For more information, contact California Citrus Mutual at (559) 592-3790.

**March 11**
CPCPP Board Meeting, Riverside/San Bernardino, California. For more information, contact CDFA at (916) 403-6652.

**March 25-27**
CRB Board Meeting and Research Project Updates, Bakersfield, California. For more information, contact the CRB at (559) 738-0246.

**May 13**
CPCPP Board Meeting, Ventura, California. For more information, contact CDFA at (916) 403-6652.

**May 21**
CRB Research Priority Screening Committee Meeting, Visalia, California. For more information, contact the CRB at (559) 738-0246.

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# PCA EXAM:

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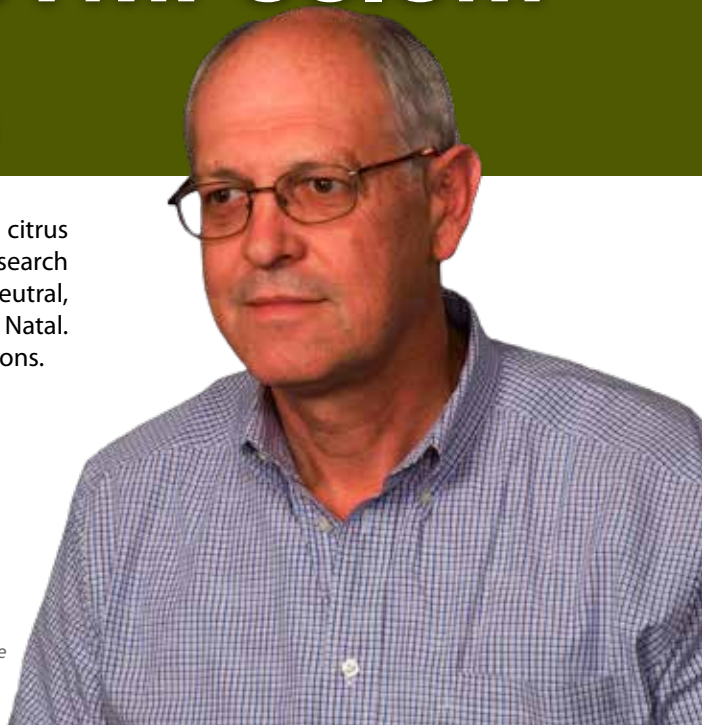
The Drakensberg mountains provided a beautiful backdrop to the site of the symposium.

# CITRUS RESEARCH INTERNATIONAL SYMPOSIUM IN SOUTH AFRICA

During August 2014, the citrus research entity of the South African citrus industry, Citrus Research International (CRI), held its 8th Research Symposium. This meeting is held every other year at the same venue in a neutral, non-citrus-producing area on the eastern side of South Africa in KwaZulu Natal. Previously, the venue rotated between the different citrus-producing regions.



*Etienne Rabe*

*Attendees packed the convention hall.*

South Africa is the twelfth largest citrus-producing country in the world, with about two million tons in production from approximately 60,000 hectares (150,000 acres). Contrast this with the California industry at approximately 3.5 million tons on roughly double the South Africa acreage (California acreage is officially 270,000 acres). Despite being a fairly small industry, South Africa ranks second in world exports behind Spain. The challenges facing South African growers, however, are quite daunting – 65 percent of the crop is exported (approximately 100 million x 15 kilogram carton equivalents). Shipping times can vary between three to five weeks to various destinations. Some countries, like the USA, also demand cold-sterilization of the fruit at -0.6°C (31°F) for up to 22 days for specific phytosanitary pests. This taxes the fruit condition tremendously.

The South African citrus industry deregulated in 1996 from a single-channel marketing system (Outspan International) as the sole marketing arm. During the single-channel system, the South African industry established a world-renowned research infrastructure to address all the challenges facing the industry. This extensive infrastructure and very experienced research staff were mostly held intact after deregulation and are now supported by a statutory box tax periodically voted on by growers.

Apart from frequent feedback to growers regionally, the organization also organizes a national symposium every second year. I have attended some of these in the past, including this last one in August. Apart from research topics presented orally and via poster that focus on progress results during the recent past, they also invite international speakers to provide wider perspectives.

Keynote speakers and topics this past year included Gerhard Backeberg, Ph.D., from the Water Research Commission on "Innovation for Irrigation Water Management;" Klaus Ekstein, Ph.D., from Bayer CropScience on how Bayer products touch our everyday lives and how the Bayer Group aims to create value through innovation, growth and high earning power; Vaughan Hattingh, Ph.D., on "Market Access and Biosecurity as Critical Components of a Sustainable Southern African Citrus Industry;" and Gerhard Verdoorn, Ph.D., on responsible pesticide use and to "Think Consumer" in order to ensure that the consumers will always recognize southern African citrus for its quality and safety.

Other keynote speakers included Andrew Miles, Ph.D., from Australia (Citrus Black Spot in Australia); Timothy Williams from California (practical experiences in the seed content of new, low-seeded cultivars, developed from

irradiation); Roy Peleg, Ph.D., from Israel (the wonder of coco coir in citrus nurseries); Ron Porat, Ph.D., from Israel (aspects affecting citrus fruit quality and flavor); and Charlene Jewell from California (use of "GRAS" salts for decay control).

My presentation was on the California research model where we do not have in-house research capacity, which is unlike the CRI where almost all core activities are handled by staff researchers and technicians. While CRB contracts fund scientists at universities, the USDA, private labs and so forth, a much smaller part of the CRI budget is spent on outside contracts. The South African industry does have a very close association with various universities by sending some of their scientists to specific departments as faculty members (horticulture, plant pathology, virology, soil science, entomology).


*Sponsors were acknowledged on CRI posters.*

*The gala dinner featured musical entertainment.*

*Those attending enjoyed one of many opportunities to network during a break in the proceedings.*

The South African industry is without question the most versatile citrus-producing country in the southern hemisphere, having the diverse climates required to produce the full range of quality citrus: navels and other oranges, grapefruit, mandarins and lemons. It usually serves as a one-stop shop for buyers from Europe and Asia.

A visit to the South African citrus industry and taking in the CRI Conference is well worth the time and effort and highly recommended. It is always an opportunity to catch up on some of the latest developments and a lot of good entertainment to boot.

***Etienne Rabe, Ph.D., is the chairman of the Citrus Research Board.***

# CITRUS STRONG

Joel Nelsen

It used to be enough for citrus producers to simply provide a highly nutritious piece of fruit in an environmentally sensitive manner. The price had to be reasonable to the customer and the returns per acre satisfactory to the grower. Life was so simple then, but not today.

The constant attacks, documentation requirements, regulation growth and assumptions by those in the media and in offices creating public policy have undermined the ability of a producer to "enjoy" the endeavor of providing food to citizens around the world. As a result, consolidation is occurring, and the number of family farmers in our great industry has shrunk.

It's not inasmuch a question of economics – the industry has enjoyed a good run this past decade. It's more a question of losing the emotional reward and fervor that comes with a satisfactory job. Has citrus farming become just a job? If it has, then it is time to get out, because not having fun results in rewards becoming drudgery as opposed to an emotional or economic high.

CITRUS STRONG
CALIFORNIA

Fortunately, most of us see the citrus industry as a path forward that contains a bright future for several reasons. This next generation believes in our industry and recognizes that farming is more complicated than ever and entails more than just working the land. Today, you have to "work" myriad audiences. One element is "working" to challenge those who erode your ability to achieve economic and emotional satisfaction.

In a phrase, there is a need to fight back. Assuring a higher price for off-setting inputs/dynamics is not fighting back. That's simply pricing your product more expensively. Too many believe the pricing conclusion is a path for the future. At California Citrus Mutual, directors believe an aggressive effort to offset misinformation is necessary. Thus, this past month, ***Citrus Strong*** made its debut.

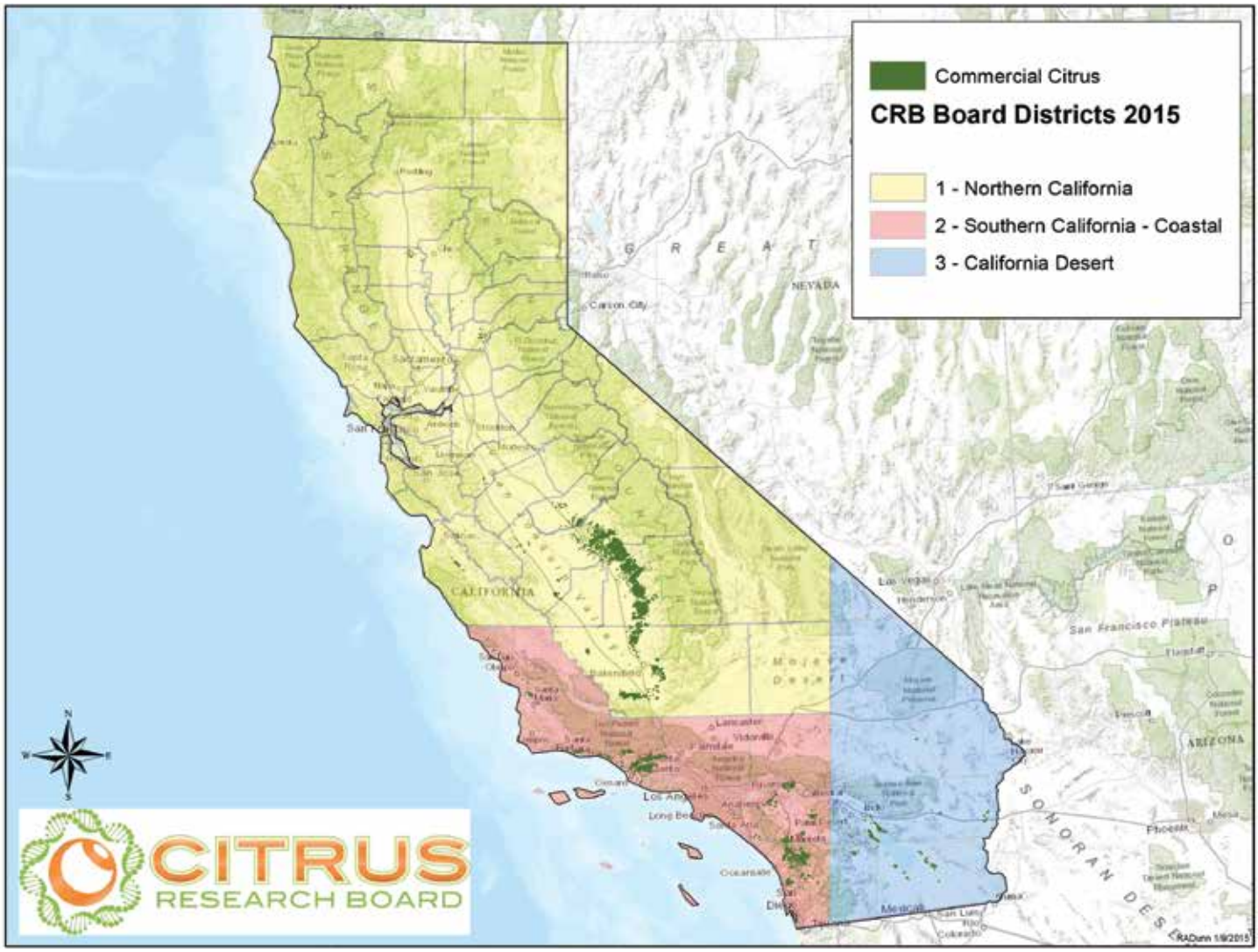
***Citrus Strong*** is a story about our industry, the people and the value it brings to our state. It's a story of sustainability, efficiency and environmental sensitivity. It's a story designed to offset the innuendos, falsehoods and assumptions that so many cast against the producer and the way of life. Others have set the agenda. All things thrown at the industry that are lies or challenges will be met via social media, via film/video, via news reports and via staged events.

Our target audiences are those in Sacramento, Washington D.C., our customer base and the media that influence those audiences. It took a year of research to determine what resonates and how best to say it and to whom. It has taken a few weeks to put the messages into form. ***Citrus Strong*** was released in Sacramento via a major event that garnered strong attendance from those who create "solutions" for alleged problems. Too often, it's a solution looking for a problem.

Farming and fighting back are synonymous for Citrus Mutual directors. Public policy advocacy is more than having a relationship. As in farming, it requires having the right tools to achieve the objective. For growers, having inputs such as healthy soil, strong trees, crop protection tools and water are the major elements. For your trade association, we have had the credibility, the relationships and the resumé to achieve objectives.

Now we need better messaging and newer tools to penetrate the mindsets of those setting public policy and offset falsehoods foisted by activists whose end-game frankly puzzles us. ***Citrus Strong*** will share, remind and emphasize why you are proud of what you do and how you benefit the economy, the environment and society.

***Joel Nelsen is the president of California Citrus Mutual.***




# CRB BOARD STRUCTURE UPDATED

*Ed Civerolo*

Several key revisions recently were implemented to the governing body structure of the Citrus Research Board (CRB). Changes were instituted in October as amendments to the California Citrus Research Program.

According to Robert Maxie, California Department of Food and Agriculture (CDFA) marketing chief, "These amendments were recommended by the Board to achieve greater equitability with the current distribution of citrus acreage among the three districts in the state and to increase participation and industry representation in Board business."

## BOARD CHANGES

Most noticeably, the CRB Board's membership structure has been revised to consist solely of full voting members, thus eliminating all of the current alternate positions.

Currently, 21 citrus industry representatives serve on the Board, comprising 15 members from District 1 in northern California, three members from District 2 in southern/coastal California and two members from the California desert area known as District 3. There also is one public member, who has voting privileges, represents the public and is not in any way involved in the production or marketing of citrus. Members of the current Board are listed on page 6.

All alternates have become full voting Board members. Effective on August 1 of this year when new terms of office commence, one currently vacant position from District 1 and one additional position from District 2 will be eliminated, thus establishing a 20-person Board for the future.

## BECOMING A BOARD MEMBER

Nomination meetings are set by the CDFA and occur in late June or early July. An official notice of the meeting is sent by the CDFA to all known citrus producer addresses in the state. The information also is communicated electronically by the CDFA and the CRB. Five positions are expiring this year in District 1, two in District 2 and none in District 3. The public member slot also is up for appointment.

It is important to note that since the CRB expends public (grower) funds, the Board must ensure the integrity of all of its funding decisions. Therefore, all appointees must file state-required financial and conflict of interest disclosures.

Anyone eligible (see sidebar) may seek nomination to the Board by requesting that his or her name be placed into the pool of candidates from the floor of the nominations meeting. Secret ballots are cast by meeting attendees who represent citrus producing entities, and tallied by the CDFA. The California Secretary of Agriculture then makes the official appointments from among those with the highest vote tallies.

## CLARIFYING TERMS OF MEMBERSHIP

Per the revised marketing order, the terms of Board membership also have been clarified.

A member's term of office on the Board will be three years beginning on August 1 of the year in which he or she was appointed, unless the appointment was made mid-term to fill a vacancy. There will be no limits to the number of consecutive terms that members may serve on the Board.

## RESPONSIBILITIES OF SERVING

The bulk of the Board's time is spent considering a broad portfolio of citrus research proposals and projects totaling millions of dollars. Members are involved in the crafting of the call for proposals, prioritizing responses, awarding funds, devising successful implementation strategies, assessing progress and providing critiques of project results.

There is a relatively high time commitment compared to many other volunteer commodity boards, but those involved with the CRB are integral in directing the response to critical citrus research needs in California and beyond. Board members are expected to attend Board meetings and to serve on several research and/or administrative committees. A typical fiscal year has five Board meetings spread around the citrus-growing districts, two three-day research meetings to select projects and receive updates, and also several individual committee meetings. In addition, attendance at conferences is highly recommended.

## REVISIONS TO QAPC

In another change that was announced by the CDFA, the Agricultural Chemical Residues Committee officially has been changed to the Quality Assurance Program Committee. A primary directive in the Marketing Order about the committee has been changed to read, "This authority shall in no event be construed to include citrus quality programs or regulations unrelated to *sanitary, phytosanitary*, pesticide or chemical residues."

The updated version of the California Citrus Research Program Marketing Order may be viewed via *www.cdfa.ca.gov/mkt/mkt*, and questions may be directed to the CDFA's Joe Monson at (916) 900-5018.

***Ed Civerolo, Ph.D., is interim president of the Citrus Research Board.***

### Qualifications to serve as a CRB Board member

- Any owner, officer or employee of an entity in California in the business of producing, or causing to be produced for market, 750 or more standard field boxes (or the equivalent) of any variety of citrus is qualified to participate in the nomination proceedings.
- An individual person is entitled to represent only one legal entity at a nomination meeting.
- In the case of a partnership, only one of the partners may vote.
- In the case of a corporation, a person affiliated with the corporation, preferably an officer, may represent the corporation.
- A married couple operating a production entity is entitled to just one vote, unless each spouse owns and operates separate and distinct entities.
- To participate in a district's nomination meeting, a business entity must have citrus production within that district. Any entity with production in more than one district must choose a single district in which to participate to vote. If a separate production entity can be proven as the operating entity in another district, the person qualified to act as the representative of that entity may vote in that district, even if he/she has voted as a representative of another entity in another district. Essentially, each separate citrus-producing business entity is entitled to one vote in the district in which it operates.
- Voting by proxy is not permitted.

*Source: CDFA Marketing Branch*


# CRB CELEBRATES FIVE YEARS OF *CITROGRAPH*

*By Chad Collin*

The Citrus Research Board (CRB) is celebrating its five-year anniversary of publishing *Citrograph* magazine. With this Spring 2015 volume, the CRB has published 31 issues since early 2010. Over that time, *Citrograph* has been a vital tool to disseminate the latest in research results, industry news and special features. *Citrograph* aims to be the "go to" citrus research publication for California and beyond. Subscriptions remain FREE for California citrus growers and bona fide industry members with advertising subsidies and CRB support.

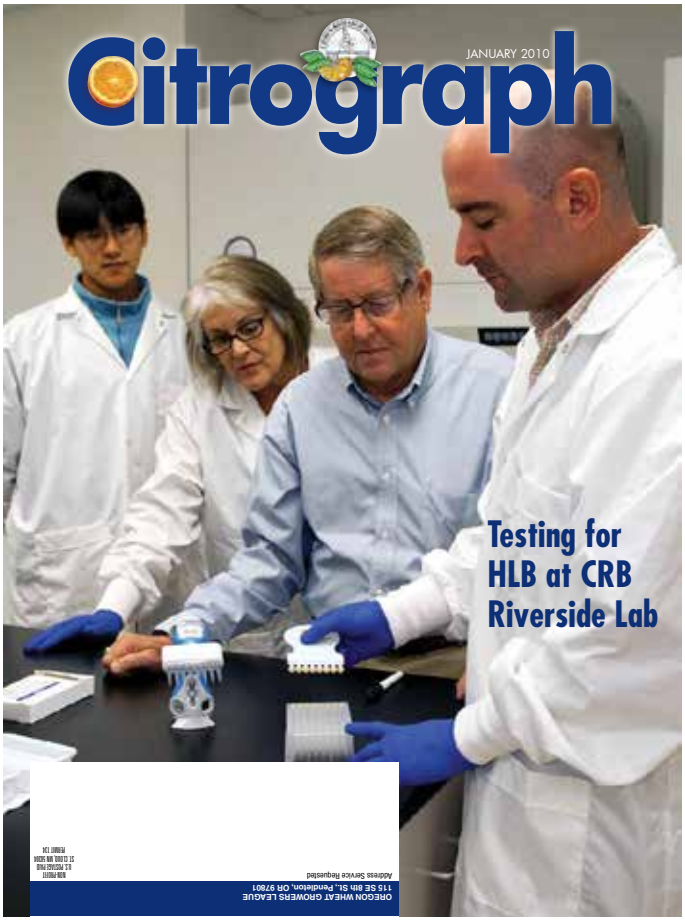



*Figure 1. January 2010 – The "Premier Issue" of CRB's* Citrograph.

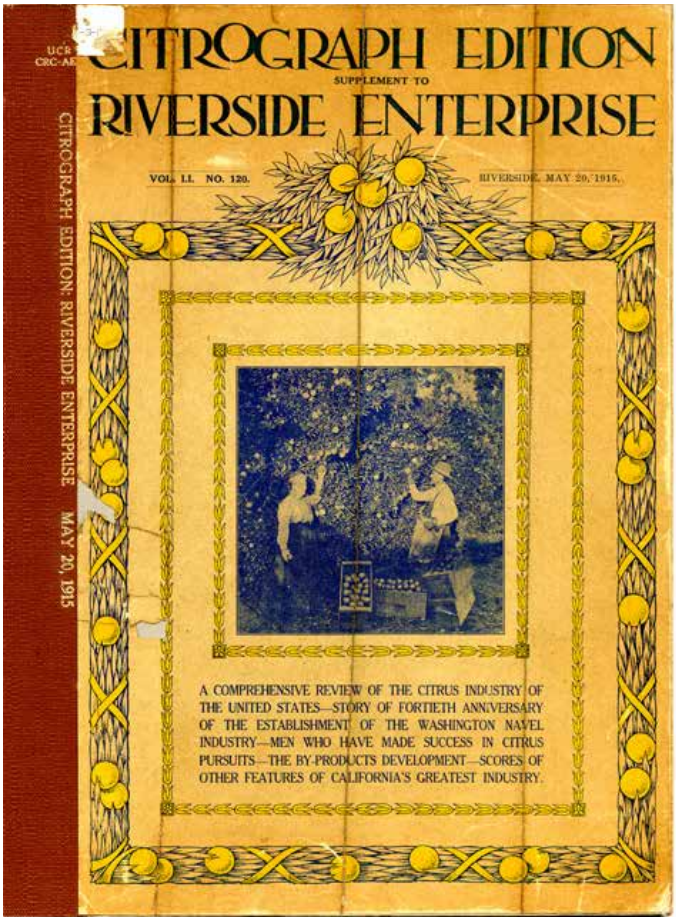



*Figure 2. May 1915 – The original* Citrograph *edition was a supplement to the* Riverside Enterprise *newspaper. It appeared on May 20, 1915. By this time, Riverside was already celebrating "the fortieth anniversary of the establishment of the Washington navel industry," as the cover states.* Courtesy of UC Riverside Libraries.

