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On the cover - Meet Bobby, a Belgian Malinois. He and Cero (not pictured) are among the citrus industry’s brightest hopes for more thorough detection of huanglongbing (HLB). The two canine sleuths began their training for HLB imprinting in June of this year. For more information, see “Canine Detection of Citrus Canker May Show HLB Application Promise” on p. 22.
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

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<td>John Richardson</td>
<td>Jeff Steen</td>
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<td>Richard Bennett</td>
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<td>Toby Maitland-Lewis</td>
<td>Jack Williams</td>
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<td>Donald Roark</td>
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<td>Jim Gorden</td>
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<tr>
<td>Joe Stewart</td>
<td>Franco Bernardi</td>
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### District 2 – Southern California – Coastal

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<td>Alan Washburn</td>
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### District 3 – California Desert

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### Public Member

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<td>Ed Civerolo</td>
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Two significant board retirements occurred at the late June and early July nomination meetings – Pauma Valley grower Earl Rutz; and Dan Galbraith, Director, Northern Operations for Limoneira Company.

With just a year under my belt here at the Citrus Research Board (CRB), I recently took the opportunity to listen to some words of experience from both Earl and Dan. I thought the perspectives gleaned from these two board members’ combined 30+ years of CRB service was especially insightful. I know their experience will prove invaluable to me as I attempt to execute the board’s vision, but I also thought of the potential value to be gained by sharing with Citrograph readers some excerpts of those conversations.

In that spirit, I will dedicate this editorial to highlights of my conversation with Earl. My next editorial will focus on Dan.
Earl Rutz was first appointed in 2002, and recently opted to forego nomination for another term. He tirelessly served as a board member and, ultimately, CRB board chairman for the 2011-12 and 2012-13 seasons. The long hours he spent fighting the Los Angeles Basin traffic between his northern San Diego County home and Visalia to carry out his board service were legion – and all as a volunteer.

**Citrograph:** Reflecting on your 12-year “career” as a CRB board member, what was the industry’s research focus when you first began your service?

**Earl:** Genetics were all the rage at the beginning of my service. There was great promise in using the development of the citrus genome as a tool to understand invasive pests and diseases of citrus. Once our various worldwide research partners developed the citrus genome, we could then begin to understand the molecular mechanisms of host-pathogen, interactions.

The genetic profile of the citrus plant itself, as well as the genetics of citrus vectors and pathogens, is of great interest and importance; and the CRB became increasingly involved in pushing this work forward over the course of my service. [Current board member] John Richardson, [former CRB President] Ted Batkin and [consultant] Victor Amoah all preceded my involvement in leading the CRB into the genetics arena, but genetics were a large focus during my early years up to today.

**Citrograph:** What was the most rewarding part of your service to the board?

**Earl:** Because of my interest in the genetics area, I was appointed as chair of the New Varieties and Genetics Committee. There was a huge profile of activity in this committee, so much so that it was later split into two committees. I then took up the chair of Genetics. This was the most rewarding to me because of the burgeoning of the citrus genome sequencing and the board’s interaction with the genetics community at the Los Alamos National Labs and, eventually, other world-class researchers at NASA’s Jet Propulsion Laboratory. Today’s work in the “omics” [metabolomics, proteomics, transcriptomics, etc.] – the molecular-level research employed to identify biomarkers, early detection and, ultimately, tree therapy – has genetics woven throughout. Virtually all of the proposals being reviewed for upcoming funding by CRB’s various science committees today involve genetics and molecular-level research.

**Citrograph:** What have you found that CRB has gotten good at during your tenure? That is, what function does it serve today in advancing citrus research that you have had a hand in developing?

**Earl:** Young researchers have gotten involved, and the process of science has been highly enjoyable to watch. Seeing the scientists collaborate in real time, the “hallway talk” of scientists interacting is critical; and the CRB with its proposal review, progress reporting and other program activity has increasingly served as the catalyst for this interaction. The CRB’s meetings and programs serve as a forum for interaction where growers’ ideas also can click with scientists. The annual New Technologies Conference that got started during my tenure was a good venue for this, as were the national ACP/HLB conferences that the CRB organized in cooperation with the federal Citrus Health Response Program.

**Citrograph:** What areas does the CRB need to get better at in advancing citrus research – perhaps an area where the progress during your time on the board has not been as great as you would have hoped?

**Earl:** There have always been challenges for commodity boards such as the CRB to get practical solutions from the (increasingly complex) basic science that the grower assessments fund. I did champion the formation of the CRB’s Research Development and Implementation Committee, which is in place today and is making some progress in interacting with the university technology transfer offices. However, more facilitation in this area is needed. How to go about this is unclear, but we have to keep at it.