# A STORIED PAST

Many growers remember reading *Citrograph* long before the CRB's "Premier Issue" **(Figure 1)** in 2010. In fact, *Citrograph* first appeared in May 1915 as a supplement to the *Riverside Enterprise* newspaper **(Figure 2)**. It evolved into a regular publication entitled the *California Citrograph* and was the trade publication for California citrus growers from 1915 to 1969. From there, it continued on as *Citrograph* magazine under various owners until production ceased in 2002 **(Figure 3)**. The University of California, Riverside recalled it as "A monthly publication devoted to the interests of the citrus industry and a study of the conditions relating to the promotion of subtropical agriculture, it contained the latest research on crop propagation, production, pest control, picking, packing, marketing and shipping, as well as advertisements for all the latest in equipment and services."

UC Riverside played a major role in the historic *California Citrograph* and used the magazine as a means to publish research papers that flowed out of the Citrus Research Center on campus. Its topics included dealing with citrus pests and disease and their control, investigation of rootstocks to support new and improved varieties, and reports on the packing, handling and shipping of the fruit to market.

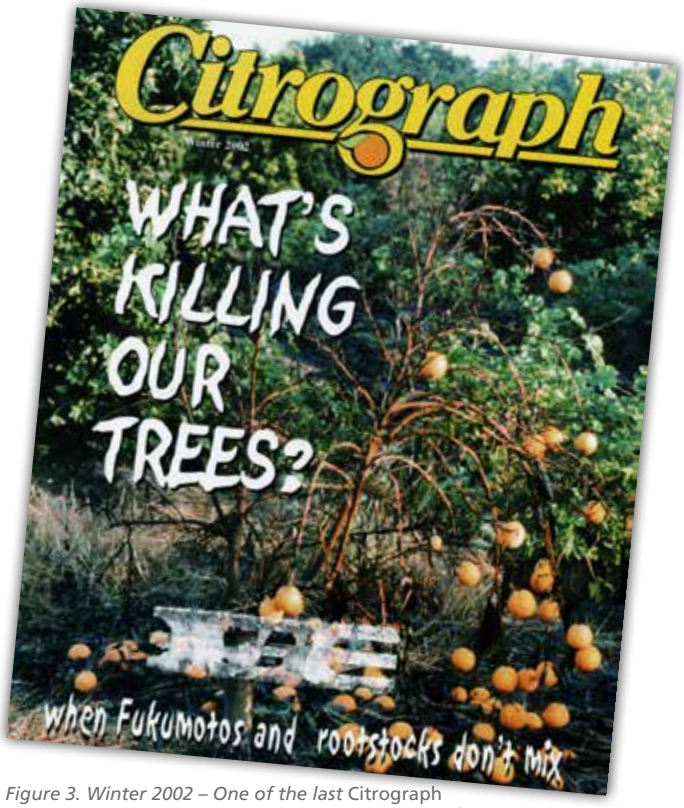



*Figure 3. Winter 2002 – One of the last* Citrograph *editions prior to the 2010 reappearance.* Courtesy of Lindcove Research and Extension Center.


*Figure 4. August 1917 .*
Courtesy of Lindcove Research and Extension Center.

*Figure 5. December 1931.*
Courtesy of Lindcove Research and Extension Center.

The publication took an eight-year hiatus until the CRB resurrected *Citrograph* after the name copyright became available. Since early 2010, *Citrograph* magazine has been the official publication of the CRB, communicating the latest in citrus research not only from UC Riverside, where many CRB-funded projects still occur, but also projects from UC Davis, University of Florida, Los Alamos National Laboratory and many more research institutions. Now, Board members and staff take pride in producing *Citrograph* and distributing it quarterly with quality content that embodies elements of a scientific journal that has "in the field" relevancy for today and the future.

"The Citrus Research Board is proud and honored to continue to produce such a valuable publication for the benefit of the industry. We pride ourselves in reporting on the most recent and important research developments that have significant relevance to the industry."

*- Dan Dreyer, CRB Communications Committee Chair*


*Figure 6. July 1944 – Many articles and ads referenced supporting the war through productive agriculture.*
Courtesy of Lindcove Research and Extension Center.


*Figure 7. May 1978.*
Courtesy of Lindcove Research and Extension Center.

Not only is this CRB's five-year publication anniversary, the ***Citrograph*** publication brand is rapidly approaching its centennial anniversary, debuting in May of 1915. See a sample of covers through the decades in **Figures 4-7**.

## INTO THE FUTURE

CRB's *Citrograph* is committed to supporting the California citrus industry by communicating research progress and solutions, as well as pertinent news. Times have changed and the science has advanced, but the topics of the past resonate with today's top research priorities to ensure a sustainable California citrus industry. The Board, editorial staff and publication team strive to serve nearly 4,000 readers (and growing) with the high standards the *Citrograph* name deserves.

## References

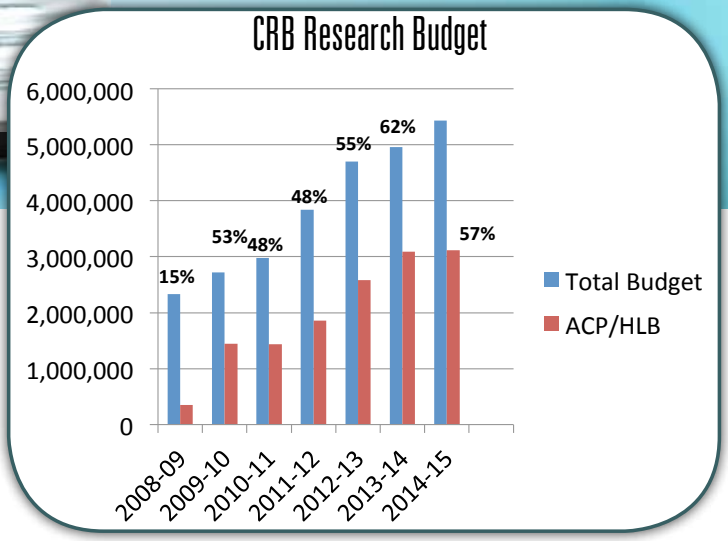
*http://library.ucr.edu/?view=collections/spcol/ces/citrograph.html*
*http://magissues.farmprogress.com/CLF/CF11Nov10/clf014.pdf*

***Chad Collin is with the Citrus Research Board, where he serves as director of Board and grower communications, and also as associate editor of* Citrograph.**

# ATTENTION RESEARCHERS:
# PUT ON YOUR THINKING CAP

## *THE 2015-2016 CRB REQUEST FOR PROPOSALS IS COMING SOON*

***MaryLou Polek***




The Citrus Research Board would like to announce that the 2015-2016 Request for Proposals (RFP) for scientific research project funding will be posted to the CRB website on **Friday, April 17, 2015**. Formulate your thoughts and assemble high-powered teams to develop projects that will benefit the California citrus industry. Although combatting huanglongbing and the Asian citrus psyllid are certainly a high priority, the CRB will entertain projects addressing other pests and pathogens, horticultural, fertilization and water issues, and food safety assurances. Matters affecting the marketplace and trade barriers also are considered a high priority.

Scientists are encouraged to work cooperatively, assembling project teams that are multi-institutional and multi-disciplinary, and that target several aspects of a desired outcome. Projects delivering practical or applied solutions that directly benefit California citrus growers are suggested.

To submit a new proposal, you are invited to visit the CRB website, *http://citrusresearch.org*, where you can download the CRB pre-proposal form. Briefly describe your research concept, complete the form and submit it electronically. New pre-proposal concepts are due **no later than 8:00 am on Monday, May 18, 2015.**

For those pre-proposal concepts that are selected to move forward, scientists will be required to submit full proposals and give a 15-minute presentation via teleconference. The deadline for the submission of full proposals is **Monday, June 22, 2015.**

New proposals will be evaluated by a Science Review Panel and screened by CRB Research Committees. If selected to move forward, principal investigators will be required to give a presentation to the entire board in person, **August 25-27, 2015.**

Final approvals will be made on Tuesday, **September 22, 2015**, and funding will commence on **October 1, 2015**.

The Citrus Research Board is a grower-supported commodity organization serving the California citrus industry under the CDFA marketing branch. During fiscal year 2014-2015, the research budget was approximately $5.5 million, with 57 percent dedicated to huanglongbing and the Asian citrus psyllid. For further information and/or questions, please contact us at: *research@citrusresearch.org*

***MaryLou Polek, Ph.D., is the vice president of science and technology at the Citrus Research Board.***

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# PROGRESS TOWARD FINDING IMMEDIATE, PRACTICAL HLB SOLUTIONS

## *The HLB MAC Group – One Year Later*

***Abby Yigzaw***

*An infested sample of flush is collected for parasitism analysis in a laboratory.*

The United States Department of Agriculture's (USDA) Huanglongbing Multi-agency Coordination (HLB MAC) Group was created in December 2013 in direct response to a request from the citrus industry to the USDA for greater urgency, support and coordination in the fight against HLB. During the last 12 months, the HLB MAC Group met each of these goals as it worked to prioritize and allocate $21 million in funding for research and field-trial projects that will soon put promising tools that fight against HLB into the hands of growers.

"From day one, our focus has been on getting growers the help they need now to combat this devastating disease," said Mary Palm, Ph.D., chair of the HLB MAC Group. "We've pushed hard this year to get promising HLB detection, control and management methods out of the labs and into large scale field trials where they can be validated and turned over to growers for use in their groves."

The HLB MAC Group made its first major announcement in May, providing $1.5 million to ramp up the release of the biological control agent, *Tamarixia radiata*, to help suppress populations of Asian citrus psyllid (ACP) in Florida, California and Texas. This also will benefit neighboring citrus-producing states. Through its work to set collective goals and priorities, HLB MAC group members all agreed that scaling up biocontrol, which is a tool that has shown promising results, would be of immediate benefit to the citrus industry. Part of the $1.5 million was instrumental in the transfer of technology for the production of *Tamarixia radiata* from the Animal and Plant Health Inspection Service's methods development laboratory to the Texas Citrus Pest and Disease Management Corporation, which repurposed Agricultural Research Service greenhouses in Weslaco for biocontrol production.

Then, in June, the HLB MAC Group made the decision to allocate $6.5 million for citrus health research projects that seem the most promising for producing tools and strategies that can help growers in the near future. This funding is supporting the field testing of antimicrobials, such as streptomycin and oxytetracycline, in Florida to gauge their effect on the HLB bacterium. It also is supporting thermotherapy technology projects, as well as a large demonstration grove in Florida to will help educate growers about best management practices that support citrus production in areas where HLB is present.

Through these and other efforts in 2014, the HLB MAC Group fostered cooperation and coordination across Federal and State agencies and industry. The MAC team focused on sharing information, making strategic decisions based on shared priorities and reducing duplicative efforts. As one example, the National Institute of Food and Agriculture's coordination with the HLB MAC Group avoided the duplication or overlap of research efforts and ensured that the greatest number of critical projects was funded.

The Group also coordinated regular communications, including weekly conference calls among State, Federal and academic biocontrol practitioners from across the United States. These calls not only help to facilitate vital information sharing, they also are enabling rapid advances in the development and use of biological control technologies for ACP. To date, the practitioners have developed common standards to measure the efficacy of biological control of ACP so that programs in different states can compare results, share information about best production and release practices and identify alternative biological control strategies, in addition to *Tamarixia radiata*, which are near the implementation stage.

"As we look ahead to 2015, we want to continue the progress we started last year and build on it to fund more projects that will get us closer to our goal of effectively battling ACP and HLB," said Palm. "We want to help the citrus industry gain the advantage as quickly as possible."

*A field insectary cage being installed over a recently hedged lime tree that is infested with thousands of ACP nymphs and where about 300* Tamarixia radiata *will be introduced for mass production.*

*A field insectary cage installed over a lime tree where* Tamarixia radiata *has been introduced and is parasitizing all ACP nymphs present. The mesh screen is removed right before the third generation of parasitoids emerge, often producing about 12,000 parasitoids.*

As part of the HLB MAC Group's efforts to help the citrus industry gain that advantage, the group cast a wide net to receive project suggestions from industry, academic, State and Federal researchers. The project suggestions selected will be funded in 2015 and focus on four critical areas:

• **early detection**, such as standardizing antibody-based detection methods, developing high throughput diagnostics using root samples and training canines to detect HLB;

• **sustainable citrus production practices**, such as the treatment of bicarbonates in irrigation water and soil, rapid propagation and widespread field testing of HLB-tolerant rootstocks and the establishment of several more demonstration groves to help showcase effective integrated management approaches;

• **treatments for infected trees**, such as field-level thermotherapy delivery systems to heat trees, kill the HLB bacteria and restore productivity; and

• **vector management**, such as a lure to attract and kill the ACP, release and establishment of several alternative biocontrol agents and new methods to increase production of the biocontrol agent, *Tamarixia radiata*.

It has been an exciting and busy year for the HLB MAC, as the group has worked toward funding near-term solutions for fighting HLB. These near-term investments will pay dividends as longer-term research continues. And it's just the start. More information on the MAC Group's efforts and announcements can be found at *http://usda.gov/citrus*.

***Abby Yigzaw is the acting assistant trade director and trade correspondence manager of the USDA's Animal and Plant Health Inspection Service.***


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# HIGHLIGHTS OF THE 3RD CTV WORKSHOP

## *OCTOBER 28-30, 2014*

*Sweet orange varieties on sour orange rootstock are susceptible to quick decline strains of* Citrus tristeza virus. *The virus causes a collapse of the vascular tissue at the bud union. Under periods of severe heat or water stress, the tree cannot pump enough water to the canopy, and the tree rapidly declines.*

***MaryLou Polek***

**This is the first of a series of articles on *Citrus tristeza virus*. The following report focuses on the workshop that was held at the University of California Lindcove Research and Extension Center (UC-LREC).**

Citrus tristeza virus *(CTV) has long haunted the California citrus industry from the time citrus was brought into the state in the late 1800s. The loss of thousands of acres of Washington navels grafted on sour orange rootstock and Meyer lemons infected with a severe strain of CTV was devastating to the citrus industry and over-all economy of southern California. When the industry shifted to the San Joaquin Valley (SJV), tolerant trifoliate hybrid rootstocks replaced sour orange and allowed the industry to thrive.*

*Still feeling the pain of CTV losses, growers in the SJV wanted added protection and worked to enact legislation to implement a statewide quarantine that is still in existence today. In addition, in 1963, the Central California Pest Control Agency was formed and later renamed as the Central California Tristeza Eradication Agency (CCTEA). The mission of this*

*organization was (and continues to be) to survey commercial citrus, test for the presence of CTV and remove any tree diagnosed as positive for CTV.*

*Periodically, the strategy of tree removal has been questioned. The Citrus Research Board assembled scientists from around the globe to discuss CTV and its ramifications to the California industry. The first workshop was held in 1995 in Visalia, California. Emeryville, California was the site of the second workshop in 2008; and in 2014, the third workshop was held at the University of California Lindcove Research and Extension Center (UC-LREC). The following describes highlights of the 2014 Workshop.*

The overall goal of this workshop was to re-evaluate and update California's response to CTV. The intended outcome was to review and recommend appropriate modifications to:

- the industry-wide effective plan for the San Joaquin Valley,
- regulatory response, and
- the UC-LREC operational plan.

Stephanos (Fanie) van Vuuren from South Africa was the keynote speaker/guest. Other speakers included Mark Hilf, USDA-ARS, Fort Pierce, Florida; John da Graça, Texas A&M University; James Ng and Georgios Vidalakis, University of California, Riverside; and Ray Yokomi, USDA-ARS, Parlier, California. Presentation topics included the management of CTV in South Africa, Texas and Florida – with and without the brown citrus aphid vector and in the presence of huanglongbing (HLB). Discussion session topics included the San Joaquin Valley suppression program, regulatory issues, UC-LREC-related issues and future strategies. Summaries of these presentations and discussion sessions will be the subject of future articles.

Participants formulated the following position statement:

*We foresee a coordinated statewide survey using all existing infrastructure and capacities for all citrus threats (HLB, CTV, CVC, leprosis or other pathogens) using multiplex detection technologies. This approach already has been approved by CDFA for the citrus nursery industry. Surveys also should include monitoring for invasive insect and mite pests. This will allow the citrus industry to proactively survey for and respond to threatening, exotic, emerging or re-emerging pathogens and pests and maximize the industry's significant investment.*

## RECOMMENDATIONS:

1. Tree removal is still recommended based on severe strains (eg. MCA13 as is currently used); this will reduce the inoculum overall, result in fewer genotypes in the field and subsequently increase the success of a cross-protection program.

2. Severe strain suppression success is dependent on an effective sampling, testing and tree removal program; and the entire state should be involved in testing to determine the significance of severe strains in each region.

3. The Citrus Pest Detection Program, operated by the Central California Tristeza Eradication Agency, should expand its program to include detection of other pathogens such as HLB and to monitor for invasive pests.

4. Develop a survey and sampling methodology for the detection of multiple pests and pathogens (Florida has implemented a "Multi-pest Survey").

5. Develop and maintain a central database of information on CTV isolates and other pathogens.

6. Institution detection methods:

   a. Short-term – continue with existing protocol of serology followed by PCR. Transition to high throughput qPCR.

   b. Long-term – multiplex detection of different pathogens to increase efficiency and expand diagnostic capabilities.

7. Research should continue to improve serological and molecular detection methods.

8. Continue to identify and characterize genotypes that are severe (eg. genotypes associated with the stem-pitting VT strain in Florida, Israel and Peru and VT and T3 in South Africa).

9. Identify specific mild strain genotypes or characteristics to prepare for mild strain cross-protection against exotic stem-pitting isolates.

10. Include commercially significant varieties in the standard host range bio-characterization.

11. Conduct a long-term field study of severe genotypes (such as California VT and T3) in commercial citrus varieties to determine the long-term tree health impacts of severe strains under SJV conditions.

*Stem-pitting strains of Citrus tristeza virus affect all citrus varieties. Although some naturally occurring stem-pitting isolates occur in California, they are not severe as this example taken in Sao Paulo, Brazil.*

12. Continue a program of breeding for tolerance or resistance to CTV and other pathogens.

13. Develop a transient CTV vector such as the one developed by William Dawson in Florida.

14. Maintain the CTV interior quarantine until all nursery production (including nursery stock) is under screen or novel CTV-management technologies are implemented.

15. Form a subcommittee to develop a voluntary citrus nursery stock certification program that is recognized by CDFA.

## THE FOLLOWING RECOMMENDATIONS ARE SPECIFIC TO UC-LREC AND ADJACENT COMMERCIAL CITRUS:

16. UC-LREC should continue to test and remove CTV-infected trees at the Center to protect research programs and the foundation block. This program should be re-evaluated each year at the Research Advisory Committee (RAC) meeting to determine if tree removal is impacting projects.

17. Continue the current Pest Control District supported program of aphid control on and around UC-LREC (citrus and pomegranates) to reduce vector populations.

18. Conduct research to improve the efficacy of the vector reduction program.

19. Continue to survey for and remove genotype-specific CTV-infected trees in the one-mile region around UC-LREC.

20. Require UC-LREC researchers to plan for a higher number of replications to account for tree loss at UC-LREC.

21. Continue to support the transgenic research as a long-term approach to protect the citrus industry against pathogens and pests.

Research conducted during the past two decades on the characterization of naturally occurring isolates of CTV has shown that most California populations of the virus are mild strains. Because of the existing management practices (Citrus Clonal Protection Program, clean nursery stock program, use of tolerant rootstocks, etc.) the tree removal strategy and the Citrus Tristeza Virus Interior Quarantine, CTV does not cause significant production issues in California, especially in the San Joaquin Valley. These practices also have prevented and minimized the spread of severe CTV strains. Continuous monitoring, survey and testing allows for the early detection of severe or exotic strains of CTV so that they can be dealt with before becoming widespread. Keeping the severe isolates rare helps to minimize virus spread by vectors (this is especially important if the brown citrus aphid is ever introduced into California).

With so much attention attributed to HLB and the Asian citrus psyllid, growers may forget or become complacent about CTV. Participants of the CTV Workshop agreed that this would be a grave mistake.