One development area I am especially proud of involves disease “signatures.” These unique molecular-level responses of the tree are critical, and when identified for specific diseases (Citrus Stubborn, for example) can lead to tools and
devices to detect critical pathogens of concern, and to distinguish one pathogen from another. I first suggested to the board that a grower’s smart phone might become a device for determining what ails a tree, particularly when various tree symptoms and diseases causing those symptoms can be masked and confused. They laughed at me, but when we suggested the concept to scientists at UC Davis and Los Alamos, they agreed it had been done in other fields (healthcare), and it could be done for citrus.

**Citrograph:** Why should growers who have not served on the CRB board consider doing so?

**Earl:** Growers should consider serving to get in touch with their industry. My view is that if they don’t, independent growers could cease to exist. “I don’t have the time” is a common refrain that I hear when I suggest to my grower friends that they should get involved. Yet I look around our current board membership, and I see that extremely busy people do take the time and find their service to be worth the time investment. So they find it of value, and it can be done. It’s not for somebody else to do, it’s for everyone to do. And I can almost promise that your grove operation will not suffer; it will likely benefit from what you learn.

**Citrograph:** What do you see as the most critical areas for the CRB as an organization to keep its eye on? What do you think the future of California citrus has in store?

**Earl:** Communicating science to growers is critical, as is keeping credibility with growers. CRB needs to keep getting better and more sophisticated in its delivery of the practical, usable science back to growers. As to the California industry, I think it will morph, becoming even more concentrated in the Central Valley, while a vestige of the industry will always remain in districts two and three. Psyllid control will lean heavily on biocontrol; the industry cannot continue to “spray and pray” our way out of psyllid finds. A genetic tree is admirable, but not all growers will be able to obtain or market that solution (or have the time to wait on a genetic solution). In the end, citrus is a good business to be in (as long as you have good soil and good water and your area does not get too cold). Location. Location. Location. Just how the industry will look in the future may change, as will the circumstances under which citrus is grown.

**Citrograph:** Any final thoughts?

**Earl:** In sum, it has been very rewarding to be part of the CRB process. The people are great. Our differences have caused us to come up with better solutions. 😊
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The Citrus Disease Subcommittee (CDS) of the National Agricultural Research, Extension, Education and Economics Advisory Board (NAREEE) recently has been reactivated as a Federal Advisory Committee. Their charge is to advise NAREEE and the Specialty Crops Research Initiative (SCRI, a grant program) on priorities that should be funded via the new Farm Bill allocation of $25 million per year for the next five years. As the name of the subcommittee suggests, any disease of citrus could be a focus of the dollars. However, resulting from its inaugural meeting, the subcommittee recommended, and the secretary agreed, to target the first plug of nearly $25 million exclusively for huanglongbing/Asian citrus psyllid (HLB/ACP) research. To my knowledge, it is the first time that the Farm Bill has directed the SCRI to earmark a very large portion of the allocated funds for the diseases of one specialty crop. Equally impressive is that the all states growing that crop represented on a Federal Advisory Committee have recommended aiming all the available funds at solving a specific problem.

To explain the general structure, the CDS comprises nine grower members from three citrus-producing states: five from Florida, three from California and one from Texas. No major decisions can be approved without a super-majority vote of six. The three California members are Justin Brown, Don Roark and myself – all current CRB board members.

The CDS recently convened in Washington, DC, to set priorities. As could be expected, the vision and approach to the HLB/ACP disease complex of the different delegations are vastly disparate. Understandably, the Florida members are very short-term focused, since they are representative of a grower base looking for immediate fixes. Florida growers need solutions tomorrow – not five years from now. Conversely, the California members have a longer-term outlook. Texas falls somewhere in the middle of this spectrum.

Hence, the voting on HLB/ACP priorities reflected the different visions. For instance, the fruit drop problem in Florida is very severe, and all of that state’s members voted this as one of the top priorities. California and Texas viewed this as a number three priority – simply because it is not an immediate threat in those states. Conversely, since Florida has varying levels of infection in essentially 100 percent of their orchards, early detection of the disease prior to visual symptoms and PCR-positive results is not important.
Needless to say, the voting resulted in very parochial priorities. The SCRI personnel undertook to outline the regional priorities in the Request for Proposals to ensure that each area is adequately represented in the eventual funding process. My personal opinion, which is not necessarily shared by all in California, is that whatever is good for Florida to solve their immediate problem will most definitely be advantageous to California in the medium- to long-term. I am less concerned about a running account snapshot that shows California risks not getting its “fair share” of monies/projects. Instead I am more inclined to look at the bigger, more long-horizon picture. If the national scientific community utilizes a good portion of the near-term SCRI funds to solve the problem in Florida, it is likely that the problem in California and Texas also will be solved. Note that I am not implying that California priorities this year or five years from now should go unfunded – only that the results from Florida-based projects (where the disease is already so widespread) also would serve California well.

The sub-committee concluded that the following research be funded:

• research aimed at controlling the vector (entomological control, attract and kill options);

• early detection technology (California-centric);

• anti-microbials to affect the vector and the bacterium; and

• genetic tolerance/resistance options that would keep plants living longer (rootstock tolerance or actual genetically-modified solutions where we have transformed plants by inserting genes imparting resistance to HLB).