***MaryLou Polek, Ph.D., is the vice president of science and technology at the Citrus Research Board.***

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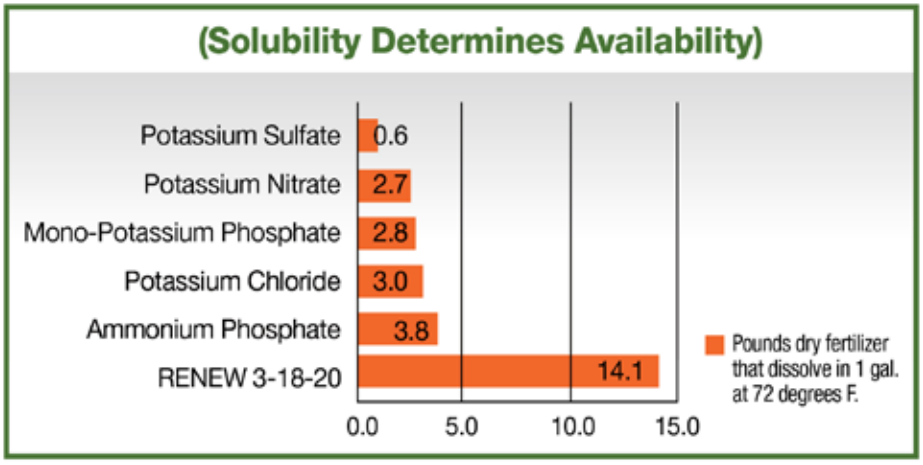


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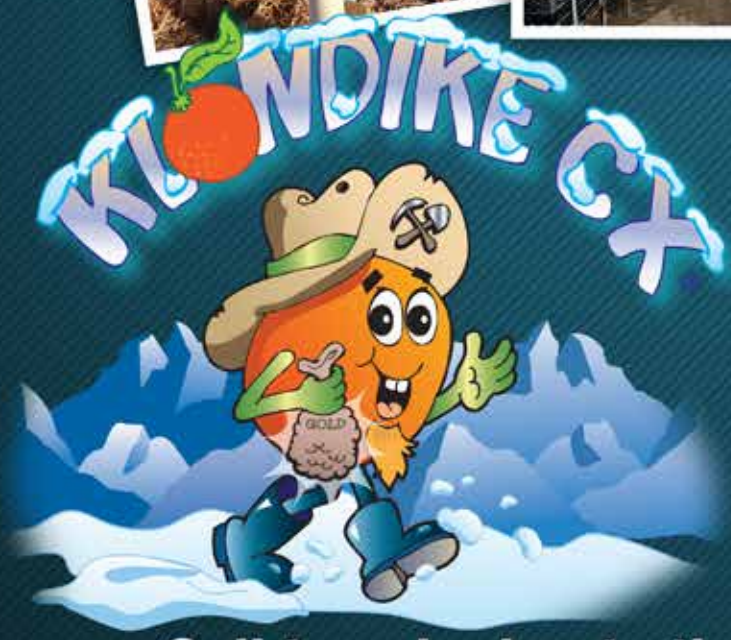


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*HLB-infected tree displaying fruit drop.*

# EARLY DETECTION OF HLB WITH METABOLOMICS

***Elizabeth Chin, Rebecca Lobo, John da Graça, Mark Hilf, Greg McCollum, Cynthia LeVesque, Kris Godfrey and Carolyn Slupsky***

Huanglongbing (HLB) is a major threat to the global citrus industry. The name literally translates to 'Yellow Shoot Disease,' emphasizing the yellow mottle of symptomatic leaves and its devastating impact on citrus trees. Infected trees have reduced fruit yield due to premature fruit drop and poor fruit quality compared to uninfected trees. Symptomatic fruit from infected trees are small in size, lopsided in shape and green in color. Even apparently "normal" looking fruit from infected trees may experience changes in flavor, often acquiring a bitter and/or metallic taste that is unpalatable to consumers. Ultimately, diseased trees die prematurely compared to uninfected trees.

Unfortunately, the symptoms of HLB can take years to appear and resemble other citrus diseases and nutrient deficiencies. Meanwhile, the Asian citrus psyllid (ACP), the insect vector associated with the disease, can still transmit the bacterium from infected trees to healthy trees even when the infected trees are asymptomatic. The long period between infection and the appearance of symptoms allows the disease to spread widely and can result in huge losses in fruit, trees and profit. Indeed, Florida's citrus industry has already been devastated by HLB with billions of dollars and thousands of jobs lost. California's citrus industry now faces the same outcome unless disease spread can be prevented by earlier detection of infection in non-symptomatic trees.

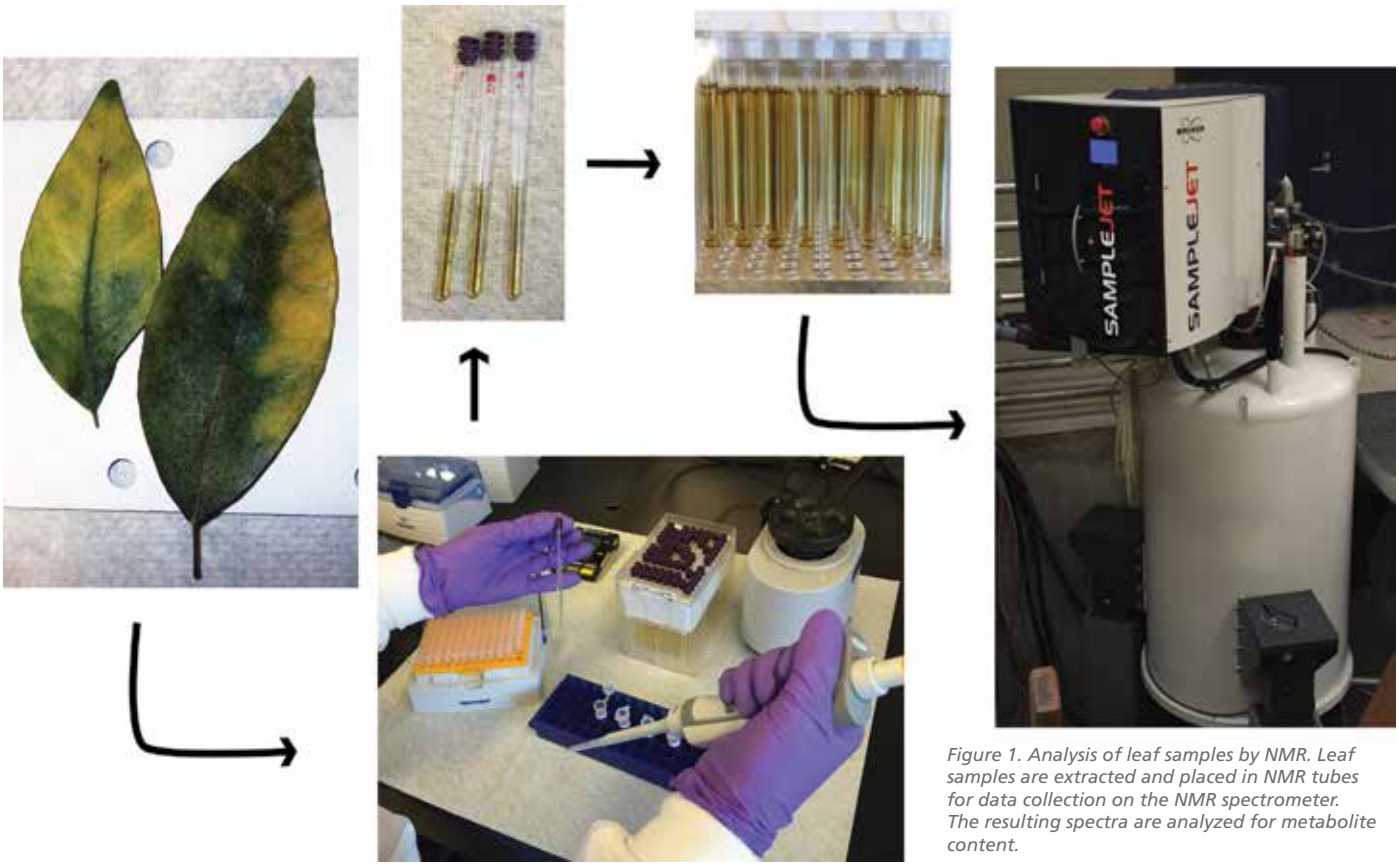


*Figure 1. Analysis of leaf samples by NMR. Leaf samples are extracted and placed in NMR tubes for data collection on the NMR spectrometer. The resulting spectra are analyzed for metabolite content.*

In the U.S., HLB is caused by the bacterium "*Candidatus* Liberibacter asiaticus" (CLas) and is spread by the insect known as the Asian citrus psyllid (ACP), or by grafting infected tissue. Resistance has not been identified in commercial citrus varieties; and if infected with CLas, these trees will eventually succumb to the disease. Research on CLas is especially challenging since the bacterium has yet to be grown in the laboratory, so there is no way to study it outside of the plant. Most efforts to reduce the spread of CLas are aimed at controlling ACP populations by trapping, use of bio-parasites or spraying insecticides. However, there are currently no fail-safe, long-term solutions for treating the disease or controlling spread in field trees other than tree removal.

Currently, the only method with regulatory approval for CLas detection is quantitative polymerase chain reaction (qPCR), which provides a measure of the amount of CLas based on detecting DNA from the pathogen. Unfortunately, CLas is not evenly distributed throughout the tree. Since the accuracy of qPCR depends on whether the sample contains the CLas bacterium, qPCR can yield false-negative results if the correct sample is not assayed. This is especially problematic in truly infected trees that are asymptomatic and, therefore, resemble healthy trees because there are no visual symptoms to guide leaf sampling. Thus, the inability to detect the pathogen during asymptomatic infection means that many trees are clandestinely carrying the disease and allowing others to be infected.

Although the bacteria may be present at low levels within the tree and difficult to detect by qPCR, the tree can detect the presence of the pathogen and mount a physiological response. It has been well documented that plants can defend themselves against stressors such as changes in temperature or water availability, as well as against insect feeding and pathogens. Initiation of the plant's defense system leads to a cascade of changes in metabolic pathways to create signal molecules, enzymes, proteins and other biochemical machinery. These changes occur soon after infection, and measuring the changes in quantity of the participating metabolites can identify the metabolic pathways that shift in the plant.

Measuring the multitude of metabolites in a sample is a daunting task, as there can be hundreds or thousands of these chemicals that are present in concentrations ranging from very low to very high. No one technique can measure all of the metabolites present in a leaf, but one of the most robust and reproducible analytical instruments for measuring metabolites that range in concentration by a factor of more than one million is the Nuclear Magnetic Resonance (NMR) instrument **(Figure 1)**. NMR works by placing a sample into a magnetic field and applying a radio-frequency pulse. The resulting oscillating electrical signals can be detected in a receiver coil. These signals are then processed into a frequency spectrum that can be matched to a library of unique metabolite signatures, allowing for the identification of metabolites present

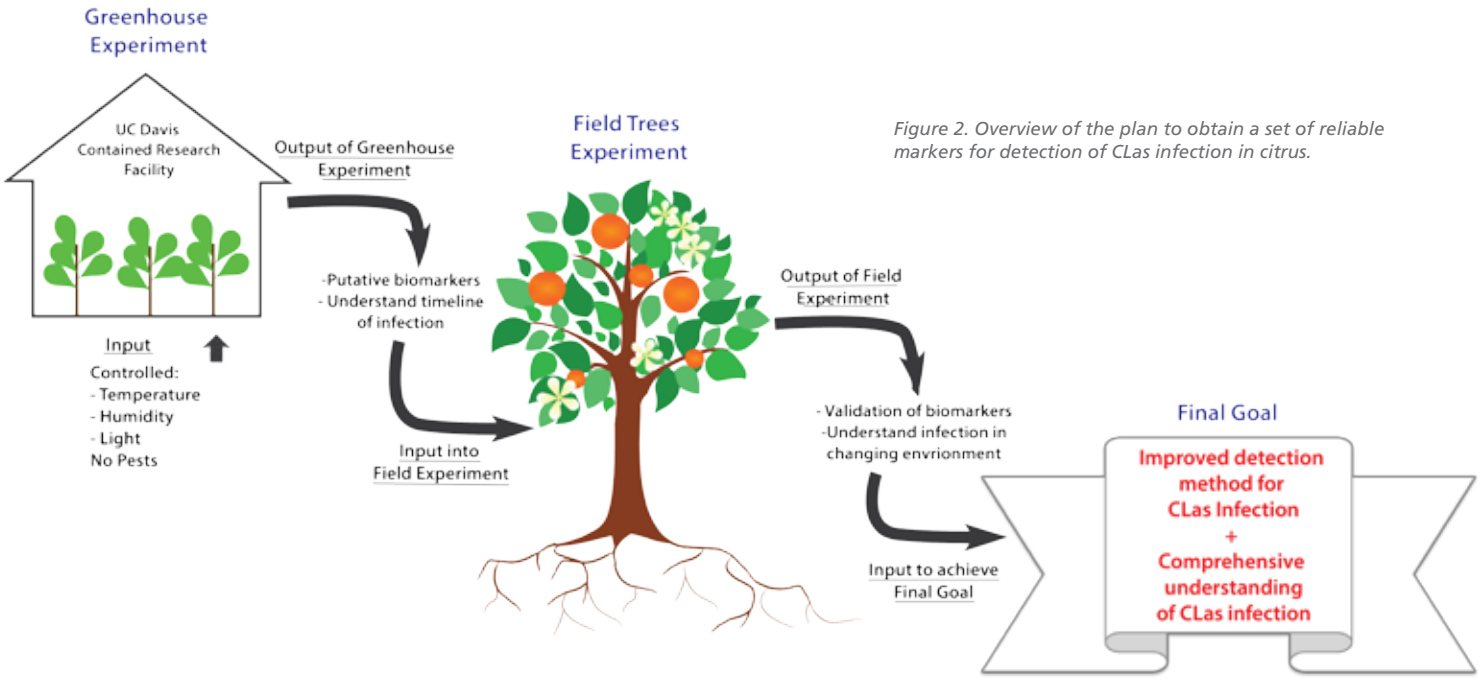


*Figure 2. Overview of the plan to obtain a set of reliable markers for detection of CLas infection in citrus.*

in a sample. Measurement of the area under the spectrum allows for quantitation. Understanding the CLas-induced 'metabolite fingerprint,' i.e. which metabolites change and by how much, can potentially be used as an improved early detection method for HLB in citrus trees.

Research in our lab has consistently shown that there is a CLas-specific metabolite fingerprint that may be used for the detection of CLas earlier than qPCR, and prior to visual symptoms. Early detection of HLB allows for early intervention that will help prevent the spread of the disease to other trees, thus reducing losses in trees, fruit and profit to the citrus industry. Greenhouse trees as well as field trees from California and Texas are being studied to gain a comprehensive overview of which metabolites change soon after CLas infection, how they continue to change during disease progression, and how this knowledge can be applied to detect CLas in mature, fruit-bearing trees soon after infection **(Figure 2)**.

## GREENHOUSE TREES

Since the CLas bacterium cannot be grown in the laboratory, we have been studying plants grown in greenhouses in both Florida, and at the University of California-Davis Contained Research Facility (for more about all HLB early detection groups in the CRF, please see "An Interdisciplinary Approach to Combat HLB" in *Citrograph*, Winter 2014).

We are investigating which metabolites change over the course of infection in several varieties of citrus to establish the earliest time at which metabolomics can detect infection. Because we are able to see changes in the metabolite patterns, these studies may enable us to determine the length of time that a tree has been infected, which will aid in monitoring the spread and help determine how long an infection has been in an area. We also have been studying the effects of other citrus pathogens, such as CTV, *Xanthomonas citri* subspecies *citri* (bacterial citrus canker) and *Spiroplasma citri* (citrus stubborn), and have determined that their metabolite profiles are distinct from CLas. These studies are building the foundation for our metabolite-based early detection method.

## FIELD TREES

In tandem with the CRF greenhouse experiment, we are validating our test using field samples collected from a grapefruit orchard in Texas where HLB has been confirmed by qPCR in a small number of trees with foliar symptoms on a few branches. The samples are being collected from neighboring and nearby trees that are most likely to be infected next. Some leaves are used for qPCR analysis in the da Graça lab while the remainder are lyophilized and sent to the Slupsky lab for metabolomics analysis. The results from this study will help us refine and validate our biomarker profiles to ensure that very few false positives and false negatives are obtained using our detection method.

Improvements for detecting CLas infection are essential to combatting the spread of HLB. Early detection of HLB allows for earlier intervention (tree removal and, perhaps eventually, treatment of the diseased tree), which will play a key role in preserving the citrus industry. Metabolomics offers a promising new strategy for the early detection of, defense against and resolution of HLB in the United States and the survival of the citrus industry.

***Elizabeth Chin is a graduate student in the Department of Food Science and Technology at the University of California, Davis; Rebecca Lobo, Ph.D., is a post-doctoral fellow in the Department of Food Science and Technology at the University of California, Davis; John da Graça, Ph.D., is a professor at Texas A&M University in Weslaco Texas; Mark Hilf, Ph.D., is a research plant pathologist at the U.S. Horticultural Research Laboratory at the USDA in Florida; Greg McCollum, Ph.D., is a research plant physiologist at the U.S. Horticultural Research Laboratory at the USDA in Florida; Cynthia LeVesque, Ph.D., is the laboratory director of the Citrus Research Board in Riverside, California; Kris Godfrey, Ph.D., is an associate project scientist at the Contained Research Facility at the University of California, Davis; and Carolyn Slupsky, Ph.D., is an associate professor with a joint appointment in the Department of Nutrition and the Department of Food Science and Technology at the University of California, Davis.***

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# WHAT'S IN A NUMBER?

## *Estimating numbers of viable* Candidatus *Liberibacter asiaticus cells in citrus and ACP*

***Greg McCollum and Mark Hilf***

Those who have anything to do with citrus, whether producing, packing, processing or just consuming, are well aware of Huanglongbing (HLB) disease and the impact it is having on citrus production in the United States. HLB is perhaps the greatest threat to sustainable citrus production worldwide. Although the devastating impact of HLB has been known in Asia for more than a century, it was not until 2004 that the disease was confirmed present in the western hemisphere; first in Brazil, then in 2005 in Florida, 2012 in Texas and 2012 in a single tree in California. Florida citrus has been especially devastated by HLB, but it is now apparent that no citrus producing region can be considered safe from the disease.

## *'Candidatus* Liberibacter asiaticus' and HLB

HLB is associated with the bacterium *'Candidatus* Liberibacter asiaticus' (CLas). This tells us a lot about the bacterium – *Candidatus* is a Latin term meaning "not cultivated or cannot be sustained in culture for more than a few serial passages." Liberibacter means "bark bacteria," and asiaticus indicates the organism was first confirmed from Asia. CLas is a bacterium that came from Asia, lives in the phloem of infected plants and has yet to be cultured *in vitro*. The unique biological niche that CLas occupies presents significant obstacles that hinder research efforts aimed at solving the HLB problem.

There is strong circumstantial evidence that CLas is indeed the HLB causal organism. However, to confirm that a microbe is truly the causal organism of a disease, it must be isolated from diseased tissue and grown in pure culture. Also, inoculum from pure culture must be used to inoculate healthy host tissue and cause disease symptoms, and the same organism must again be isolated from the inoculated plant. This process is known as fulfilling Koch's postulates. Although considerable research efforts have been devoted to isolating and growing CLas sustainably in culture, none of these efforts have been successful to date. However, even without fulfilling Koch's postulates, it is well accepted among the citrus research community that CLas infection does lead to the infection known as HLB.

## CLAS INFECTION IS CONFIRMED BY DETECTION OF CLAS DNA

In addition to preventing absolute confirmation that CLas is the HLB causal organism, inability to culture the bacterium also prevents enumeration of viable CLas cells in infected citrus tissue.

Ever since the pioneering work by Louis Pasteur and Robert Koch at the end of the nineteenth century, the detection of viable microorganisms has been carried out by cultivation and enumeration of colony forming units (CFUs) *in vitro*. Almost all judgments on pathogen infection and efficacy of antimicrobials are based on bacterial growth on solid agar medium followed by CFU counts. The ideal scenario in most applications of microbial diagnostics is that only viable cells are detected. In the case of plant pathogens, only viable cells are likely to cause disease. The term 'viability' has been subject to many discussions and is typically referred to as the ability of cells to replicate. Because *in vitro* cultivation of CLas cells is currently not possible, counting CFUs on agar petri dishes to estimate viable cells is not an option.

At this time, the only certain method to confirm CLas infection, and thereby confirm that a tree is succumbing to HLB, is detection of CLas-specific DNA. Detection of the diagnostic CLas DNA sequences is accomplished by a method known as polymerase chain reaction (PCR); and in particular, a version of PCR known as quantitative PCR (qPCR) is frequently used, since this method quantifies the number of bacterial cells in a sample. PCR is used to specifically and repeatedly copy the diagnostic DNA sequence, eventually generating enough of the DNA to be detected. PCR is extremely sensitive and specific; a single molecule of target DNA typically can be amplified 10 billion-fold to produce enough for detection. PCR amplification is a very reliable test to confirm that CLas DNA is present in a plant sample; but since DNA is a stable molecule and can be amplified from even dead cells or fragments, PCR cannot differentiate live cells and dead ones.

The ability to enumerate live CLas cells from citrus as well as ACP would have significant impact in research aimed at evaluation of host plant resistance, impacts of transgenes on pathogen viability, effects of antimicrobials, screening germplasm for effects on CLas colonization, CLas transmission and infectivity studies.