The GMO option elicits a lot of emotion and debate, but we need to spend money in this field of research in the event our other research avenues come to naught.

An enormous amount of money has been spent in the last five years on HLB/ACP efforts in all the affected states. In California, most of it went toward regulatory aspects, trying to keep the movement of ACP at bay. In Florida, the funding was allocated to reduce the impact of HLB. The bulk of HLB-related money to be spent in the next five years from the Farm Bill funding will be used to seek research solutions.

We can only hope that there is a short-term option in play for Florida within this time frame and real progress toward long-term solutions for the benefit of California.

The California NAREEE CDS members are well aware of the need to ensure that solutions are achieved in the very near future. We take the charge seriously. For good measure, let’s all cross our fingers that a solution is found, or as we might say in my native South Africa, “Let’s hold our collective thumbs for answers soon!”

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

In California, we want to have an early handle on whether trees are infected, and not only once we visually see the symptoms. This will allow early removal of inoculum and limit the spread of the disease. [Note: as you may be aware, HLB-infected trees can be infectious from the time of inoculation of the disease by an infected psyllid, without showing any visual symptoms or being detectable by the conventional laboratory (PCR) test. Such undetected non-visual infection can be dormant for up to three years without being evident to the human eye, all the while serving as a reservoir for transmission]. This line of research is thus clearly of utmost importance in California, where the disease is not known to have taken hold, versus Florida, where it is simply out of control.
TEXAS GROWER DECISIONS ON TREE REMOVAL TODAY COULD PROVE CRITICAL

The following is a condensation of an opinion column that appeared recently in the Texas Citrus Mutual E-Weekly. Ray Prewett emphasized that some of the statements are his opinions, which are not official board positions and may or may not be supported by definitive science.
Today, members of the Texas citrus industry are making decisions that they may not have the opportunity to make later. At some point, those choices will not matter as much as they do right now. One of the most important decisions is what to do about removing infected trees. Given the current low incidence of huanglongbing (HLB)-positive trees detected in groves and residential areas, property owners should continue to remove infected trees. This is a best management practice – not a dictate mandated by the state or federal government. If infected trees are left in a grove or a backyard, we may not have the opportunity to make a different decision down the road. At the very least, the impact of removing these trees for an over-all inoculum reduction strategy is greater right now than it will be when there are a lot more infected trees.

One of the facts we must face is the increase in the number of sites for HLB-positive citrus trees and Asian citrus psyllid (ACP). As of this writing, we have 14 groves and 76 residential sites affected. This number of finds is concerning. With latency, we know there are more infected trees still unidentified.

Does the above constitute a widespread, high infection rate? Adjacent to this column is a map showing the general locations (not all the individual sites) where the disease has been confirmed. While there is reason to be very concerned about the variety of locations, the number of known infected trees is still a very small percentage of the total number of groves and backyards in the Rio Grande Valley. The highest number of infected trees in recently detected positive areas did not exceed six trees in any grove. In addition, the positive trees have been found in clusters and not scattered.

The Texas Citrus Pest and Disease Management Corporation (TCPDMC) is actively pursuing a grant to demonstrate that if growers remove infected trees AND do all the other right things, including early detection and effective ACP control, they can survive in the face of citrus greening. Unfortunately, even if this grant request is approved in the next
What can we do in the meantime? How important is tree removal? Can the Texas citrus industry be successful in slowing the spread of citrus greening without removing infected trees? We recently asked a Florida citrus scientist that question, and the essence of his answer was, “I do not know of any place in the world where an industry 'successfully' dealt with a vector-transmitted disease (for any crop) without an effective program to reduce the amount of the inoculum.” We should take this statement very seriously, because it is not possible to kill all ACP. If the inoculum stays in the grove or residential site, ACP will spread it.

Texas citrus growers (and the industry as a whole) are facing many critical decisions in the next few months. Ultimately, individual growers must decide what is best for them, but Texas Citrus Mutual and the TCPDMC are here to help. Growers are not in this fight by themselves. The following are a couple of the more important questions growers are facing, along with assistance we are offering now or in the near future:

- **What if the source of ACP is from a neighboring abandoned grove or from someone’s backyard citrus trees?**

  We have just received a sizable grant from USDA to ramp up production of *Tamarixia* parasitoids at Moorefield and at a new location in Weslaco. We are looking forward to being able to use this tool, particularly in residential areas adjacent to groves.

- **What if I want to do a survey of my grove to determine whether or not it has diseased trees?**

  It is obvious that individual growers have the primary responsibility for detection of the disease. The industry is in the process of offering an intensive training course for those who scout groves. With several recent new finds, it is clear that we need to look more often and in more places for this disease. Early detection and inoculum reduction, as well as psyllid management, are all key to winning the battle with HLB.

Texas can learn from Florida’s experience and avoid some of the same mistakes. We have talked to many growers and scientists in Florida who said they should have acted more quickly and taken HLB more seriously on several fronts, including psyllid control and tree removal.

What is the bottom line for the Lone Star State? Recently, we have found several new locations with citrus greening; but compared to the situation in Florida, the rate of infection in Texas is still quite low. We still have a window of opportunity to slow the spread of the disease. The question is whether we are ready and willing to take advantage of that opportunity. The clock is ticking, and we do not have much time to make decisions to effectively combat greening. However, I am an optimist, and I believe we can make a difference IF we act quickly and aggressively.

*Ray Prewett is the president of Texas Citrus Mutual.*
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The Citrus Research Board (CRB) and University of California Cooperative Extension (UCCE) recently held grower seminars throughout California for industry members. The three seminars were hosted in Santa Paula, Riverside and Exeter on June 27, July 15 and July 17, respectively.

Grower turnout was positive, as the seminars not only offered an opportunity to learn and earn continuing education units, but also to connect with fellow citrus growers and industry members. Citrograph magazines were on hand with free subscription sign-up for California citrus growers.
The half-day seminars offered a breadth of topics varied to best suit each region. UCCE and University of California, Riverside (UCR) provided many of the speakers. Scientific topics included export challenges due to plant disease and pest problems (Jim Adaskaveg of UCR, Joe Morse of UCR, Beth Grafton-Cardwell of UCCE); strategies for dealing with water shortages (Ben Faber of UCCE, Mary Lu Arpaia of UCCE); and an introduction to genetic engineering and possible application in citrus (Peggy Lemaux of UCCE).

Specific to Riverside, biological control of ACP was added to update southern California growers on efforts to rear and release *Tamarixia radiata*, a wasp that is a specific biological control agent of ACP (Mark Hoddle of UCR). Compelling non-research topics also were featured at all three locations, including labor issues facing the California citrus industry (Laura Brown of California Citrus Mutual) and an update on CRB’s strategic direction (Ken Keck of the CRB). Another
Riverside-only topic highlighted the importance of coordinated treatments of ACP in commercial groves in relation to residential ACP detections (Tina Galindo of CDFA, Alan Washburn of CPDPC).

The seminars also introduced and promoted the recently published Citrus Production Manual. The 434-page manual was developed by the University of California–Agriculture and Natural Resources to provide a comprehensive reference on citrus production. For ordering and more information, visit www.anrcatalog.ucanr.edu or call 800-994-8849. You can also find more information on pages 46 and 47.

The partnership of CRB and UCCE has promoted many citrus education seminars over the years. The organizations strive to provide relevant and timely information that benefits growers by prioritizing, investing in and promoting sound science.

When’s the next seminar? Sign up for CRB’s e-mail notifications at www.CitrusResearch.org/signup. Notifications include seminar announcements, Citrograph and official CRB correspondence. The CRB will not share your email address, and you may unsubscribe at any time.

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The seminar in Santa Paula began with UCCE Farm Advisor Ben Faber presenting to the group.

Riverside seminar at the California Citrus State Historic Park.

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Canine detection of citrus canker may show HLB application promise

Proven success with detection of citrus canker in Florida may provide new approaches for HLB management, but much still needs to be learned...

Lori Berger
Detection dogs have been best known for assistance in criminal investigations, primarily sniffing out illegal drugs and explosives. Pest control companies have used dogs for locating bedbugs and termites. Agricultural regulatory agencies’ use of dogs to sniff out insect pests has proven successful at airports and other ports of entry, preventing potentially devastating pests from gaining a foothold in domestic food production. Dogs also have been used to detect medical conditions such as cancer and blood sugar levels for diabetes. The use of combat dogs in military efforts in Iraq and Afghanistan was featured as the cover story of the June 2014 issue of National Geographic.

While making use of a canine’s extraordinary sense of smell is not a new concept, researchers are finding additional valuable applications that may benefit the citrus industry.

**TRAINING DOGS FOR CANKER DETECTION**

In Florida, highly trained detection dogs are being prepared to move into a new sector – finding disease-infected trees before symptoms are visible to the human eye. Research into citrus canker detection has been underway since 1999, with lead USDA-ARS scientist Tim Gottwald, Ph.D., noting that the concept has been proven for detection of citrus canker with greater than 99 percent accuracy. Gottwald, research leader and plant pathologist/epidemiologist at the U.S. Horticultural Research Laboratory in Fort Pierce, Florida, said dogs have been found to be accurate at detecting citrus canker in randomized new plantings of trees with varying levels of infection. Five dogs have been undergoing training and testing at the research site. Working with professional dog trainers, these dogs have sniffed out trees in the earliest stages of canker infection. In June 2014, Gottwald’s lab embarked upon initial steps to begin imprinting two dogs for huanglongbing (HLB) detection. During early training, the two dogs have already demonstrated the ability to differentiate HLB-infected trees from healthy trees.