## MODIFIED PCR VERSION ALLOWS DISTINCTION BETWEEN VIABLE AND NON-VIABLE BACTERIAL CELLS

Within the last decade, there has been considerable interest and research by environmental and food safety microbiologists into methods for distinguishing between live and dead bacterial cells in water and food products. One approach for distinguishing live from dead bacterial cells that has come out of this research is the use of the DNA intercalating dyes Ethidium monoazide (EMA) and Propidium monoazide (PMA) prior to DNA extraction **(Figure 1)**. DNA intercalating dyes

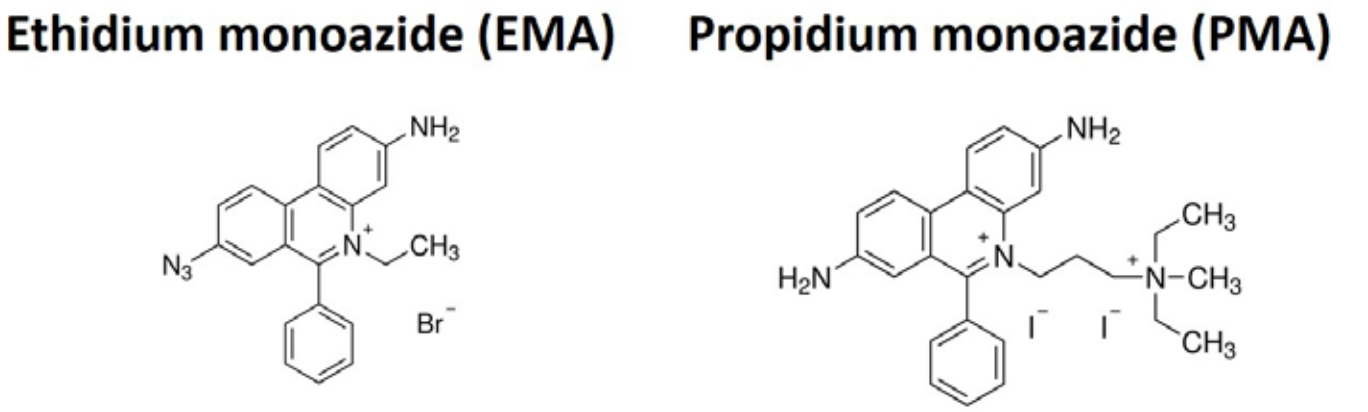



*Figure. 1 Chemical structures of the two DNA intercalating dyes used in the research.*

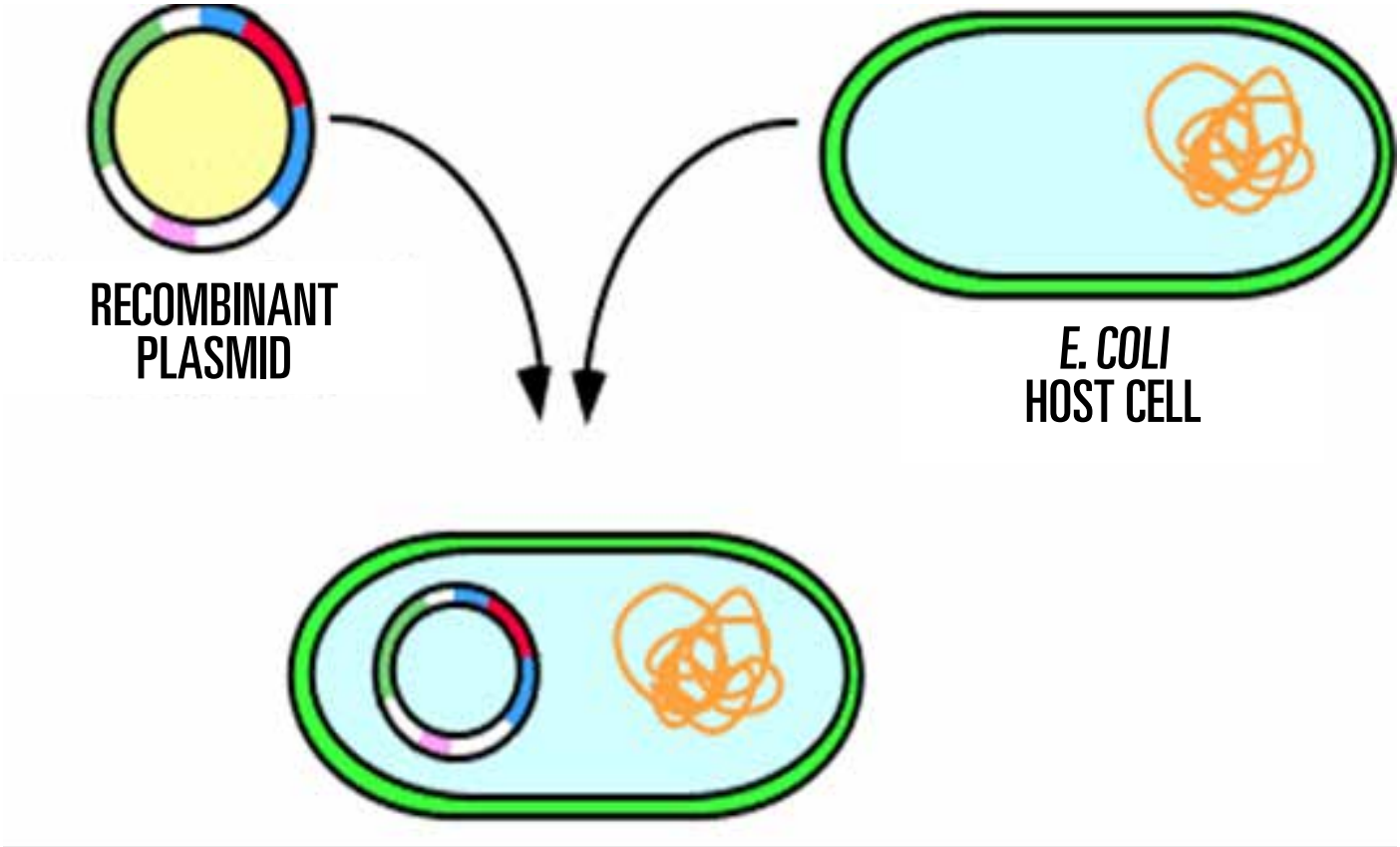



*Figure. 2. A model system to test the effects of DNA intercalating dyes. CLas target DNA sequence inserted into circular piece of DNA (plasmid), plasmid incorporated into bacterial cell* (Escherichia coli).

destroy DNA from dead bacterial cells in a population of live and dead cells. Following treatment with intercalating dyes, only DNA from live cells is amplified.

Although intercalating dyes have proven effective for enumerating live bacteria in liquid culture and in water samples, the technique is much more challenging for application with bacteria restricted to tissue, such as CLas. Due to the recognized importance of distinguishing live from dead bacterial cells and the very limited research that has been reported on this topic, we have endeavored to utilize DNA intercalating dyes to distinguish between live and dead CLas cells from citrus and ACP.

## RESEARCH FINDINGS

*A model system.* Our first step in this research was to create a model system that would allow us to determine the effects of DNA intercalating dyes on PCR amplification of CLas DNA. We used standard molecular biology protocols to: 1) produce a circular piece of DNA (plasmid) that contains the CLas target DNA sequence, and 2) insert the plasmid into *Escherichia coli* (*E. coli*) bacterial cells **(Figure 2)**. These plasmid-containing cells can be grown in liquid culture and produce abundant amounts of the CLas target DNA. Using *E. coli* transformed to produce CLas DNA provides the closest thing we have to an actual culture of CLas itself.

*How do DNA intercalating dyes affect amplification of CLas DNA?* Specificity and efficacy of EMA- and PMA-qPCR were determined using both purified plasmid containing the CLas DNA target sequence and *E. coli* cells transformed with the same plasmid. Results with our model system confirmed that both EMA and PMA treatments are specific for the CLas target sequence. PCR amplification of the CLas target from plasmid DNA was inhibited 100 percent by both EMA and PMA. These results provide evidence that the DNA intercalating dyes do in fact eliminate amplification of CLas DNA that is free of live cells. If cells are heat killed prior to dye treatment, amplification is inhibited 100 percent. During the course of these experiments, we also optimized variables in the protocol to give greatest sensitivity in the assay and the widest working range.

*Viable CLas titer in citrus tissue.* Our first experiments to estimate CLas titer in citrus tissue were conducted with citrus seed coat vascular bundles. We chose these bundles as the

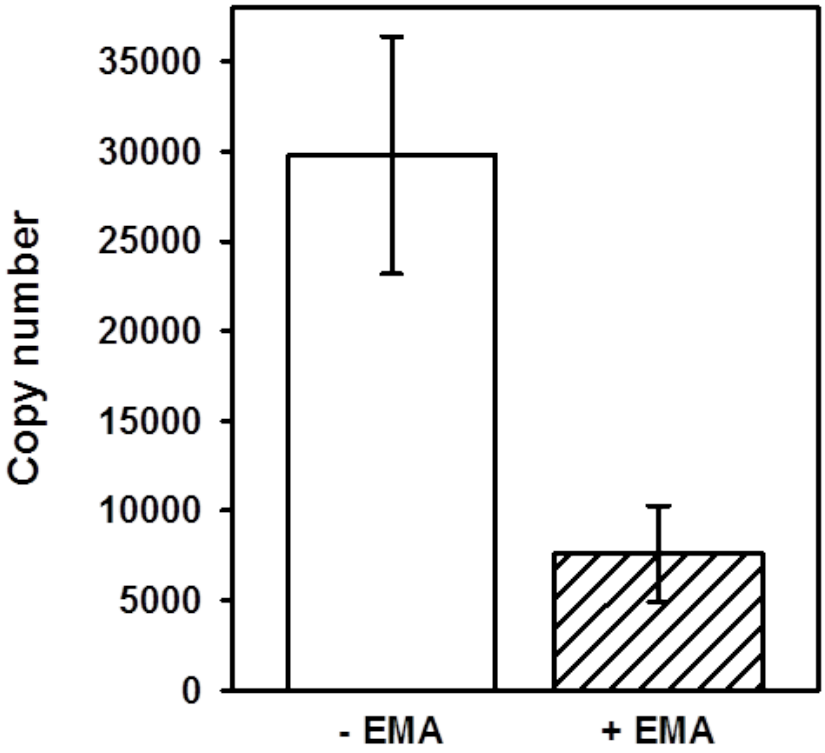



*Figure. 3 Estimation of viable CLas cell numbers in citrus seed coat vascular bundles. CLas copy numbers for EMA-treated tissue was about 25 percent of those obtained with non–EMA-treated tissue, suggesting that 25 percent of the cells are viable.*

experimental material because they are a rich source of CLas DNA. In addition, there is some question regarding whether CLas can be transmitted via seeds and whether it is viable CLas cells or merely CLas DNA that accumulates at the end of the phloem pipeline. In experiments conducted with DNA extracted from seed coat vascular bundles that had been treated with EMA prior to qPCR, viable cells were estimated to be about 25 percent of the CLas copy number of that in DNA from non-treated seed coat vascular bundles **(Figure 3)**. These results suggest that a great proportion of the CLas DNA recorded in citrus seed coats is not from viable cells.

We also have conducted experiments to estimate numbers of viable CLas cells in citrus leaf and petiole tissues. The classic foliar symptom of HLB is a distinct blotchy mottle. We analyzed leaves that displayed this symptom to varying extents and leaves that were a more healthy green **(Figure 4)**. We were surprised that our results suggested that with increasingly

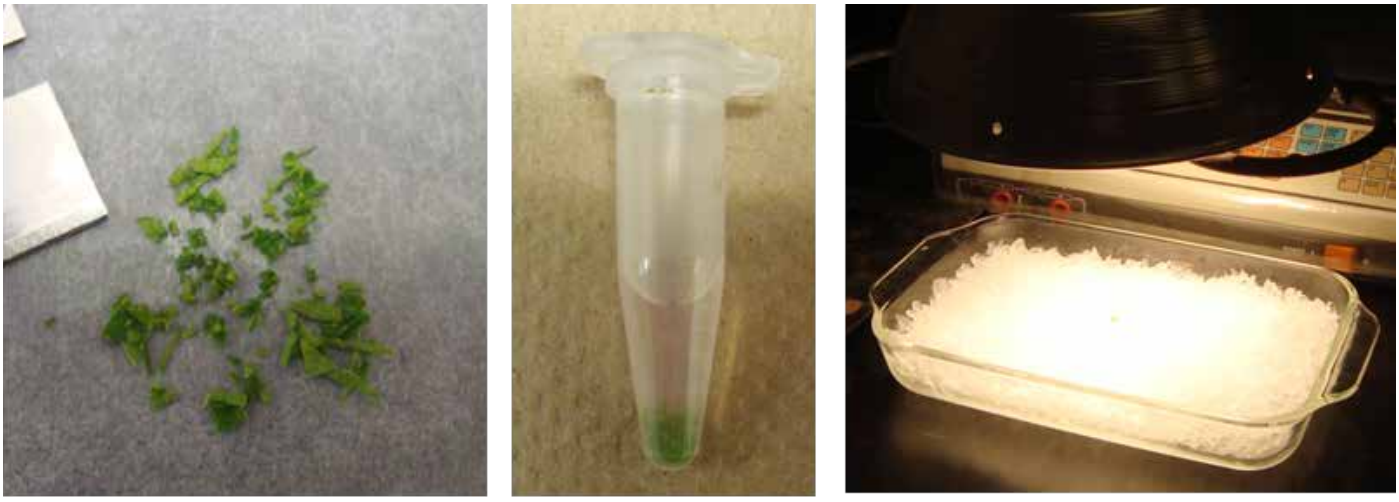


*Figure. 4. Treatment of tissue with intercalating dyes prior to DNA extraction: 1) chop into fine pieces; 2) incubate in water or dye solution; 3) expose to bright light. These steps activate DNA intercalation and destroy dye that does not bind to DNA.*

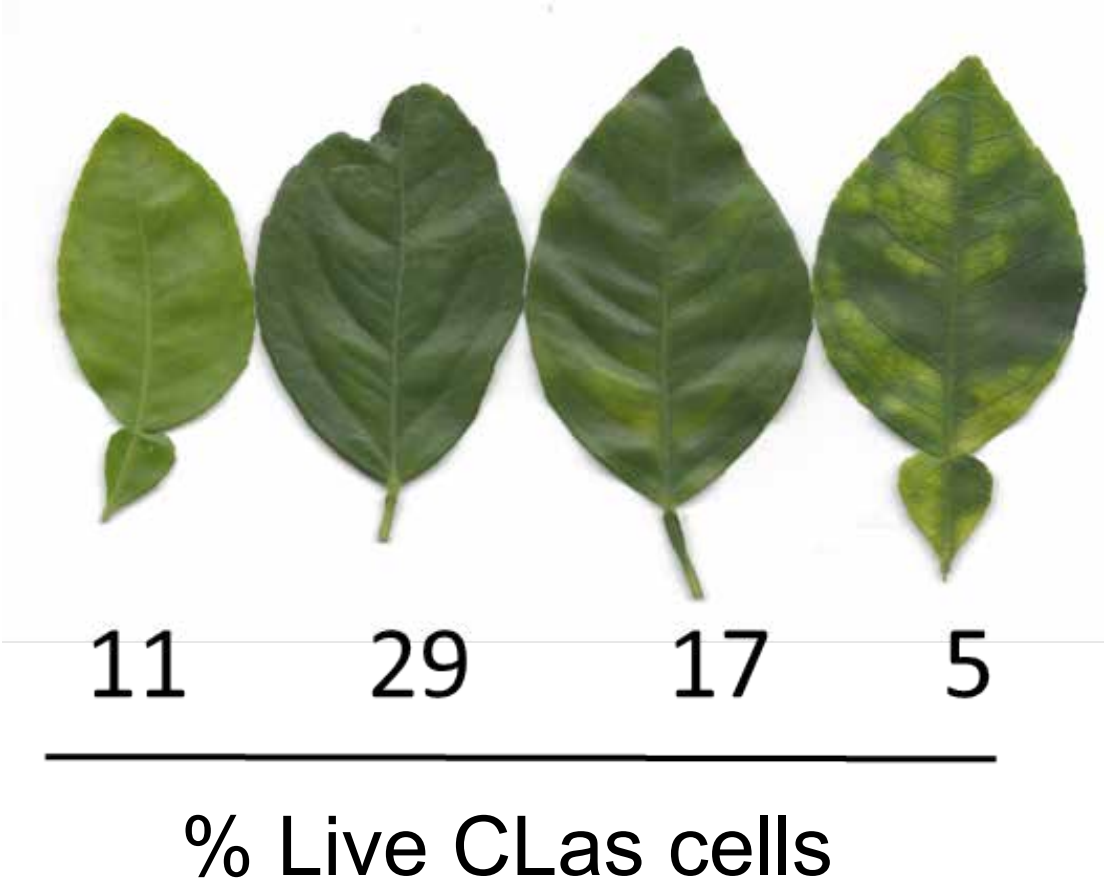



*Figure 5. Estimates of percentage of live CLas cells in citrus leaves expressing different symptoms of blotchy mottle.*

severe mottling, the percent of live CLas cells decreases. The well-developed blotchy mottle symptom may represent an older infection in that tissue; hence, it would be reasonable to have fewer live bacteria, especially if nutrient flow to the leaf is affected **(Figure 5)**. However, these results are not always consistent, and we are working to refine sampling and assay protocols that will provide more consistent and predictable results.

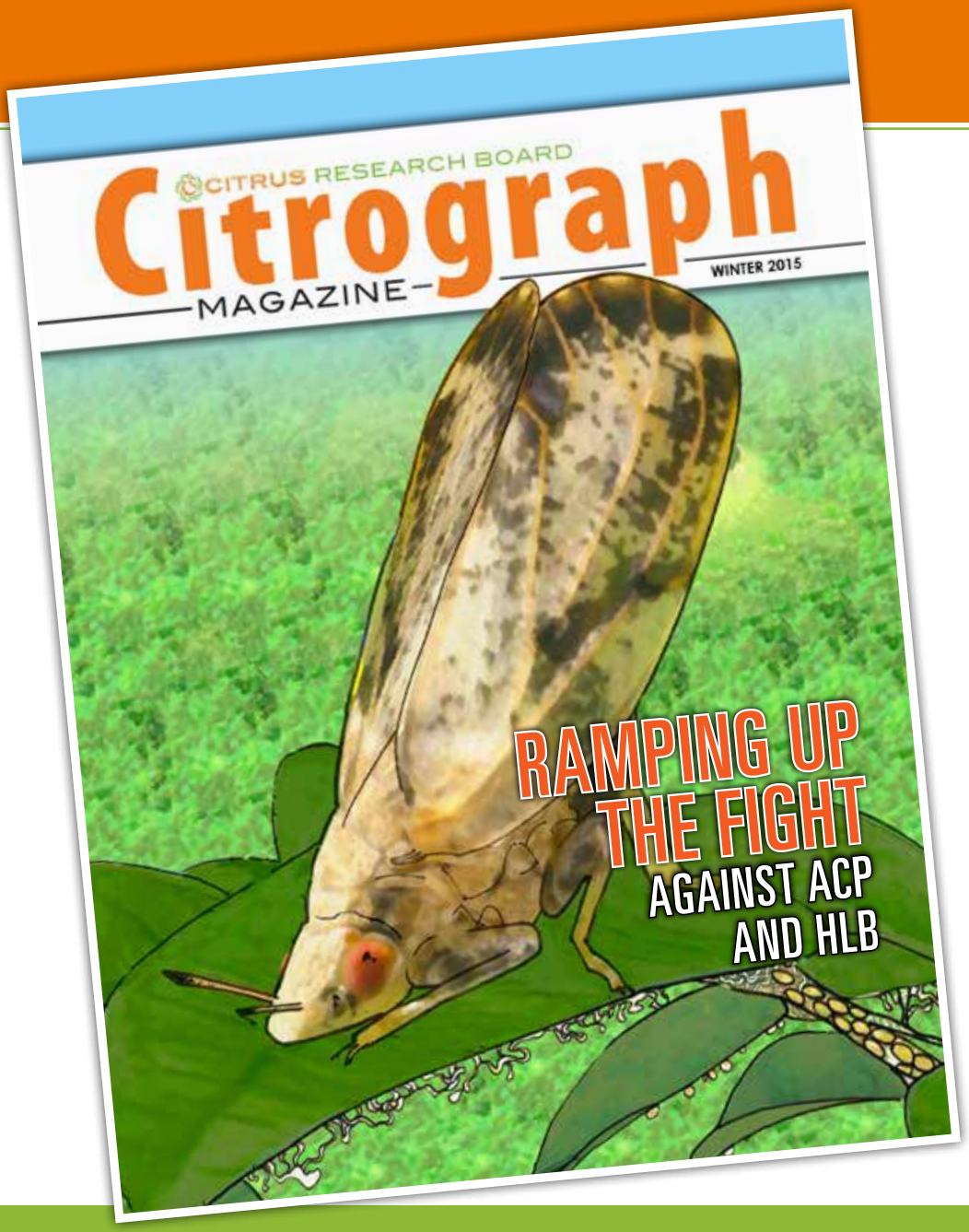
*Viable CLas titer in ACP.* We also analyzed adult psyllids, and based on EMA-PCR, we estimated that viable cells were only about five percent of the CLas population in ACP. Although five percent sounds like a low number, it represents about 100 cells per 1/1000th of a gram or about 100,000 per gram of tissue. Our results show quite clearly that viable CLas cells are a much smaller fraction of the total number of bacteria estimated by PCR. If this is a consistent character of the bacterial population in infected trees, this may have important relevance to how effective the transmission of CLas is from ACP to citrus.

## FINAL COMMENTS

Our research is providing new insight into the complex interaction between the three components of HLB disease: citrus, CLas and ACP. Being able to quantify numbers of viable CLas cells out of a population of live and dead cells is important for developing solutions to the HLB problem. We were particularly surprised to find the low number of apparently viable CLas cells in both citrus tissue and in ACP. These results suggest that numbers of living CLas cells are much fewer than are estimated by qPCR, a finding that could be important to researchers trying to isolate and culture CLas.

The results also will help us answer some basic questions, such as how many bacteria are needed to infect a citrus tree. It is without question that the number of live CLas transmitted by ACP will impact the likelihood of a successful infection and the eventual development of HLB disease. Developing reliable estimates of viable CLas cells in citrus will allow researchers to better evaluate the impact of treatments being developed to rid infected plants of CLas. This method will be an important research tool until it becomes possible to cultivate CLas in the laboratory and enumerate live CLas cells by the standard plate count method. The results of our research will validate the utility of DNA intercalating dyes for distinguishing between live and dead CLas in citrus.

***Greg McCollum, Ph.D., is a research plant physiologist and Mark Hilf, Ph.D., is a research plant pathologist, both at the USDA Horticultural Research Laboratory in Ft. Pierce, Florida.***




## REACH COMMERCIAL CITRUS GROWERS IN CALIFORNIA, ARIZONA AND BEYOND

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*Technician Ashley Shepherd observes recovery in citrus shoot tips after cryopreservation.*

# WIDESPREAD APPLICATIONS OF CITRUS CRYOPRESERVATION

***Gayle M. Volk***

*Micrografted citrus shoot tip exhibits regrowth after liquid nitrogen exposure.*

Citrus cryopreservation technologies have been developed with the generous support of the Citrus Research Board (CRB). Citrus genetic resources can now be successfully cryopreserved, which means that they can be placed into long-term storage at liquid nitrogen (LN) temperatures. Tiny shoot tips (1 mm) are excised from vegetative budwood and treated with solutions that allow them to tolerate extreme conditions (-150º to -196ºC) for years. The technology is currently being used to provide a safety back-up to critical and vulnerable U.S. citrus collections.

Cryopreservation technology was specifically developed to address the immediate need to have secure long-term back-up storage for citrus collections that are currently maintained as potted or field trees in industry, university or USDA collections. As cryopreservation protocols are implemented, materials are secured at the National Center for Genetic Resources Preservation in Fort Collins, Colorado.

The technology is established and applicable to a diverse range of genetic resources. In addition to its value for long-term conservation, cryopreservation has been shown to be a technology that can be used in other important applications. This article focuses on opportunities that may be possible, now that citrus cryopreservation techniques are available to the research community.

## PROPAGATION SOURCES

Citrus rootstocks are often propagated using tissue culture techniques. These plants in tissue culture are disease-free, and many identical clones are generated. However, sometimes slight (or significant) genetic changes may occur after many subculture or propagation events, as a result of a process termed "somoclonal variation." When cultivars are cryopreserved, they are kept in an inactive (yet living) state for extended lengths of time. Since cryopreserved materials are kept in a non-propagative state, there is a reduced risk of incurring genetic changes that can result from extended tissue culture regimens. As necessary, cryopreserved shoot tips can be recovered from LN and propagated, thus providing a fresh source of clean material that hasn't undergone numerous subcultures.

## PATHOGEN ELIMINATION

The process of cryopreservation may eliminate pathogens from plant tissues. When infected materials are cryopreserved, testing has revealed that sometimes (or under certain conditions) only the non-infected cells survive, thus rendering the resulting plant pathogen-

*Rodrigo Latado, Ph.D., measures the dry weight of citrus shoot tips, as part of an experiment to determine the moisture levels of cryopreserved materials.*

free. When testing has assured that plantlets are not harboring pathogens, they can be transported, by permit, for use in other laboratories. These cleaned-up materials can be subsequently multiplied and made available to others. In addition, materials that are in the cryopreserved state can be kept pathogen-free, without the threat of disease or pathogen infection that may occur if plants are in the field.

## GENETIC IMPROVEMENT

New genetic technologies will facilitate the transfer of specific citrus genes from one plant to another, while still maintaining most of the genetic integrity of the desirable cultivar. There are examples whereby the process of cryopreservation enhances the transformation efficiency and plant regeneration after LN exposure. While these applications are not fully developed for citrus, the possibility exists that a combination of traditional techniques, as well as cryopreservation technologies, will facilitate the development of new cultivars.

## BREEDING

Citrus pollen can also be cryopreserved for extended lengths of time. Thus, breeding programs can store and then exchange pollen of desirable cultivars that may not bloom at the same time. In some cases, pollen can also be shipped between countries, which may provide access to novel genetic resources currently not available in the United States.

Now that citrus cryopreservation is a reality, the application of this technology for varietal improvement, propagation, pathogen elimination and conservation has become possible. In effect, the CRB has created a tool that breeders, researchers and industry can use to improve the efficiency, availability and profitability of citrus for years to come.

***Gayle M. Volk, Ph.D., is a research plant physiologist with the USDA-ARS National Center for Genetic Resources Preservation in Fort Collins, Colorado.***

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*Figure 1. Protein Interaction Reporter technology is being applied to identify protein interactions within the Asian citrus psyllid (ACP), which are critical to the insect's ability to transmit and spread Liberibacter. Interacting proteins are locked in place with a specialized cross-linker (red molecule linking green and gray protein representations). Mass spectrometry analysis reveals the identity of the cross-linked regions. Protein interactions between ACP and Liberibacter are being investigated as targets for the development of specific citrus greening control agents.* ACP image used with permission of Michael Rogers, Ph.D., University of Florida.

# NEW SOLUTIONS TO PREVENT THE SPREAD OF HLB

## *Blocking Liberibacter transmission by the ACP*

***John Ramsey, Juan Chavez, James Bruce and Michelle Cilia***

## PROJECT SUMMARY

*Control of the Asian citrus psyllid (ACP) vector is the most effective way of preventing the spread of the bacterial pathogen implicated in causing huanglongbing (HLB), also known as citrus greening disease. The citrus greening bacterium has to spread throughout the body of the psyllid vector before it can infect a healthy tree. This transmission process is regulated by highly-tuned interactions between psyllid and bacterial protein molecules. Proteins are critical molecules in all life on earth because of their intricate and highly specific shapes that allow two or more proteins to interact to perform some function, similar to how a lock and key fit together, resulting in a lock opening. Pairing the right "locks" and "keys," the citrus greening-associated bacteria gain entry into psyllids to spread from plant to plant. By understanding how the shapes of the bacterial and psyllid proteins fit together, we can design inhibitors that will prevent Liberibacter transmission by psyllids. This approach has been used to develop inhibitors of many human pathogens, including HIV.*

*Our team is using Protein Interaction Reporter (PIR) technology, developed in the laboratory of James Bruce, Ph.D., to characterize the shapes of the protein interactions that regulate transmission of the citrus greening bacterium by psyllids. Our team will provide the HLB research community with information on these interactions on a scale that cannot be achieved using traditional approaches to measure protein interactions. Each interaction has the potential to be a new, economically beneficial target for the development of a novel inhibitor compound that prevents transmission.*


## INTRODUCTION

The Asian citrus psyllid (ACP) is the only insect capable of transmitting the bacteria associated with HLB, *"Candidatus* Liberibacter asiaticus" (CLas) in North America. Specialized interactions unique to pathogen transmission within the psyllid represent promising targets for specific and effective disease control. Rendering the ACP incapable of transmitting the HLB-associated Liberibacter is a promising strategy to stop the spread of the disease in California and elsewhere. Implementation of such a strategy relies on an enhanced understanding of the mechanism of transmission of the disease-associated bacterial pathogen within the insect vector.

Work underway in the laboratories of Michelle Cilia, Ph.D., of the USDA-ARS/Boyce Thompson Institute for Plant Research at Cornell University and James Bruce, Ph.D., of the University of Washington is focused on understanding how Liberibacter moves through the ACP from the moment it is ingested from an infected plant and subsequently egested into a healthy plant. Using Protein Interaction Reporter technology (PIR), a cutting edge tool developed in Bruce's lab for analysis of protein interactions, researchers in the Cilia and Bruce labs are working to peer inside the insect to identify components of the pathogen or insect that are required for successful pathogen transmission. Strategies to block transmission of Liberibacter are being developed based on new understanding of protein interactions between Liberibacter and the psyllid during transmission. The objective of this work is to design specific inhibitors of protein interactions critical for bacterial pathogen transmission and ultimately to provide growers with novel and robust tools to prevent the spread of citrus greening disease.

## CIRCULATIVE PATHOGEN TRANSMISSION BY INSECT VECTORS

As the sole insect vector capable of transmitting the HLB-associated bacterium in the United States, the ACP is the primary target to control disease transmission. Bacterial pathogens from the genus '*Candidatus* Liberibacter,' which are associated with the disease, must complete a complex journey through the insect vector before infecting a healthy plant. To complete this circulative transmission pathway, Liberibacter must cross multiple tissue barriers -- for example, the gut and

the salivary glands -- within the insect and circumvent the insect's immune system. Transmission would not be possible without cooperation between Liberibacter and the ACP. During their co-evolution, it is likely that the pathogen has exploited essential proteins or other biomolecules of the vector to enable its transmission. The initial goal of this research program is to identify protein interactions between Liberibacter and the ACP and to characterize the function of these interactions in the spread of HLB.

## PROTEOMICS AND MASS SPECTROMETRY

The revolution in genomics technology has advanced biological research by enabling rapid genome sequencing of relevant plant, pathogen and insect genomes. Knowledge and understanding of genomics opens the window to how DNA codes for specific proteins. Concurrently, technological advances in the use of mass spectrometry have provided (or allowed for) the highly accurate and sensitive identification of proteins extracted from a biological sample.

Together, all the proteins of an organism are referred to as the "proteome," and proteomics is the study of how an organism's proteins function and interact with one another. A mass spectrometer is the central tool that our team uses to measure proteins and protein interactions. Proteins are in many cases the central biomolecules involved in performing the specialized cellular functions enabling productive interactions between pathogens and their plant hosts and insect vectors during transmission. Mass spectrometry-based proteomics is being applied in the Cilia and Bruce labs to study HLB-associated Liberibacters in both the trees and in the ACP.

A previous *Citrograph* article described the first aim of our CRB-funded project – to identify specific protein changes in citrus trees in response to infection with Liberibacter to establish early stage biomarkers of disease. The second aim of our research is to identify protein interactions between Liberibacter and the ACP during circulative transmission of the pathogen within the vector, using Protein Interaction Reporter technology.

## PROTEIN INTERACTION REPORTER TECHNOLOGY

A revolutionary method for detection of protein interactions in living cells, called Protein Interaction Reporter technology, has been developed in the Bruce laboratory. The central element of this technology is a novel protein cross-linker which is capable of penetrating live cells and binding to specific surface-exposed protein components. The cross-linker locks proteins together as they exist in living cells and enables our team to study interacting proteins in great detail using mass spectrometry. The locking of protein surfaces to one another is achieved by two reactive groups tethered to the core of the cross-linker that form covalent bonds with neighboring protein surfaces. These binding events may serve to cross-link neighboring regions within one protein – the intra-molecular cross-links reveal potentially valuable information about the three-dimensional shape of the protein surface. Understanding a protein's shape is critical to understand its function. Alternatively, each reactive group of the cross-linker may bind to neighboring regions of two interacting proteins.

Mass spectrometry analysis of the cross-linked proteins is subsequently used to identify the sequences of the interacting proteins. A critical difference between this methodology and other techniques used to identify protein interactions is that, using PIR, we can map exactly where protein interactions are occurring within interacting proteins **(Figure 1)**. This enables precise modeling of the shape of the protein-protein interaction, provides insight into the function of the interaction and facilitates the development of inhibitors of the interaction, which will be tested for their efficacy in blocking transmission.

Our team is using PIR technology to capture connections between proteins of the pathogen and the insect, in order to increase the understanding of the intricate protein connections required for the insect to successfully transmit the disease. Previously, Bruce has applied this technology using living human cells to identify protein crosslinks in all major cellular compartments. Also, the Cilia and Bruce labs have collaborated in the discovery of structural features of the aphid-transmitted *Potato leafroll virus*, which provided insight into the transmission mechanism of this virus by its insect vector.

## RESEARCH MILESTONES

Analysis of Liberibacter using PIR technology is greatly facilitated by the preparation of an enriched sample of pathogen cells from either the plant host or insect vector. With the help of Mark Hilf, Ph.D., a research plant pathologist at the USDA-ARS in Fort Pierce, Florida, we have implemented a protocol to produce an enriched sample of intact, live Liberibacter cells from a sample of infected ACP. Insects are blended in a homogenization buffer that maintains the integrity of microbial cell walls and retains interactions between CLas and associated psyllid cells. The cells in the insect homogenate are fractionated (separated) based on their size to generate a sample enriched for Liberibacter cells. In addition to pathogen cells, this method isolates endosymbionts associated with the ACP. Endosymbionts are other bacteria living within the insect that benefit the insect in some way (like the bacteria that inhabit the human gut) and may themselves be promising targets for vector control.

Samples enriched for Liberibacter and ACP endosymbionts were treated with the PIR cross-linker, and the reaction products were analyzed for protein interactions using mass spectrometry. A schematic of this workflow, from sample preparation of

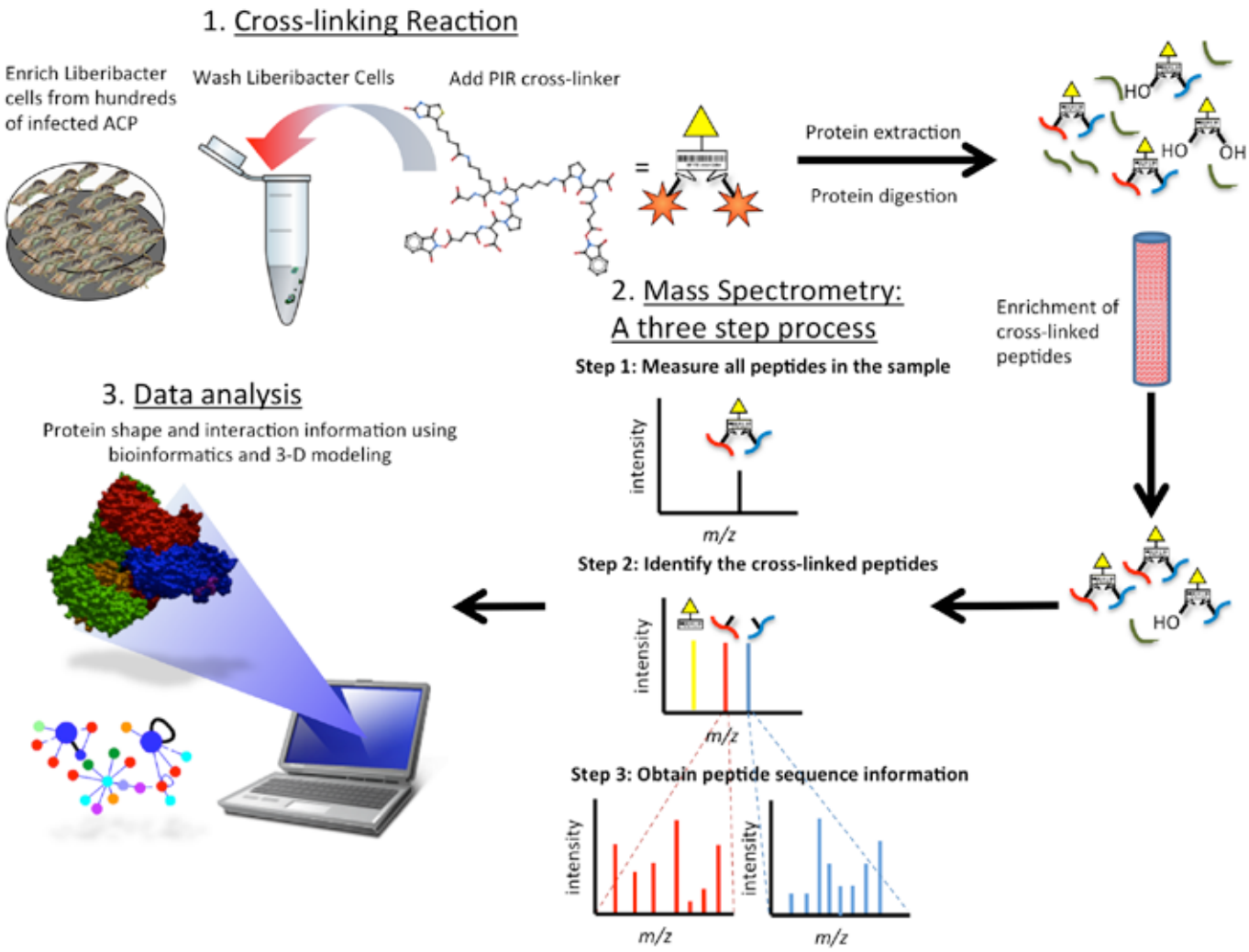


*Figure 2. Schematic representation of Protein Interaction Reporter workflow. Several hundred Liberibacter-infected adult ACPs are processed to generate a sample enriched for intact Liberibacter cells. The PIR cross-linker is added to the enriched sample, and interacting proteins are cross-linked in a reaction that proceeds at room temperature. During the subsequent protein extraction and digestion of the protein with trypsin to create short peptide fragments, the cross-linker keeps the proteins locked as they were within living cells, essentially capturing a protein interaction snapshot. Peptides bound together by the cross-linker are purified from the sample using chromatography and are injected into the mass spectrometer for determination of the sequences of the bound peptides. Analysis of the cross-linked regions enables three-dimensional modeling of the predicted topology of the protein complex. In the next phase of the research, inhibitors of protein interactions predicted to be important for Liberibacter transmission, including interactions between Liberibacter and ACP proteins,will be designed and tested for their efficacy as novel disease control agents. While many traditional tools allow researchers to focus on protein interactions within microbes or hosts, Protein Interaction Reporter technology enables our team to probe the interface between host and pathogen where the most promising sites for disruption may occur.*

Liberibacter-infected ACP to identification of cross-linked proteins, is shown in **Figure 2**. Promising initial data include the detection of protein interactions between Liberibacter proteins, in which two copies of the same protein come together to form what is called a protein homodimer. Based on the role that this protein has been predicted to play in related pathogens, we hypothesize that this interaction may be critical for transmission of Liberibacter by the ACP. The functional consequences of this interaction are currently the subject of follow-up experiments, including engineering the expression of the Liberibacter protein in experimentally tractable bacteria to perform additional analysis of the protein's structure, function and interacting partners.

Furthermore, initial data revealed protein interactions between the ACP and its associated bacterial endosymbionts. Based on sequences found in its genome, the endosymbiont *Carsonella* is predicted to provide the ACP with metabolic products, including amino acids, which the ACP may not obtain sufficiently from its diet. This prediction is supported by the PIR data. The role of the *Wolbachia* endosymbiont in the ACP is not clear and, in some cases, contradictory. In some insects, *Wolbachia* benefits insect reproduction, while in other instances, *Wolbachia* has been used as an insect biocontrol agent due to its deleterious effects on insect reproductive biology. Deciphering specific interactions between these organisms will serve as the basis for understanding what roles these insect-associated microbes may play in the ability of ACP to successfully spread HLB.

## CONCLUSION

The interface among the ACP, CLas, associated endosymbionts and citrus trees is of critical importance in determining whether this pathogen or these disease-related pathogens will be spread successfully by its insect vector. Ongoing research in the Cilia and Bruce labs enabled by CRB funding has the power to reveal details about these interactions which will expand our

understanding of how the ACP is able to transmit Liberibacter from an infected tree to a healthy one. There are many ways that transmission can be unsuccessful, and our research objective is to determine these points of vulnerability in order to design effective and specific control agents. In addition to avoiding non-target effects associated with broad spectrum insecticides, development of control agents that specifically block Liberibacter transmission will exert less pressure on the insect to develop resistance, as treatment will stop the spread of disease without killing the ACP.


## Acknowledgements

We would like to thank the Citrus Research Board for funding this research. The authors also would like to thank Jaclyn Mahoney and Jared Mohr, junior research scientists in Cilia's lab, who provide the team with invaluable contributions towards achieving all CRB-funded research objectives. Further, the authors thank Kathy Moulton and David Hall, Ph.D., at the USDA in Fort Pierce, Florida, for their contributions towards enabling our team to rear the ACP used in these experiments.



***Michelle Cilia, Ph.D., is research molecular biologist in the USDA-Agricultural Research Service, an assistant professor at the Boyce Thompson Institute for Plant Research, and an assistant professor of plant pathology and plant-microbe biology at Cornell University in Ithaca, New York. James E. Bruce, Ph.D., is a professor of genome sciences and a professor of chemistry at the University of Washington in Seattle, Washington. John Ramsey, Ph.D., is a post-doctoral associate in Cilia's lab, and Juan Chavez, Ph.D., is a staff scientist in Bruce's lab.***



**CRB Project Number 5300-153A/B**


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## Glossary

**Circulative Pathogen Transmission:** Pathogen transmission method within an insect vector in which the disease-causing pathogen must cross gut and salivary gland barriers for successful transmission.

**Covalent Bond:** A chemical bond between two atoms that is characterized by shared electron pairs. Covalent bonds are robust interactions between atoms resistant to non-specific disruption.

**Mass Spectrometry-Based Proteomics:** Analytical chemistry technique to identify proteins based on the masses of their component peptides.

**Protein Interaction Reporter Technology:** An analytical method using a protein cross-linker and mass spectrometry to identify protein interactions within and between living cells.

**Protein Cross-Linker:** Chemical designed to contain two linked reactive groups that bind to the surface of associated proteins and capture a snapshot of protein interactions.