**A WET AND BUSY INFORMATION TECH SYSTEM**

Dogs possess up to 300 million olfactory receptors in their noses – far more than the six million found in a human nose. When dogs inhale, airflow splits in two directions with about 12 percent going to a recessed area in the back of the nose that is dedicated to olfaction. Within the recessed area, the air and odors are filtered through structures called turbinates that sieve odor molecules based on different chemical properties. Olfactory receptors within the tissue that lines the turbinates “recognize” the molecules and dispatch signals to the brain for analysis (Figure 1).

![Figure 1. A look inside a complex system. Image from the Journal of the Royal Society Interface used with permission from Brent Craven, Ph.D., Department of Mechanical and Nuclear Engineering, Pennsylvania State University.](image-url)
While humans force out odors when they exhale, dogs can usher in new odors allowing them to sniff almost continuously. Dogs can also use each nostril independently, which helps them determine which nostril an odor entered and also determine a directional source of the odor.

The immense surface area of canine nasal cavities is made up of multiple layers of cells called epithelial cells (e.g. the skin of the entire body is composed of epithelial cells), that are well supplied with receptor cells. The act of “smelling” is when volatiles bind to receptors and information is passed on to the brain. Some scientists estimate up to one-third of the canine brain is wired for olfactory sensing. The continual “sniffing” our own pet dogs do is simply data collection and cataloguing this vast library of volatile chemical information continually wafting through their environment.

**IMPRINTING CANINES**

While dogs possess the ability to detect scent, imprinting the odor and training them to search for specific molecules can be done in various ways.

Imprinting involves using a reward system. Trainers use a variety of methods to present the dogs with target odors (volatiles) directly or in containers of some kind. When the target odor is correctly identified by the dog, the dog is rewarded by a few minutes of play with a favorite toy such as a rolled up towel or a rubber ball with a handle known as a “kong.” Trainers prefer dogs with strong predatory behaviors, who have undergone obedience training. A verbal search cue should be chosen that would be used exclusively for odor detection. Trainers begin with scented objects in full view and then move to hiding the objects and adding blank, or non-target objects, into the mix. The main point, trainers say, is to keep changing the environment so that the dog becomes highly conditioned in hunt drive and does not become bored with the task. Imprinting the initial odor is typically done in just a few days. At that time, the dog should be ready to locate the target odor in a new environment 90 percent of the time with no handler involvement and show a change in behavior at the source.

**ADDRESSING THE OBSTACLES**

Canine ability to focus on a single scent, while literally dozens of different scents are present, is notable. Dogs have been used to detect mussels on boats at public watercraft ramps and to find bumblebee nests. When it comes to citrus canker detection, Florida growers have expressed significant interest, but believe obstacles to the program’s success remain.

Florida citrus growers applaud the canine canker detection research, but look forward to the concept being expanded for practical, large-scale use in commercial operations. To cover that state’s citrus acres, growers will need many trained dogs to be available for area-wide detection efforts. Growers say they would be open to pooling resources to pay for canker detection dogs if they become available in large enough numbers.

In enclosed areas such as citrus packing houses, canker detection dogs have been particularly adept at sniffing out individual pieces of fruit that have canker. However, their accuracy diminishes if the dogs become overheated. A dog’s nose generally works best (or is most sensitive) in cool, calm weather. Odors become more volatile at higher temperatures; and wind can dilute and disperse odors over a large area, camouflaging their source.

While canine canker detection ability has been proven and implemented, USDA researchers are at the beginning stages of considering what data will be needed to establish a similar approach for the detection of HLB. A challenge specific to HLB detection is the confidence in knowing that the plants being used to train and test the dogs are truly positive. The time from infection (by psyllid or graft-inoculation) to disease detection can range from several months to

Trained Belgian Malinois dogs have resulted in a greater than 99 percent success rate with citrus canker detection. Here, Bady (Coast to Coast K9) searches on the run and then alerts on a canker-infected citrus tree in a randomized field test.
several years. Therefore, it must first be determined through time-sequence experiments exactly at what point in the infection process a canine can detect the disease.

MEASURING THE DOGS’ ABILITY TO “GET IT RIGHT”

Summarized research results from multiple USDA-ARS trials by Gottwald’s team in Florida are provided in the Table 1 and show the promising results of using canine detection in citrus. Sensitivity is the ability of the dogs to correctly identify canker-infected trees/fruit. Specificity is the ability of the dogs to correctly identify uninfected trees/fruit. Precision is the ability of the dogs to correctly identify infected trees/fruit considering the entire test population of infected and uninfected trees/fruit. Finally, accuracy is the ability of the dogs to correctly identify infected and uninfected trees/fruit considering their entire test population. Thus, accuracy is the measure of overall performance of the dogs to “get it right.”