**Protein Homodimer:** A protein complex composed of two identical protein molecules.

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*Hundreds of Asian citrus psyllid nymphs on young flush of citrus. ACP nymphs produce copious amounts of a sugary-waxy substance on which ants love to feed.*

# LIFE OF THE ACP

## *Field experiments to determine natural enemy impact on ACP in southern California*

***Erica J. Kistner and Mark S. Hoddle***

## PROJECT SUMMARY

*The efficacy of ongoing biological control programs targeting Asian citrus psyllid (ACP) population growth and spread in southern California is poorly understood. In addition to biological control by the parasitoid,* Tamarixia radiata*, ACP numbers also may be reduced by naturally occurring predators. By examining ACP survival and mortality factors across different life stages in the field, we determined that both parasitism and predation limited ACP population growth.*

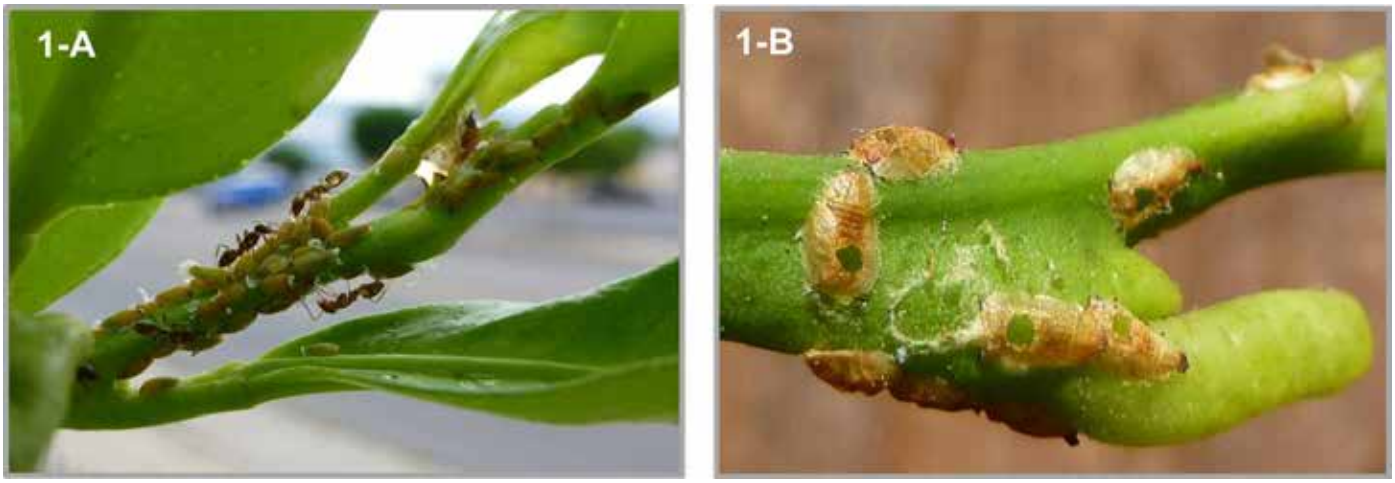



*Figure 1. (A) Argentine ants tending Asian citrus psyllid nymphs on experimental colonies. These ants may interfere with biocontrol efforts by protecting ACP from* Tamarixia *and predators. (B) The remains of Asian citrus psyllid nymphs killed by* Tamarixia. *The circular holes near the head indicate where the adult wasps emerged from their mummified hosts.* Photos by Mark Hoddle.

## URBAN ORCHARDS OF SOUTHERN CALIFORNIA

Since its accidental introduction in 2008, the invasive Asian citrus psyllid, *Diaphornia citri*, is now widespread throughout southern California including San Diego, Imperial, Riverside, Los Angeles, Orange and San Bernardino counties. ACP populations appear to be flourishing in countless unmanaged residential citrus trees. These urban orchards provide a safe haven where ACP populations may grow free from insecticide applications that would likely be used in commercial citrus orchards.

In time, urban ACP may spill over into neighboring commercial citrus groves. This immigration threatens commercial citrus because ACP may spread the bacterium responsible for huanglongbing (HLB) from infected urban trees. Classical biological control, the deliberate introduction and establishment of host-specific co-evolved natural enemies from the pest's home range, is an important ACP management strategy being employed in urban residences where spraying is not feasible.

In 2011, scientists from the University of California at Riverside UC Riverside began releasing *Tamarixia radiata*, a host-specific parasitoid of ACP sourced from Punjab, Pakistan, with the intent of suppressing urban ACP populations across southern California. As of July 2014, the California Department of Food and Agriculture has released ~700,000 individuals. This parasitoid has multiple stable populations and has even spread to sites where it was never released. Despite these release efforts, the efficacy of *Tamarixia* in controlling urban ACP population growth and spread remains unknown. Preliminary results of bi-weekly ACP population density data across 27 sites over a two- to four-year period in Riverside and Los Angeles counties suggest that *Tamarixia* may be limiting ACP populations. However, these urban sites are home to both naturally occurring enemies (e.g., *Tamarixia*) and allies (e.g., ants) of ACP. Consequently, management of ACP and HLB in urban southern California is complex and multifaceted.

## NATURAL ENEMIES AND ALLIES

In addition to *Tamarixia*, ACP may have other enemies, including naturally occurring predators. Generalist predators, including lady beetles, are known to significantly reduce ACP numbers in Florida, but their impact in California is unknown. To complicate matters further, the Argentine ant, another invasive pest, may be helping ACP thrive in southern California. These ants have been observed to protect ACP colonies from their enemies (including *Tamarixia*). In exchange, colonies of ACP nymphs provide ants with honeydew, a sweet waste product that nymphs excrete **(Figure 1A)**. This type of beneficial relationship between two species is known as mutualism. All of these different species interactions (e.g. parasitism, predation and mutualism) vary across ACP life stages.

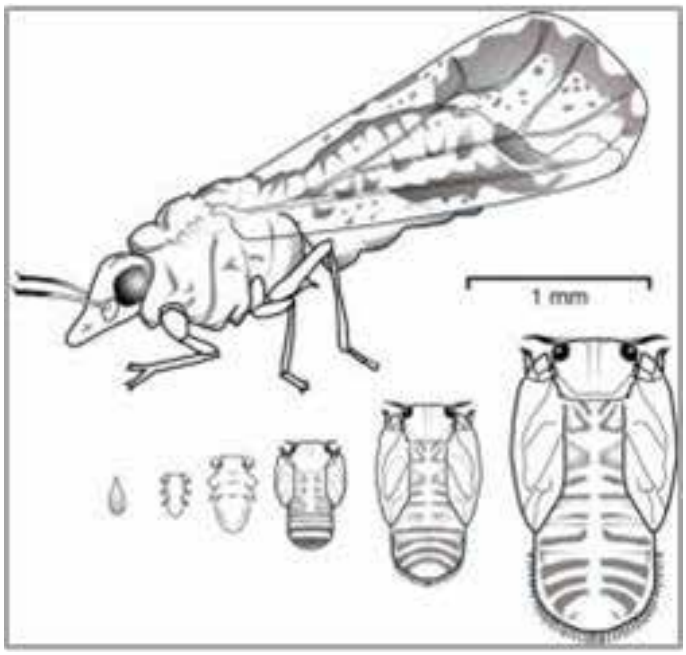



*Figure 2. Asian citrus psyllid,* Diaphorina citri, *life stages. UCNFA, G.O. Conville 1970.*

*Figure 3: Experimental ACP cohorts at the UC Riverside Biocontrol Plot. Each potted citrus plant is home to families of ACP (known as cohorts) whose survival is monitored from egg to adulthood.* Photo by Ruth Amrich.

# ACP LIFE CYCLE

ACP nymphs hatch from eggs and transition through five juvenile life stages (known as instars) before reaching adulthood **(Figure 2)**. Parasitism by *Tamarixia* usually occurs during the fourth and fifth instars while generalist predators may indiscriminately consume eggs and nymphs. ACP adults have wings and can fly. Their generation time is short with development from egg to sexually mature adult taking ~two to four weeks at 80ºF to 70ºF (or 27ºC to 21ºC).

# ACP LIFE TABLE EXPERIMENTS

To access the impact of natural enemies on ACP population growth, experimental ACP cohorts on small citrus plants currently are being monitored at two sites within Riverside County, California, where *Tamarixia* has been released and/or established (August 25, 2014 - current date). The UC Riverside Biocontrol Plot is equivalent to an organic citrus grove, while the second site (Lochmoor) is a residential site. Additionally, ACP cohorts were established at a residential site (Jurupa Valley) on November 1, 2014.

ACP cohorts of ~200 eggs are established in the lab on four citrus plants that are placed at field sites to assess the impact of natural enemies on these experimental ACP cohorts **(Figure 3)**. Four experimental treatments are evaluated to assess natural enemy impact on immature ACP:

(1) Potted plants are completely enclosed with a fine mesh bag to exclude all natural enemies. This treatment acts as a control to determine ACP survivorship rates in the absence of natural enemies. We expect survivorship rates to be high in this treatment since nothing is able to access and feed on ACP.

(2) Potted plants are enclosed within a coarse mesh bag to prevent access by large predators (e.g., ladybugs) while still allowing entry of small natural enemies like *Tamarixia*. This treatment provides information on how much mortality *Tamarixia* alone can inflict on ACP if these parasitoids can find ACP cohorts in the field.

(3) A sticky barrier is applied to potted plant bases to prevent access to ACP by walking natural enemies (e.g., spiders). Only natural enemies that can fly (e.g., ladybugs and *Tamarixia*) will be able to land on these plants to attack ACP eggs and nymphs.

(4) Potted plants are fully exposed, thereby allowing free access to ACP life stages by all natural enemies (e.g., walkers and flyers). Plants are examined every other day using a 10x lens, and numbers of ACP by life stage are recorded per treatment. Predators observed on ACP patches or trapped in tangle-foot barriers are identified. Parasitism by *Tamarixia* is easy to detect in the field since emerging adult wasps leave a circular exit hole in the body of the deceased ACP host **(Figure 1B)**.

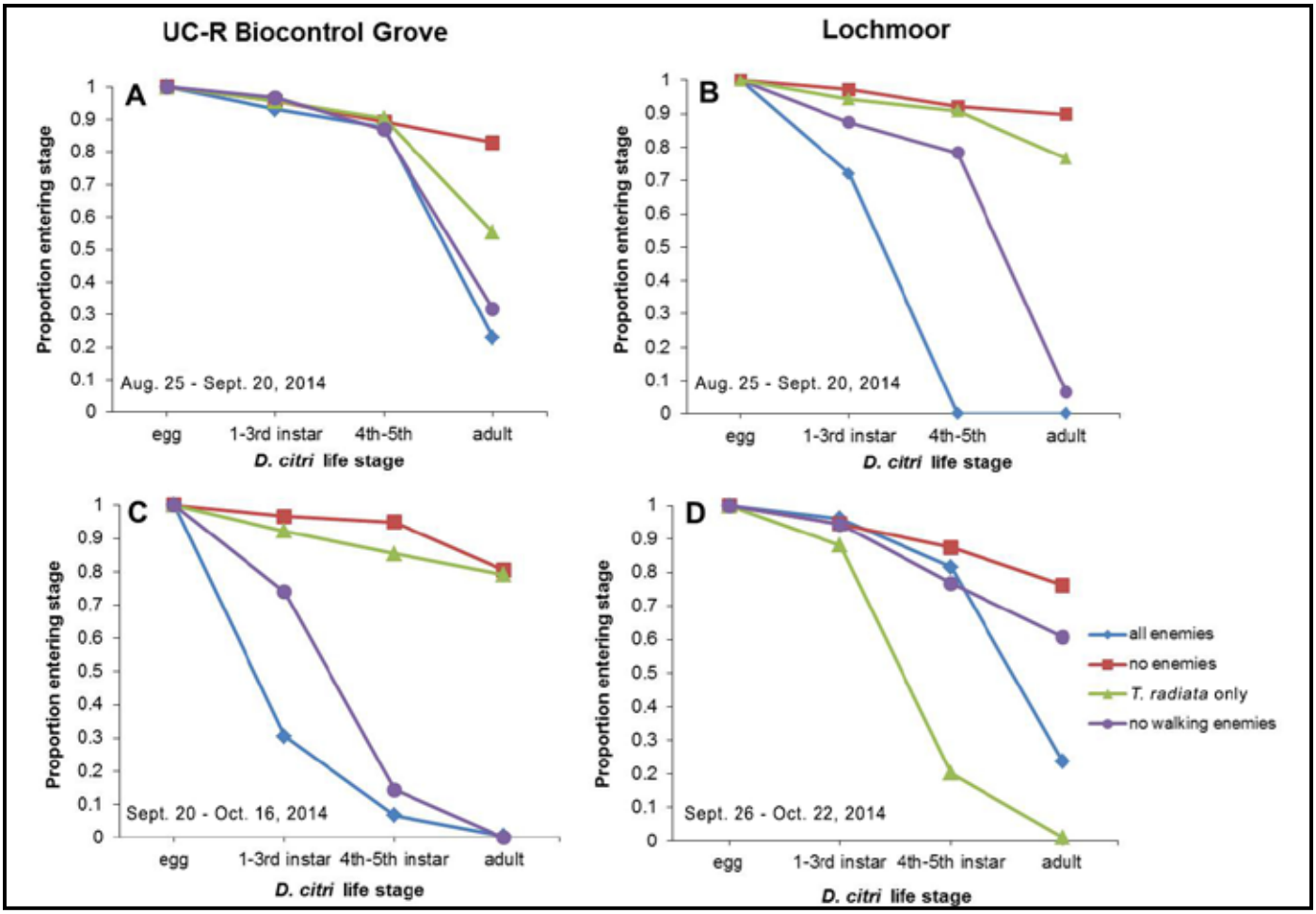



Figure 4. Survival of *D. citri* life stages across exclusion treatments at UCR Biocontrol plot (left column) and private residence Lochmoor (right column).

# RESULTS TO DATE

Overall, our preliminary results suggest that both *Tamarixia* and predators reduce experimental ACP numbers and may help reduce urban population growth over time.

# ACP SURVIVAL AND REPRODUCTION

Survival of experimental ACP cohorts plummets when they are exposed to natural enemies **(Figure 4)**. Natural enemies can reduce ACP survival by six-fold in some instances **(Figure 4C)**. In contrast, ACP do well in the absence of enemies (e.g. control treatment) with as many as 82 percent of all individuals surviving to reach adulthood over the summer and fall.

Surviving adult females exhibited higher net reproductive rates when protected from all natural enemies **(Table 1)**. ACP cohorts exposed to natural enemies yield

| Cohort | Treatment | $R_o$ | MT | $r_m$ | $T_d$ |
|---|---|---|---|---|---|
| **Aug-Sept 2014** | No enemies | 259.56 | 25.9 | 0.21 | 3.22 |
| | No walking enemies | 172.35 | 21.6 | 0.21 | 3.26 |
| | All enemies | 71.36 | 21.9 | 0.19 | 3.56 |
| | *T. radiata* only | 98.99 | 23.4 | 0.22 | 3.14 |
| **Sept-Oct 2014** | No enemies | 198.6 | 28.5 | 0.18 | 3.7 |
| | No walking enemies | - | - | - | - |
| | All enemies | 1.18 | 20.3 | 0.01 | 85.05 |
| | *T. radiata* only | 195.4 | 27.8 | 0.19 | 3.65 |
| **Oct-Nov 2014** | No enemies | 107.38 | 43.5 | 0.101 | 6.45 |
| | No walking enemies | - | - | - | - |
| | All enemies | - | - | - | - |
| | *T. radiata* only | 75.0 | 44.8 | 0.096 | 7.19 |

*Table 1. Comparison of life table parameters of* D. citri *cohorts across exclusion treatments at UC Riverside Biocontrol Plot: Ro , net reproductive rate (per capita rate of population growth); MT, mean generation time (in days); rm , intrinsic rate of increase; Td , doubling time (in days) for a population. If Ro > 1, then the population is growing. Treatments with no individuals surviving to adulthood have no life table values.*

far fewer adult females and are often completely destroyed before they could reproduce. Our results strongly suggest that ACP population growth is severely limited by the activity of natural enemies.

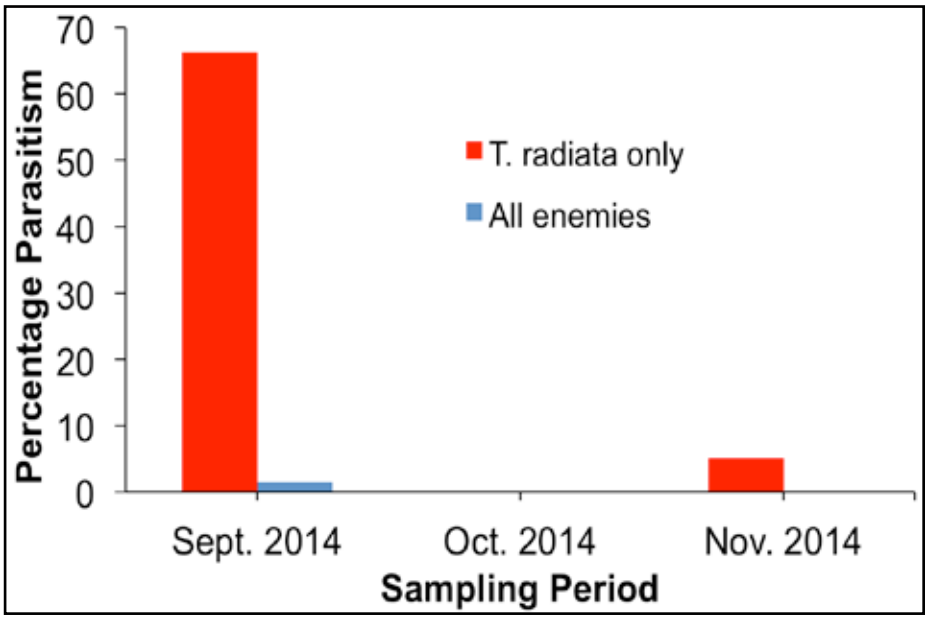



*Figure 5. Percent parasitism of ACP cohorts exposed to all enemies (blue bars) vs. exposed to only* Tamarixia *(red bars) at the UC Riverside Biocontrol Plot over September-November 2014.*

## BIOLOGICAL CONTROL VIA PARASITISM

*Tamarixia* parasitism rates decrease when exposed to naturally occurring enemies. For instance, at the UC Riverside Biocontrol plot, ACP cohorts exhibited a 66.3 percent parasitism rate when ACP cohorts were protected from all insects (both allies and enemies alike); and only a 1.4 percent parasitism rate was observed when cohorts were fully exposed in September 2014 **(Figure 5)**. We suspect this reduction in biological control may be the result of ACP's mutualistic relationship with Argentine ants. These ants have been observed tending experimental ACP colonies which likely protects them from natural enemies.

## NATURAL ENEMIES AND ALLIES

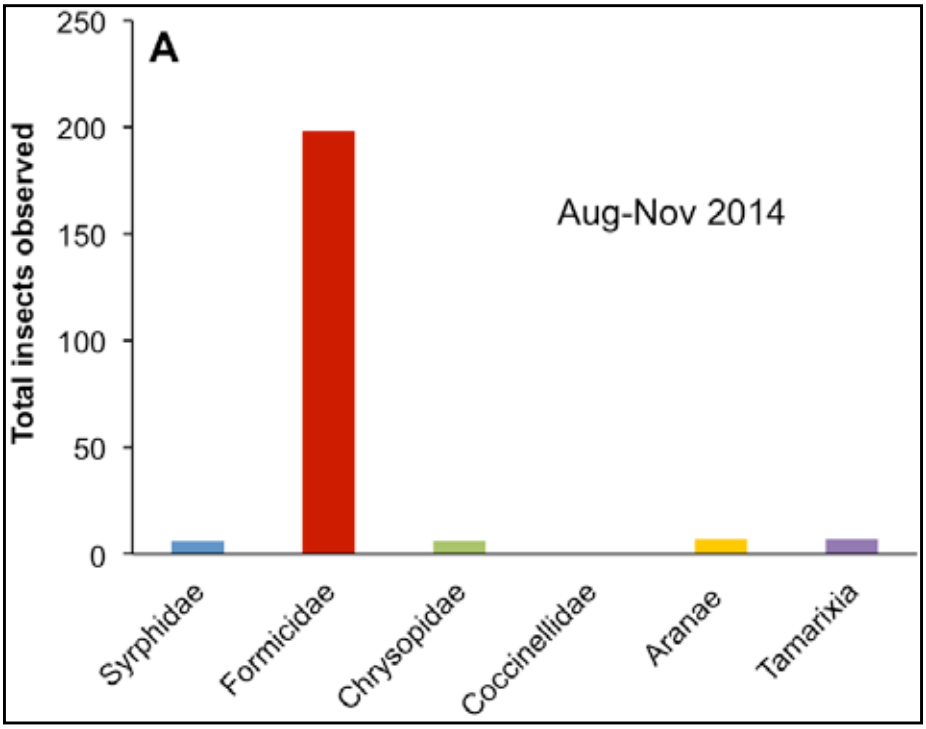
Argentine ants (Formicidae) are the most commonly observed insect interacting with ACP, but generalist predators also are abundant. Thus far, hover fly (Syrphidae) and green lacewing (Chrysopidae) larvae have been the most commonly observed predators, but spiders (Aranae) and lady beetles (Coccinellidae) also have been seen on experimental ACP colonies **(Figure 6)**. Hover fly and green lacewing larvae are voracious and may consume more than 100 ACP nymphs within 48 hours. ACP mortality from these hungry larvae can reach as high as 93 percent in some instances. As we continue these studies, it is anticipated that predator abundance and diversity will change during the winter and spring months.

## PROMISING PRELIMINARY RESULTS

The initial results of these life table experiments are very encouraging. Our results suggest that biological control by *Tamarixia*, as well as predation by naturally occurring generalist predators, are helping to limit ACP numbers at urban sites. These biocontrol agents may help prevent the future spread of HLB in California by reducing ACP populations, which, in turn, will help to protect our commercial citrus production areas from ACP and HLB. The experiments will continue until the end of 2015 to compare seasonal variation in ACP densities and natural enemy diversity and abundance through time. Argentine ants are prevalent at all experimental sites, and their interactions with ACP and natural enemies are subject to ongoing investigations.

***Erica Kistner, Ph.D., is a postdoctoral scholar, and Mark Hoddle, Ph.D., is an extension specialist in biological control and the director of the Center for Invasive Species Research in the Department of Entomology, University of California – Riverside.***

This project is supported in part by CRB Project 5500-191, USDA-APHIS-MAC Group and USDA-APHIS-CHRP. We are grateful to Michael Pazzani at UC Riverside Research and Economic Development.



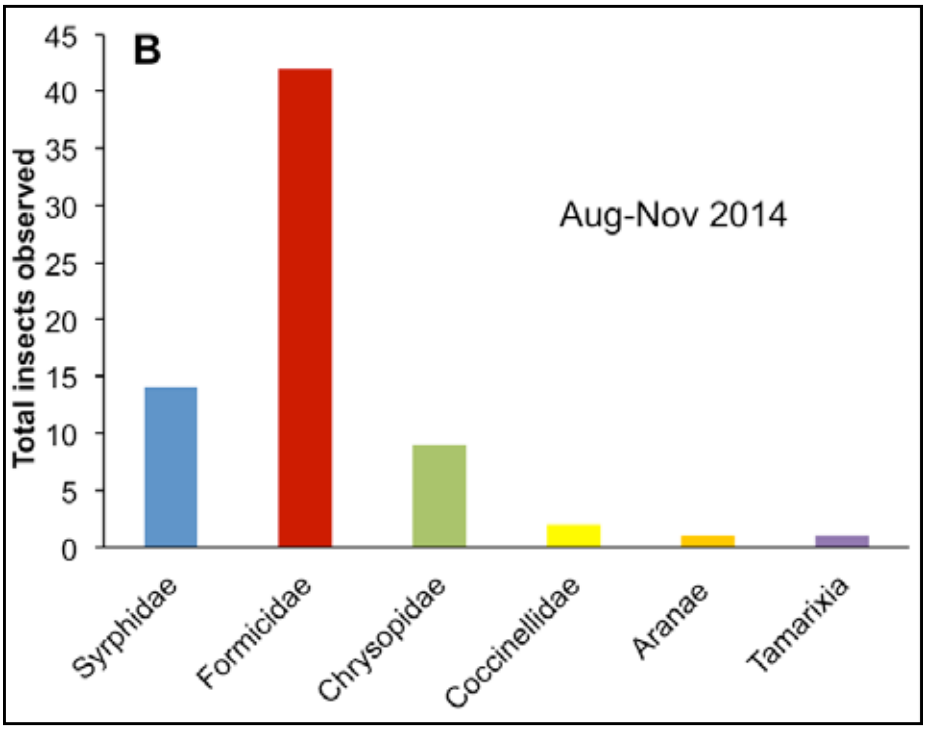




*Figure 6. Insects observed on* D. citri *cohorts on potted Citrus* volkameriana *or trapped on sticky barrier at UC Riverside Biocontrol plot (A) and private residence Lochmoor (B).*

## Glossary

**Classical biological control:** Importation and establishment of specialist natural enemies to suppress pest populations to less damaging levels.

**Parasitoid:** An organism that, during its development, lives in or on the body of a single host individual, eventually killing that individual by consuming it.

**Generalist predators:** A predator that consumes a range of prey species.

**Mutualism:** A relationship between two species of organisms in which both benefit from the association.

**Manipulative experiment:** An experiment in which the experimenter manipulates the system of study in order to uncover causal relationships.

**Life tables:** Tables on survivorship and fecundity rates of individuals within a population.

**Cohort:** A group of individuals all born in the same time period.

**Control:** An individual, group, event, etc., that is used as a constant and unchanging standard of comparison in scientific experimentation. Treatments are compared to controls to determine the effect of the treatments of interest.

*Figure 1. An adult male (black parasitoid on the left) and female (yellow parasitoid on the right)* Diaphorencyrtus aligarhensis *on a citrus leaf.*

# ENLISTING A SECOND NATURAL ENEMY SPECIES FOR ACP BIOCONTROL

## *First Official Release of* Diaphorencyrtus aligarhensis *in California*

***Mark S. Hoddle, Allison Bistline-East, Christina D. Hoddle and Michael Lewis***

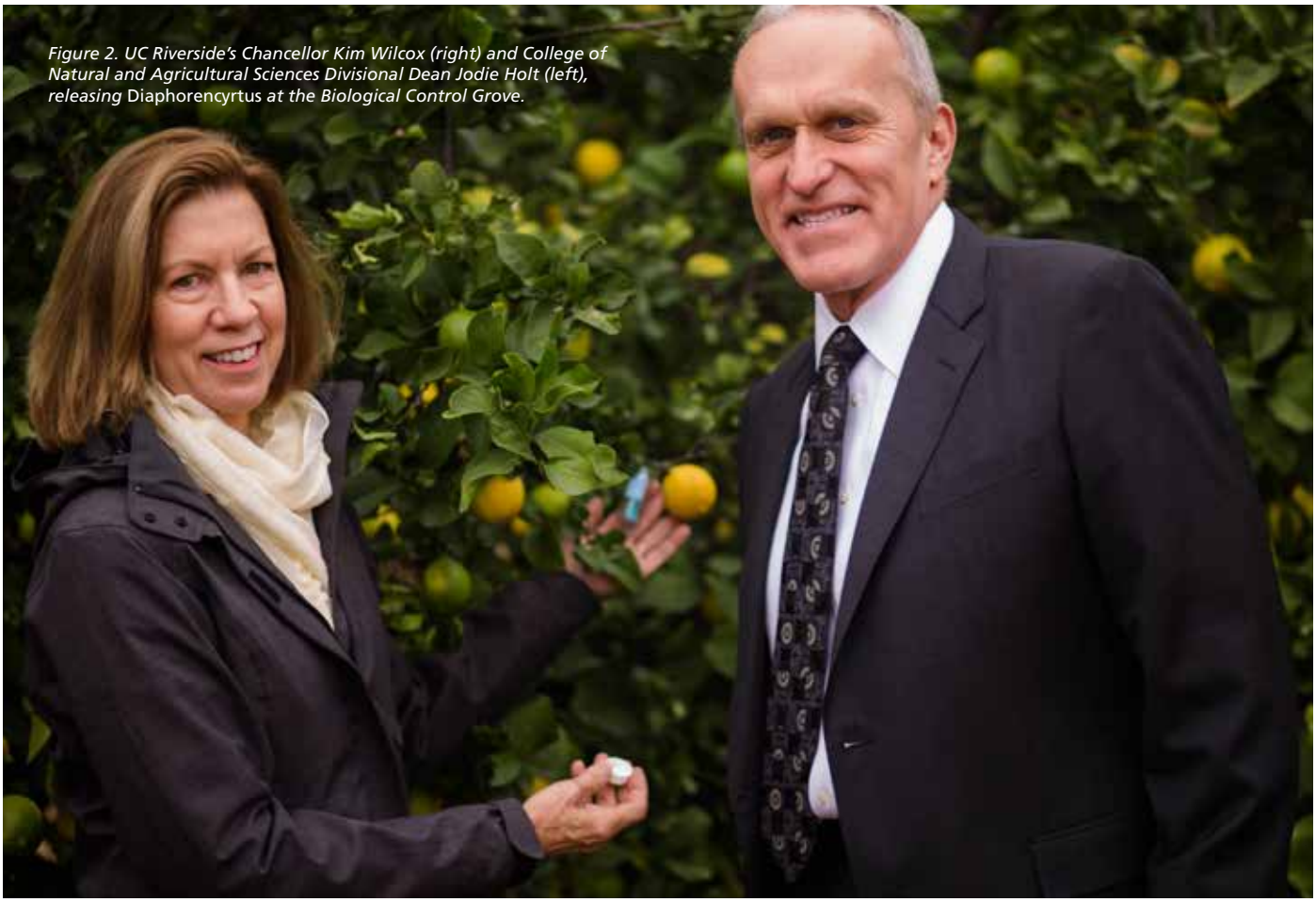


*Figure 2. UC Riverside's Chancellor Kim Wilcox (right) and College of Natural and Agricultural Sciences Divisional Dean Jodie Holt (left), releasing* Diaphorencyrtus *at the Biological Control Grove.*

## PROJECT SUMMARY

*A second species of natural enemy of Asian citrus psyllid (ACP),* Diaphorencyrtus aligarhensis*, imported from Punjab, Pakistan, was officially released on December 16, 2014, at the Biological Control Grove at the University of California, Riverside. It is anticipated that this natural enemy will be complementary to* Tamarixia radiata*, a parasitoid that also attacks ACP nymphs that was released in California in December 2011.*

The classical biological control project targeting Asian citrus psyllid (ACP) took another significant step forward in mid-December 2014 when 556 *Diaphorencyrtus aligarhensis* (Hymenoptera: Encyrtidae) **(Figure 1)** were released at the University of California Riverside (UCR) Biological Control Grove. The release occurred at 9:00 a.m. and was attended by approximately 40 people representing UCR, the Citrus Research Board (CRB), the California Department of Food and Agriculture (CDFA), local pest control advisors and media representatives.

The first of 15 vials that contained parasitoids was opened by Chancellor Kim Wilcox **(Figure 2)**; the remaining vials were distributed amongst attendees, opened and tied to branches of lemon trees. The road to releasing *Diaphorencyrtus* in California was long. The parasitoid was first collected from Punjab, Pakistan, in March 2011 and recovered from ACP nymphs regularly until the sixth and final collecting trip was completed in April 2013. A total of 1,023 *Diaphorencyrtus* were collected in Pakistan and returned to the quarantine facility at UCR and used to establish seven isocage lines.

## *DIAPHORENCYRTUS* SAFETY TESTING

Host range testing to determine the host specificity of *Diaphorencyrtus* took almost 18 months to complete. This process involved exposing the parasitoid to seven non-target species that included native psyllid species and psyllids used as weed biocontrol agents to determine the parasitoid's host range (i.e., the number of psyllid species it can attack) and host specificity (i.e., which psyllid species are most preferred for parasitism). Results from no-choice and choice tests

were used to prepare an 84-page Environment Assessment Report that was submitted to USDA-APHIS for review on November 1, 2013. The results of experiments demonstrated that *Diaphorencyrtus* has a very strong preference for ACP nymphs and likely poses little environmental risk.

After a lengthy review process, USDA-APHIS issued a Finding of No Significant Impact (FONSI) on October 26, 2014. On November 24, 2014, USDA-APHIS issued the official release permit, P526P-14-04034, to move *Diaphorencyrtus* out of quarantine for release and use in California as a biocontrol agent of ACP. The first official release of this parasitoid occurred on December 16, 2014 at the UCR Biocontrol Grove with the liberation of 556 parasitoids. The sex ratio of this Pakistani parasitoid is ~50 percent female and ~50 percent male.

*Figure 3. Diaphorencyrtus ovipositing in a third instar ACP nymph.*

*Diaphorencyrtus* is the second parasitoid species sourced from Punjab, Pakistan, to be released in California for the classical biological control of ACP. The first parasitoid species, T*amarixia radiata* (Hymenoptera: Eulophidae) (sex ratio is ~75 percent female and 25 percent male), also sourced from Punjab, Pakistan, was released on December 20, 2011 at the UCR Biocontrol Grove. Since this initial release, more than 850,000 *Tamarixia* have been mass reared and released by UCR and the CDFA. This parasitoid appears to have successfully established in California and is capable of spreading into new areas on its own.

## *DIAPHORENCYRTUS* BIOLOGY

*Diaphorencyrtus* is an endoparasitoid that can parasitize second through fourth instar ACP nymphs, but second and third instars seem to be preferred **(Figure 3)**. All parasitized ACP nymphs, regardless of stage that is parasitized, continue to feed, develop and molt to the fifth and final instar before they turn into mummies. Curiously, *Diaphorenyrtus* is unable to develop in late stage fourth instar (i.e., nymphs that are older than 7.5 days of age), as for some unknown reason, parasitoid eggs don't hatch. Fifth instar ACP nymphs are also unsuitable for oviposition because the cuticle may be too thick to pierce with the ovipositor, and defensive twitching by these large nymphs may deter ovipositing females.

Development from egg to adult parasitoid emergence takes about 16 days at 77ºF (25ºC). During this developmental period, parasitoid larvae passes through four larval instars, or developmental stages, before reaching a pre-pupal stage that transitions into the pupa. Developmental times for *Diaphorencyrtus* eggs, first and second instar larvae are about two days; the third, fourth and pre-pupal stages last around one day, while the pupal stage takes seven days to complete. During the third instar, *Diaphorencyrtus* larvae use secretions from the tip of the abdomen to anchor themselves within the thoracic region of the ACP nymph's body cavity. Once secured, parasitoid larvae then position themselves with the posterior of their bodies aligned with the head of the ACP nymph and the larval head or anterior region facing the posterior of the host. Therefore, when adult *Diaphorencyrtus* emerge, they chew an exit hole at the posterior end of the ACP mummy through which they escape. In contrast, *Tamarixia* chews an exit hole in the anterior or head region of the ACP nymph to emerge **(Figure 4)**.

*Diaphorencyrtus* females obtain protein for maturing eggs by host feeding on ACP nymphs. To host feed, females use their ovipositor to pierce the body of the ACP nymph. Hemolymph or insect blood, leaks from these wounds and is eaten by females. The trauma of being stabbed and then fed upon is often sufficient to kill ACP nymphs. Additionally, adult *Diaphorencyrtus* may gain nutrition from eating ACP honeydew, the dried white material that is exuded by feeding nymphs **(Figure 5)**.

*Figure 4. Asian citrus psyllid mummies showing the position of exit holes chewed by adult* Diaphorencyrtus *(left, hole is in posterior of the ACP mummy) and* Tamarixia *(right, hole is in anterior of the ACP mummy).*

*Figure 5. An adult female* Diaphorencyrtus *feeding on Asian citrus psyllid nymph honeydew.*

## DIAPHORENCYRTUS RELEASE PLAN

Releases of *Diaphorencyrtus* are planned for areas where *Tamarixia* has either not been released or surveys indicate that selected areas near *Tamarixia* release sites have little or no activity associated with this parasitoid. The reason for this strategy is to minimize competition between *Diaphorencyrtus* and *Tamarixia* so as to give *Diaphorencyrtus* the best possible chance to establish. To select sites for *Diaphorencyrtus* releases, we are working closely with the CDFA to identify suitable sites that encompass a variety of different climatic conditions. For example, *Tamarixia*-free sites in the Coachella Valley have been identified, and scouting is underway to look for suitable release areas in Riverside and Los Angeles counties.

Past experience suggests that establishing more than one natural enemy of a citrus pest in California can increase the chances of successful biological control. Perhaps one of the best recognized cases is that concerning the cottony cushion scale, *Icerya purchasi*, with the predatory beetle, *Rodolia cardinalis*, and the parasitic fly, *Cryptochaetum iceryae*. Surveys indicate that the beetle provides control in arid desert interior regions, while the fly dominates in cooler coastal areas where citrus is grown.

An additional factor that needs consideration is the number and frequency of releases needed to establish *Diaphorencyrtus* in California. Invasion biology can offer some help with this – a general rule of thumb indicates that multiple small releases or several large releases can increase the likelihood of establishment greatly. For example, large numbers of natural enemies released (i.e., >30,000) have resulted in natural enemy establishment approximately 80 percent of the time; releases of less than 5,000 individuals had colonization rates of 10 percent; and intermediate release numbers had a 40 percent establishment rate. Release frequencies of more than 20 attempts of

more than 800 individuals were more likely to establish, and establishment likelihood is low when <10 releases were made of fewer than 800 individuals. To increase the likelihood of establishing *Diaphorencyrtus*, these generalizations suggest that significant effort should be invested in making multiple releases of relatively large numbers of natural enemies.

Some advanced planning will be needed to prepare sites for releases. For example, it is likely that ant control will be needed as ants protect ACP from natural enemies. Also, releases should be made when ACP stages are abundant for parasitism and host feeding, and floral resources or some other food subsidy (e.g., softscale honeydew) are available as a carbohydrate source for newly released parasitoids and their offspring. Site security needs to be ensured to minimize preventable accidents such as pesticide sprays or pruning of trees, which could accidentally eradicate incipient *Diaphorencyrtus* populations.

## WHAT CAN WE REALISTICALLY EXPECT FROM *DIAPHORENCYRTUS*?

*Diaphorencyrtus* populations sourced from Taiwan, Vietnam and China (all female colonies) have failed to establish in Florida despite multiple release efforts involving more than 11,000 parasitoids. Reasons for this are unknown, but could be due to heavy pesticide use to control ACP, lack of synchrony between releases and ACP life stages suitable for parasitism, competition from *Tamarixia*, and possible predation of parasitized nymphs. Other factors may include low genetic diversity (because these parasitoids in Florida are all female, they don't reproduce sexually) and too little investment put into release and establishment efforts.

In many countries, *Tamarixia* and *Diaphorencyrtus* coexist (e.g., Vietnam, China and Taiwan); and in Pakistan, the results of ~ 2.5 year-long surveys in Kinnow mandarin and sweet orange suggest that average year round parasitism of ACP nymphs by *Diaphorencyrtus* is ~ 20 percent while *Tamarixia* accounts for ~30 percent parasitism each year. In Saudi Arabia, *Diaphorencyrtus* may be the only parasitoid species attacking ACP nymphs infesting Mexican limes with maximum parasitism rates of 64-71 percent being recorded.

It is impossible to predict what level of ACP suppression *Diaphorencyrtus* is likely to provide in California. It is anticipated that if *Diaphorencyrtus* establishes, it will complement the activity of *Tamarixia*, thereby increasing over-all ACP mortality. If *Diaphorencyrtus* does establish, it may possibly have eco-climatic preferences different to that of *Tamarixia*, which may allow it to provide control in areas where *Tamarixia* is not effective. The only way to determine these potential outcomes is through a multi-year research program that tracks the establishment, spread and impact of *Diaphorencyrtus* on ACP in urban and commercial citrus production areas in California.



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***CRB Project 5500-191***

*ALL PHOTOS COURTESY OF MICHAEL LEWIS, CENTER FOR INVASIVE SPECIES RESEARCH, UC RIVERSIDE.*


*Standard yellow sticky trap.*

# NOVEL ATTRACTANT AND TRAP FOR MORE SENSITIVE ACP MONITORING AND DETECTION

***Darek Czokajlo, Lukasz Stelinski, Kris Godfrey and Mamoudou Setamou***

The Asian citrus psyllid (ACP), *Diaphorina citri*, vectors three phloem-restricted bacteria in the genus *Candidatus* Liberibacter, which have been associated with huanglongbing (HLB), otherwise known as citrus greening disease. Citrus trees infected by HLB produce small, misshapen fruit characterized by bitter taste, rendering the juice and related products unmarketable. Infected trees gradually decline, drop much of their fruit load and ultimately die.

ACP was first reported in Florida in 1998, but has invaded many more regions, which include all citrus growing areas of the continental U.S., Puerto Rico and Hawaii. HLB was first discovered in Florida in 2005 and is now well-established. It has been confirmed in several commercial groves in Texas and in only one residential tree in California. Whereas the psyllid has been detected in several areas in Arizona, neither psyllids nor plant material has tested positive for Liberibacter.

Current sampling protocols for adult ACP rely on passive sticky traps, which capture ACP by incidental or random encounters of flying adults with these sticky surfaces or by tap sampling in which tree branches are shaken to dislodge psyllids onto a sticky surface held below the branch. This renders adult psyllid monitoring for forecasting or evaluating insecticide applications inaccurate. In 2008-09, a new ACP trap was developed through the joint research of Mamoudou Setamou, Ph.D., of Texas A&M University in Kingsville and Darek Czokajlo, Ph.D., of Alpha Scents, Inc. (unpublished data) **(Figure 1)**. We have found that this lime green trap captures significantly more ACP than the standard yellow traps commonly used. The un-baited, yellow sticky traps currently used for monitoring ACP adult populations are only marginally effective without an attractant.

Currently, a semiochemical-based lure (chemical used for communication between individuals) to attract ACP is not commercially available. ACP exhibits strong preference for citrus volatiles and aggregate and lays eggs exclusively on young unexpanded leaves. Thus, plant-related chemicals are crucial signals used by adults for plant selection. In addition, there is evidence documenting that mate location in ACP is mediated by a volatile sex pheromone and hydrocarbons emitted from the cuticle or outer surface of the insect. In this research, we evaluated the potential of using individual and

**Figure 1. ACP captures on different color sticky traps Weslaco, Texas, 2008**

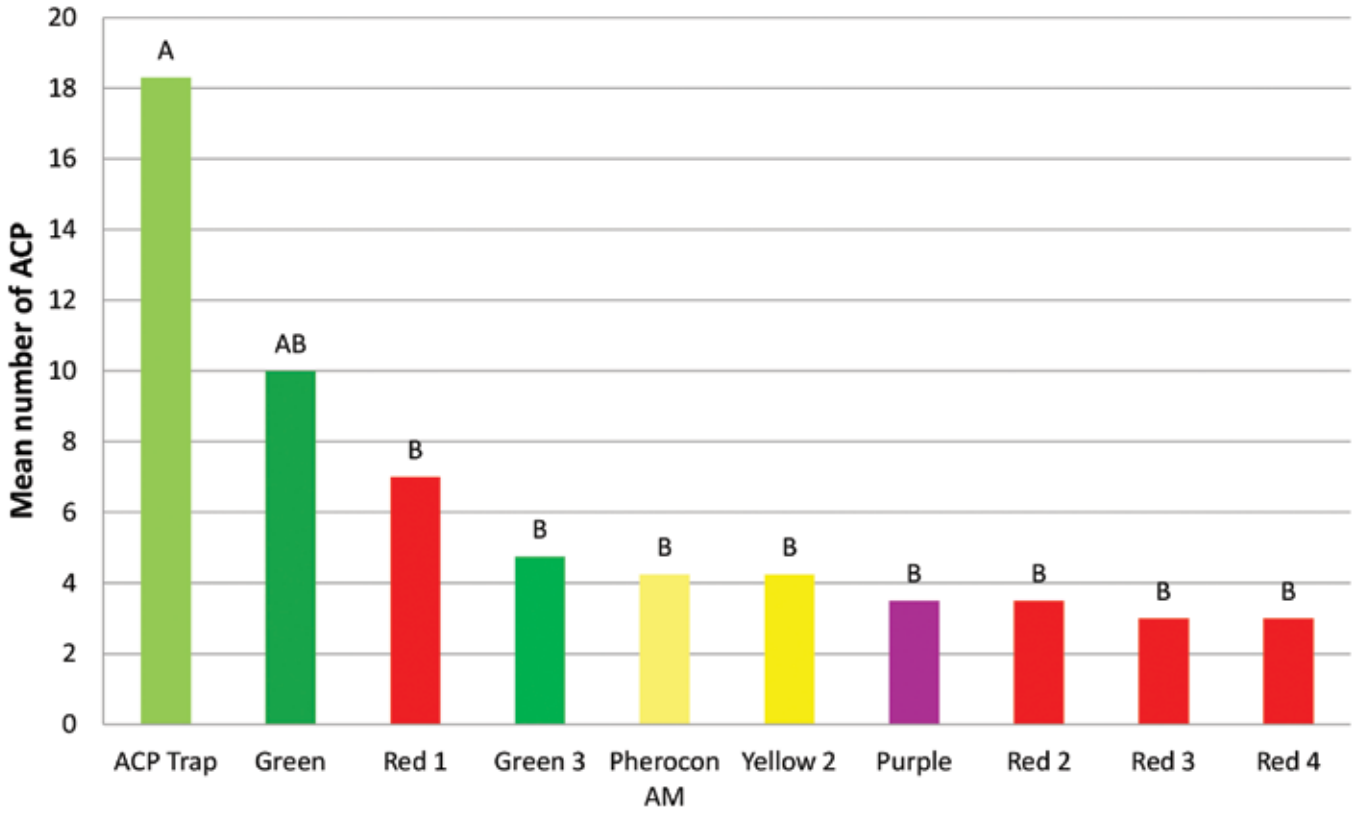


*Figure 1. Monitoring low populations of ACP with different color sticky traps, Texas A&M University-Kingsville, Weslaco, Texas, October 25-November 6, 2008.*


*Figure 2. ACP lure on Alpha Scents green ACP trap*

blends of plant volatile and pheromone compounds as field attractants to improve monitoring. The attraction of adult ACP to traps baited with synthetic compounds was compared to captures on un-baited (control) ACP traps.