The simulated new plantings consisted of 100 two- to three-foot tall trees, with one to five percent of the trees infected with citrus canker. Packing house trials consisted of 100 boxes of grapefruit with ~40 pieces of fruit in each box and only one or two fruit with very few lesions placed in a few target boxes.

Plantation trials were the most challenging because they required human technicians to scrutinize thousands of leaves on each tree to verify the presence of citrus canker. Often, the dogs alerted on a tree that human inspectors did not previously find infected. This led to a laborious evaluation of those trees to determine if they were indeed infected, but previously had been missed by the human team. This frequently was the case. Thus, the dogs could do in seconds what required human inspectors many minutes per tree to accomplish, and the canines did it with much improved accuracy.

<table>
<thead>
<tr>
<th>Combined Results over Multiple Tests</th>
<th>General Description of Testing</th>
<th>Sensitivity: True Positive Rate (TPR)</th>
<th>Specificity: True Negative Rate (TNR)</th>
<th>Precision: Positive Predictive Value (PPV)</th>
<th>Accuracy: (ACC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simulated New Planting</td>
<td>100 2-3 ft. tall trees; 1-5% infection</td>
<td>0.8771</td>
<td>0.9960</td>
<td>0.9177</td>
<td>0.9902</td>
</tr>
<tr>
<td>Existing Plantation</td>
<td>Citrus grove trials; trees 4-10 years of age; 5-10% infection</td>
<td>0.9333</td>
<td>0.9878</td>
<td>0.8750</td>
<td>0.9832</td>
</tr>
<tr>
<td>Packing House</td>
<td>Boxed grapefruit with 1 infected fruit per box</td>
<td>0.7882</td>
<td>0.9948</td>
<td>0.9054</td>
<td>0.9825</td>
</tr>
</tbody>
</table>
CRB, PARTNERS TO REQUEST MAC FUNDING FOR CANINE HLB DETECTION

As described in several Citrograph reports (Winter and Spring 2014), the United States Department of Agriculture earlier this year announced the formation of the HLB Multi-Agency Coordination (MAC) Group. Congress made available $21 million for MAC-approved projects that have the promise of making a difference for growers in combatting HLB within a two-year window.

A project proposing the use of canine sensory training of 20 dogs for HLB detection is currently in development. This proposal will be considered for funding through the MAC funding process. Gottwald’s collaborators in this plan are MaryLou Polek, vice president of science and technology with the Citrus Research Board; Eliezer Louzada, plant pathologist at the Texas A&M Citrus Research Center at Kingsville; and Liz Baldwin and Jinhe Bai, product chemists at the USDA, ARS, Fort Pierce, Florida.

Validation trials would be conducted to determine the sensitivity of canine detection relative to current detection methods. In addition, validation would be conducted to demonstrate canine detection sensitivity of HLB at various stages of disease development. These stages include shortly after initial infection, through the cryptic period when there are no visual symptoms and plant tissue is PCR-negative, to when PCR-positive but still asymptomatic, and finally stages of symptomatic infection.

Canine detection of HLB, as in canker detection, is dependent on the recognition of the presence and/or absence of complexes of unique volatiles from infected plant material. Dogs can detect these volatile signatures in a matter of seconds and alert handlers to the presence of HLB. Currently, the CRB is supporting research at the University of California at Davis to characterize volatile organic compound signatures of HLB for early diagnostic markers for field detection (Citrograph: March/April 2012, pp. 54-56, and Winter 2014, pp. 28-34).

Pre-symptomatic detection methods would be especially useful in low incidence areas such as California, Texas and Arizona. In Florida, early detection would benefit those growers who are pushing out highly infected groves and re-planting with tested, clean nursery stock. Early detection and rapid response (e.g. removal of infected trees) are imperative for maintaining areas free of HLB. There is growing consensus among growers and citrus industry leaders that a detection tool such as the use of dogs could contribute to the long-term productivity of the national citrus industry.

Acknowledgement
Sincere appreciation is expressed to Tim Gottwald, Ph.D., USDA-ARS, Fort Pierce, Florida, for providing much of the background information, and to Coast-to-Coast K-9 and J and K Canine Academy for collaboration in the above-mentioned studies and for providing information and photographs for this article.

“...the dogs could do in seconds what required human inspectors many minutes per tree to accomplish, and the canines did it with much improved accuracy.” – Tim Gottwald, USDA-ARS scientist

USE OF DETECTOR DOGS

Agricultural products
Arson investigations
Cadaver location
Chemical weapon location
Concealed person location
Criminal tracking
Crime scene investigations
Currency location
Drugs and paraphernalia
Endangered species poaching detection
Explosives detection
Gas leak detection
Guns and ammunition
Medical diagnostics (melanoma, diabetes)
Land mine location
Rotten power pole location
Pest detection
Search and rescue missions
Wildlife location

To observe a working dog in a Florida citrus grove, go to www.citrusresearch.org/canine-training
Sources


Tim Gottwald, Research Leader/ Plant Pathology: Horticultural Research Lab  www.ars.usda.gov/pandp/people/people.htm?personid=2071

CDFA Detector Dogs  www.cdfa.ca.gov/plant/dogteams/index.html

Lori Berger, Ph.D., is an entomologist and agri-business consultant in central California. www.agbusinessresources.com
The 2nd International Hemipteran-Plant Interactions Symposium was held at the University of California, Riverside campus June 22-25, 2014. This conference brought together 150 international scientists from the disciplines of plant biology, plant pathology and entomology to discuss advances made in understanding the interactions between:

1. plant hosts and their pathogens,
2. plant pathogens and their insect vectors,
3. plant hosts and insect pests, and
4. endosymbiotic bacteria within insect vectors.

Hemipteran insects include many of the world’s most economically damaging pests – aphids, psyllids, whiteflies, leafhoppers and thrips. Some of these pests are the vectors of devastating citrus diseases such as tristeza, huanglongbing and stubborn.

IN THE BEGINNING

A Polish entomologist, Beata Gabrys, reported on the ancient origin of aphids. Despite their modern day hosts of angiosperms (flowering plants), fossil records of the Triassic period indicate that aphids actually originated feeding on gymnosperms (conifers) or cone-bearing plants such as pine needles.
trees. Since aphids are phloem feeders and the anatomy of phloem tissue (sieve elements) is very different between angiosperms and gymnosperms, this begs the question of what drove the evolution of aphids to such a drastic change in host plant preference in the first place? This question remains unanswered.

DECEPTIVE VIRUSES

Mark Mescher from Switzerland described experimental results showing how certain plant viruses induce deceptive signaling to deter the insect vector from feeding on incompatible plant hosts. This causes the insect vector to move on to a different host plant, thereby increasing the odds for successful transmission. He also showed a video of a worm parasite of crickets. The worm actually prefers an aquatic environment for a portion of its life cycle. When the worm is ready to emerge from the cricket body, it elicits a chemical signal that makes the cricket determined to find water. Upon finding a water source, the cricket jumps in, the parasitic worm exits and the cricket drowns.

Newly-hired UC Davis faculty member Clare Casteel also claimed that a virus can promote its own transmission by manipulating the physiology of its host plant to attract its aphid vector. Further, she showed that the aphid’s fecundity (reproductive rate) increased when it was carrying Cucumber mosaic virus (CMV). Another virus, Turnip mosaic virus (TuMV), suppresses callose deposition, a major plant defense. This lack of callose formation increases free amino acids, which are the major source of nitrogen for the aphid.

INSECTS SPARRING WITH THEIR ASSOCIATED PLANT HOST

Plants intrinsically have structural (cell walls, waxy cuticles) and chemical barriers for protection against insect and plant invasion. Plant cells maintain their rigidity due to turgor pressure (for example, celery kept in a refrigerator for an extended period of time becomes limp when the cells lose their turgor pressure). Oxygen levels also play a key role as a chemical barrier; the level of oxygen at the leaf surface is only eight percent, compared to 42 percent in phloem tissue. Insects have had to adapt by developing mouthparts to penetrate plant cell walls and internal pumps to access nutrients from the sugar-rich phloem sap under high pressure and high oxygen concentrations. One way insects have overcome this, as described by Xiangfeng Jing (Cornell University), is by the formation of aquaporins or water channels. These water channels prevent the insect from becoming dehydrated as it uses the water to dilute the sap sugars, thus decreasing
the osmotic pressure. This allows for the sugars to leave the phloem and travel through the insect’s mouth parts (stylet), like sucking through a straw.

Saskia Hogenhout from the United Kingdom described “Zombie Viruses.” (See http://onwardstate.com/2012/12/17/penn-state-scientist-discovers-zombie-virus/) Certain viruses and phytoplasmas take control over their host plant and turn them into ‘zombies’ by producing effector molecules. These molecules alter the plant’s ability to develop normally. We know one result as phylloidy or witch’s broom, the proliferation of leaf tissue (Photo1). This dramatic increase in leaf production favors insect colonization and egg-laying, whereby, the insect benefits.

INSIDE THE BIG FISH IS A LITTLE FISH

An endosymbiont is any organism that lives inside another organism. In some cases, the host organism cannot survive without its symbiont, in which case, we call it an obligate symbiont. A facultative symbiont is not essential for survival of the host; however, there is evidence that over evolutionary
and chloroplasts. Nancy Moran from the University of Texas showed that there are indications that hosts have acquired bacterial genes from their symbionts. Little is known about endosymbionts, as they cannot survive outside of their hosts, making them very difficult to study.