To test potential semiochemicals for their ability to attract adult ACP, the number of ACP captured on baited green traps was compared to those captured on non-baited green traps. Traps were placed on the outer canopy of citrus trees at five feet above the ground along the edge of all four sides of groves. Trees were spaced about 24 feet apart; only one trap per tree. Formulations of active compounds were placed directly on traps in a controlled release device **(Figure 2)**. All treatments were replicated eight times at three different sites in Florida, Texas and California. In Florida, the lures were tested in Valencia and Hamlin orange groves. In Texas, evaluations were conducted in lemon groves. In California, trapping was conducted in residential trees.

There were two experiments. In Experiment One, lures were tested in June and July 2014; and in Experiment Two, they were conducted in September and October 2014. Traps were checked and replaced weekly, and the number of ACP captured on traps was counted.

For Experiment One, Alpha Scents formulated a total of eight different lures composed of host plant volatiles and ACP-produced compounds. Five lures were blends of host-plant volatiles only, and three lures were blends of plant volatiles and ACP-made compounds. All eight blends were tested in Texas, and seven blends were tested in Florida and California.

Based on results from Experiment One, we made another seven blends of plant host volatiles for Experiment Two. Three lures were blends of host-plant volatiles only, and four lures were blends of plant volatiles and ACP compounds. All blends were tested in Texas, and five blends were tested in Florida and California. The composition of the blends is not revealed to protect proprietary information. Data were subjected to analysis of covariance and a permutation test to determine statistical differences between means, although this data is not presented here.

Several of the blends tested resulted in a significant increase in the number of psyllids trapped as compared to un-baited control traps **(Figures 3-6)**. Specifically, in Florida the experimental blends MS1, MS1L, MS1AL and MS1HC appeared most promising and promoted greater capture of ACP on traps as compared with un-baited traps. Despite the lower numbers of ACP populations in Texas during June and July of 2014, significant differences in the effectiveness of blends in luring adult ACP were observed. Consistent with data obtained in Florida, traps baited with the experimental blends MS1A and MS1L captured significantly more ACP than the un-baited ones. During the September-October 2014 study period in Texas, higher ACP densities were recorded. Consequently, the number of ACP captured dramatically increased, and marked differences were recorded in the performance of experimental blends tested. Traps baited

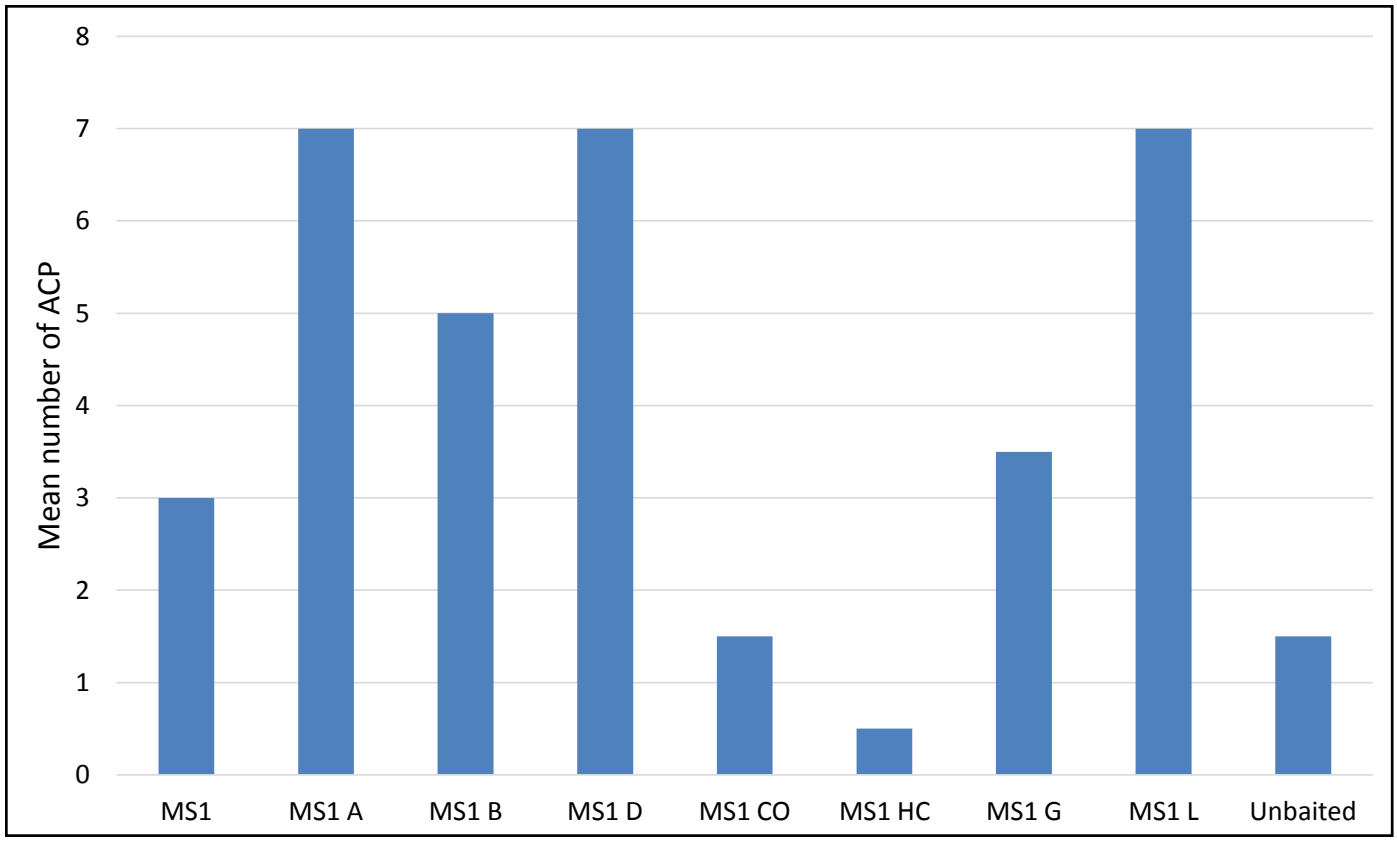


*Figure 3. ACP captures, Weslaco, Texas, July 2014.*

with the MS1A, , MS1L and MS1AL lures captured significantly more ACP adults. In California, most of the baited traps captured more ACP than un-baited traps, but the data were inconclusive (data not shown). Because traps were deployed in residential trees rather than commercial groves, problems were encountered during this trial including an unequal number of traps recovered. Therefore, the California data could not be analyzed to obtain a meaningful interpretation. Although our results indicate that these new lures increase the capture of ACP as compared with un-baited controls, the data were not consistent between states. Specifically, the data from California did not indicate efficacy as clearly as in Florida and Texas. These field trials need to be repeated in order to clarify this discrepancy. One possible explanation is the differences in ACP population density between the three states. Methods used to conduct trials will be the same at each location in the future to avoid inconsistencies in results.

An effective trapping system is paramount for quarantine programs, such as those currently in place in California and Texas. Quarantine programs rely heavily on the detection of pests at very low population levels in order to successfully eradicate targeted pest with insecticides. Given that visual color-based traps are only attractive to ACP from a short distance, an attractive lure that would capture ACP more effectively from longer distances and when psyllid populations are low would increase the confidence level in survey results. Pesticides used for eradication purposes could be used more judiciously.

An effective lure for monitoring ACP would also be useful in areas of the country where ACP are endemic and eradication is no longer possible, such as in Florida. Growers rely heavily on insecticides to reduce ACP populations in order to reduce the spread of the pathogen that causes HLB. Monitoring for the pest is difficult, time consuming and expensive. However, the increased need for insecticide sprays to control ACP is also very expensive, and the potential for the development of insecticide resistance in ACP populations already has been proven scientifically in Florida. Therefore, a more effective trap and lure system would be useful to more accurately estimate the number of psyllids in a given area. Pesticide applications could be based on ACP population density rather than on a calendar basis, and, therefore, greatly reduce the number of applications.

In summary, several promising blends for the capture of ACP were determined in this investigation. These blends hold potential as possible commercial lures for improved monitoring of ACP. Three to four times higher trap captures were achieved using various blends as compared to un-baited traps. These blends need to be further tested in larger field studies for confirmation of their effectiveness and possible refinement of their final formulations. All experimental lures

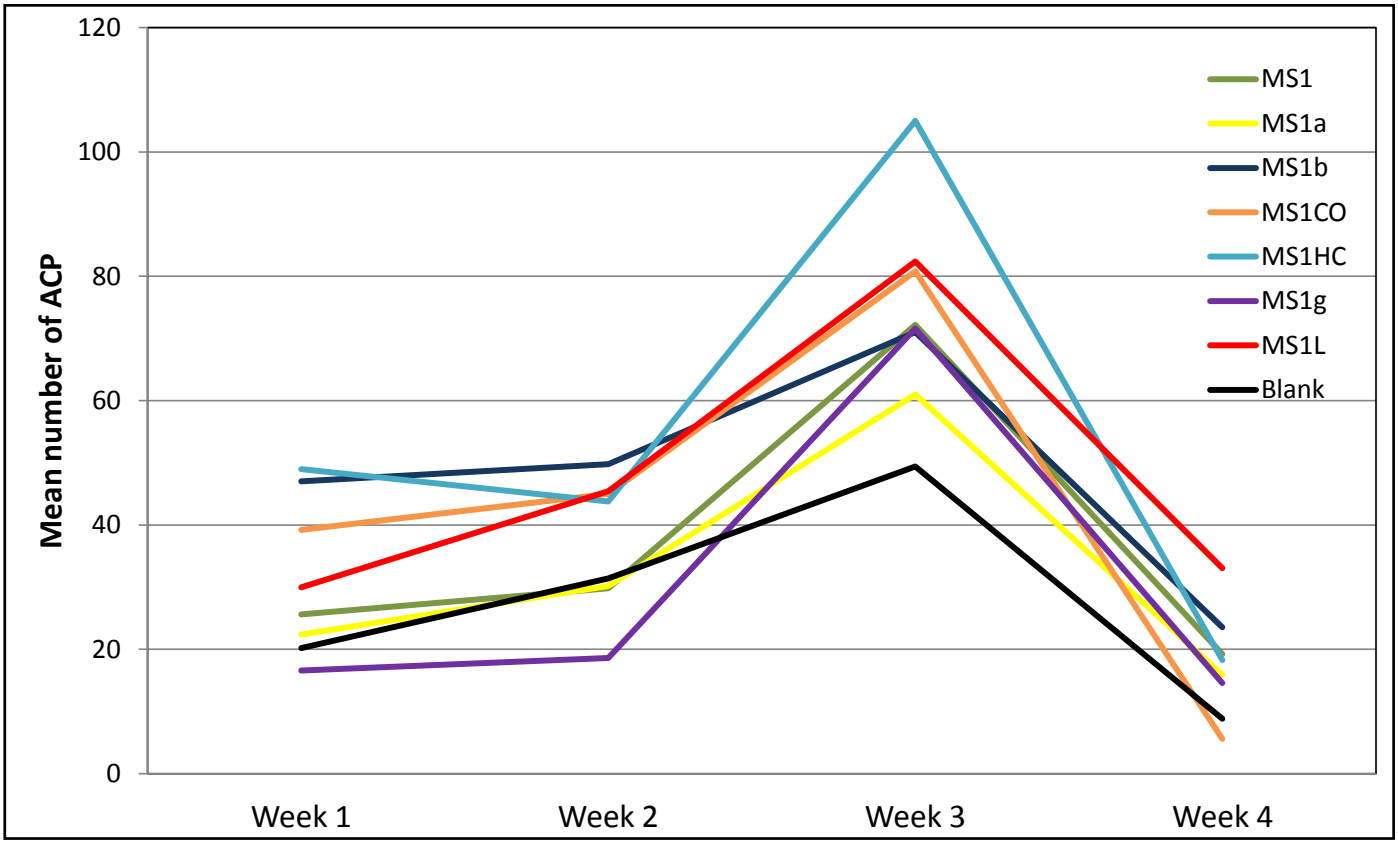


*Figure 4. ACP captures, Lake Alfred, Florida, July 2014.*

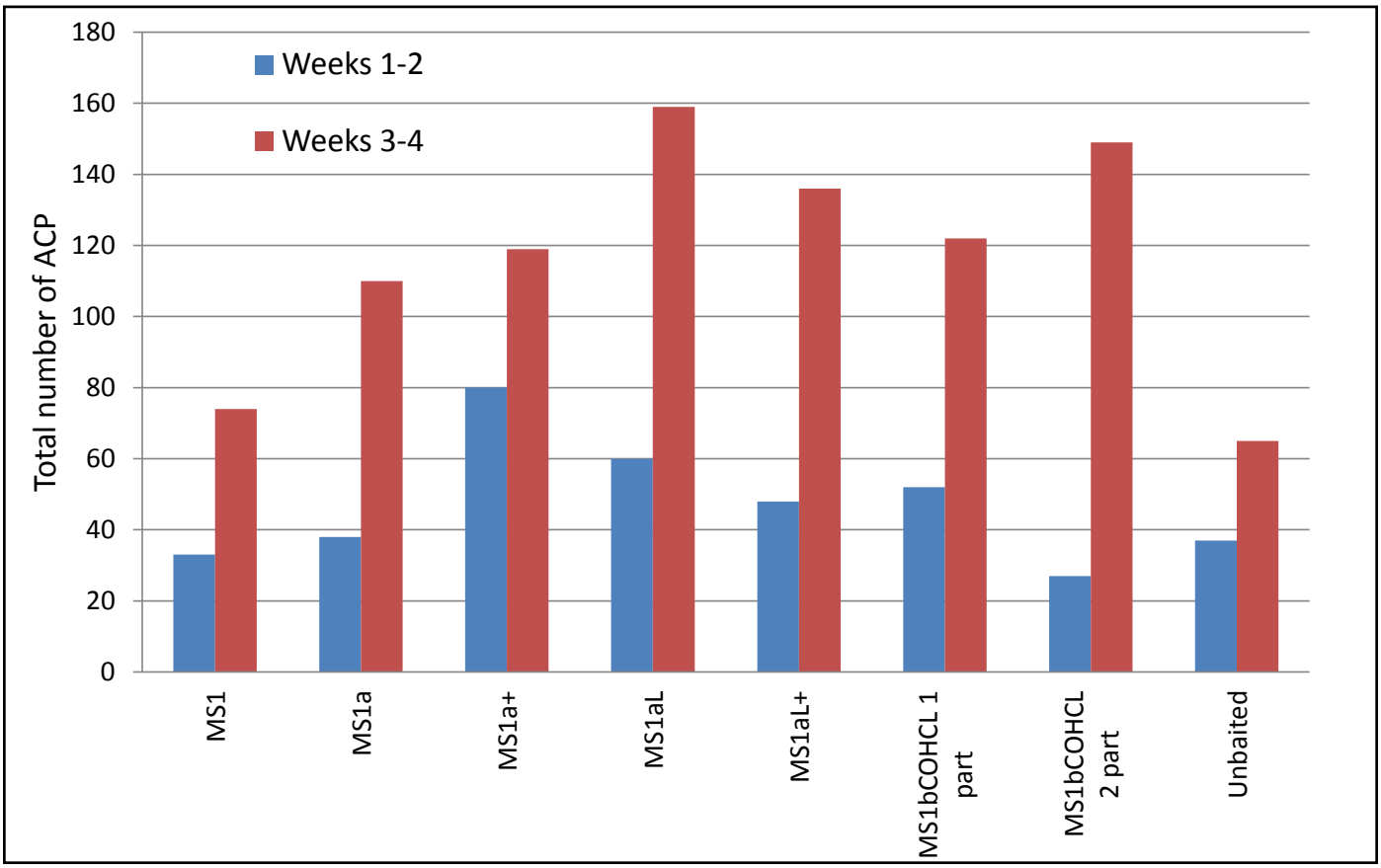


*Figure 5. ACP captures, Weslaco, Texas, September - October 2014.*

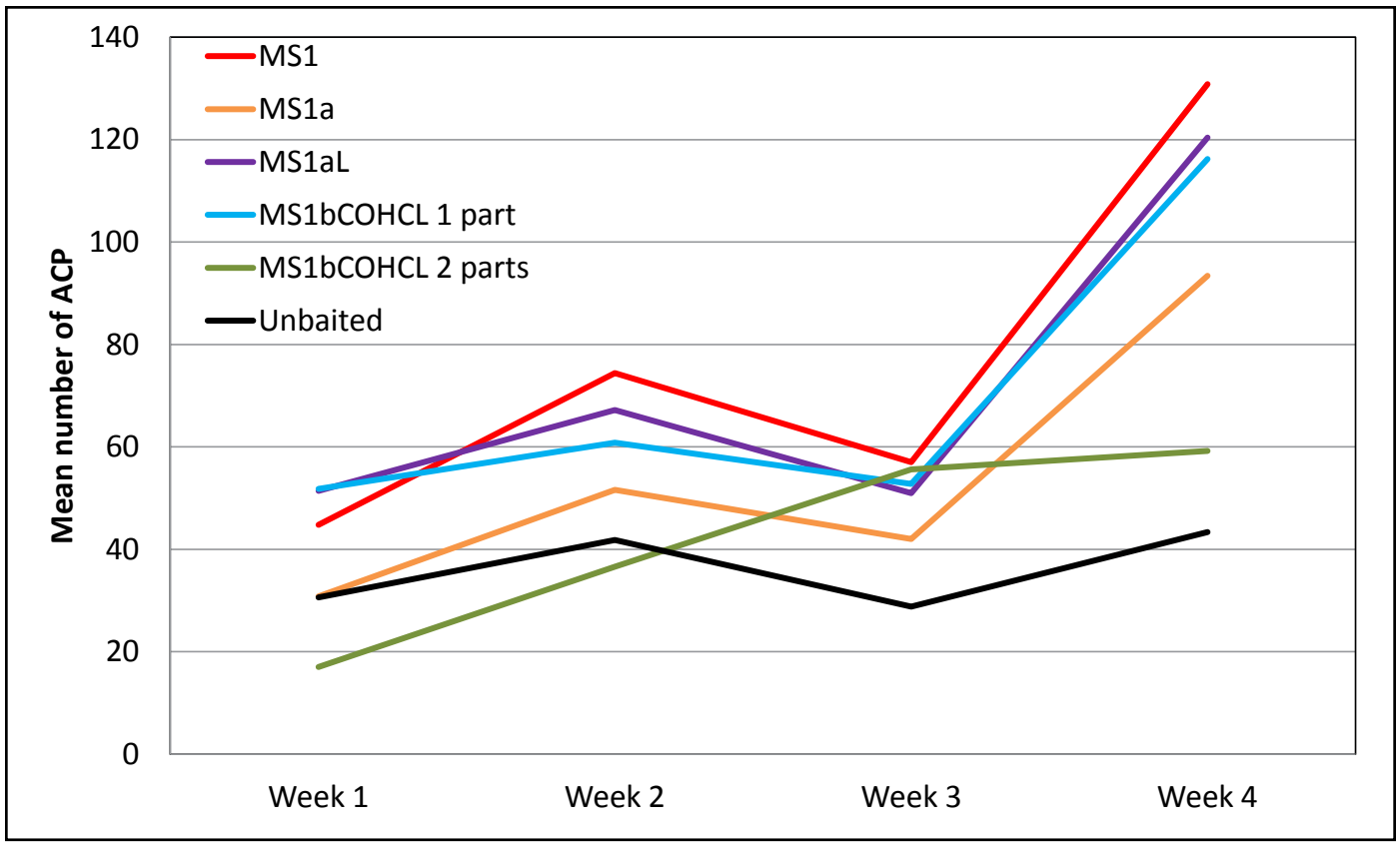


*Figure 6. ACP captures, Lake Alfred, Florida, August - September 2014.*

were active in the field for more than four weeks. Alpha Scents' ACP trap attracts three to four times more ACP than standard yellow trap; and in addition; the lure will attract three to four times more psyllids. Taking this into consideration, we can state that monitoring ACP with Alpha Scents' ACP trap and lure is six to eight times more effective than the currently-used un-baited yellow traps. The commercial ACP lures will be available from Alpha Scents in the 2015 season.

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