Phloem-feeding hosts benefit greatly from the presence of symbionts. Whereas the phloem sap is high in sugars and carbohydrates, it lacks many amino acids. These internal life forms generously provide their insect host with essential amino acids (amino acids that the host cannot manufacture), making their relationship one of nutritional dependency. They also can affect the heat tolerance of their hosts. In laboratory assays, Martha Hunter from the University of Arizona discovered that sweet potato whiteflies containing Rickettsia produced more progeny, had a greater number of offspring that survived into adulthood, produced more female offspring and developed more rapidly than non-infected whiteflies. Furthermore, her research team determined that whiteflies manipulated the host signal hormones, salicylic acid (SA) and jasmonic acid (JA), both of which are involved in plant defense. The whiteflies induced an increase in the production of SA and a decrease in the production of JA.

The Asian citrus psyllid (ACP) is known to contain at least two such symbionts – Wolbachia and Carsonella. Wolbachia is being studied to determine its role in the ACP’s ability to transmit the pathogen causing huanglongbing (HLB). If Wolbachia can be manipulated, perhaps transmission can be prevented, along with the subsequent spread of the disease. Sulcia is associated with glassy-winged sharpshooters (GWSS). Both ACP and GWSS have a range of color variants. Research is underway to determine if their endosymbionts are responsible for this variation in color.

**INSECT VS. VIRUS**

Stephane Blanc from France reported on his results that do not fit well-accepted views. Certain families of plant viruses do not express their genes or replicate within their insect vector; they simply traverse cellular barriers (stylet, salivary glands, gut). Blanc’s results on the interaction between Faba bean necrotic stunt virus (FBNSV) and its aphid vector indicate otherwise. FBNSV is a multipartite virus; its genome is composed of eight single-gene encoding segments, each encapsidated individually. This research team intended to answer the question of whether the aphid acquires all eight segments during feeding and does it egest all eight segments during transmission. Their results showed that the frequency of viral gene segments within the source host plant was significantly different from that of the insect within 12 hours after acquisition of the virus during feeding. This suggests an interaction between FBNSV and its aphid vector that modifies the genetic composition of the viral population.
Several scientists provided evidence that viruses can enhance their own transmission. Maria Sánchez-Guzmán from Spain reported that when whiteflies feed on tomato plants, signaling via JA is induced within the plant, deterring whiteflies from laying their eggs on that plant. The whitefly then moves to a different plant. However, the induction of JA appears to be the target of Tomato Yellow Leaf Curl Virus (TYLCV). The virus benefits because the whitefly will feed on many plants, not just one; thereby, the virus is transmitted to several plants by one insect.

Cauliflower mosaic virus (CaMV) and Turnip mosaic virus (TuMV) were studied by Martin Drucker’s team in France. Both of these viruses are vectored by aphids. As soon as an aphid lands on an infected host plant, CaMV virions alter their form, allowing for enhanced acquisition of the virus by the aphid. Frequently, when plants are attacked by an insect or pathogen, they respond by producing a sudden burst of hydrogen peroxide as an initial line of defense. This substance is short-lived, as high concentrations can be detrimental to both the attacker and the host. In lab studies, when host cell protoplasts were incubated in the presence of hydrogen peroxide, the transmission of TuMV was drastically increased. This indicates that the virus uses the hydrogen peroxide signal to somehow prepare itself for more insect acquisition and subsequent transmission to a new host.

Scientists worth their weight always will provide you with as many new questions as their discoveries. This symposium held true to this statement.

- Aphids puncture many plant cells before finding the “right” phloem cell for sustained feeding. In contrast, whiteflies seem to target a phloem cell on their first try. What is the evolutionary meaning? What are the benefits to the insect, to the pathogen?
- How do endosymbiont genomes regulate gene expression in their hosts? How do insects manage their endosymbionts?
- How can insects both contribute to and exploit resources of the pathogen they transmit?
- Is a healthy ecosystem one that is rich in parasites?
- What is the role of small molecules (double-stranded RNA, small interfering RNA, micro-RNA) in plant-host interactions?
- There are many changes in the emission of volatile organic compounds (VOCs) by a plant when fed upon by an insect predator and when infected by a pathogen. What does VOC chemistry mean? How can it increase our understanding of host-pathogen-vector interactions?

Attendees were left to ponder the following thought – the next time you sneeze, just think to yourself, “Am I being controlled by an airborne virus?”

Photo 4. Three generations of scientific researchers: Conference organizer and CRB-funded researcher Michelle Cilia (left) enjoys the lunch break with (clockwise from top) Alana Jacobson, Stewart Gray and George Kennedy. Kennedy was Gray’s and Jacobson’s major professor, and Cilia worked for Gray as a post-doc.
## GLOSSARY

**Aquaporins:** Channels in the membrane of cells that allow for the passage of water and other small molecules; playing a critical role in controlling the water contents of cells. This is a passive process that follows the direction of osmotic pressure across the membrane.

**Bacteriocyte:** A bacteriocyte (Greek for bacteria cell) is a specialized structure that houses endosymbionts within an organism. It protects and allows for the passage of endosymbionts in insect eggs, i.e. the mother transmits her endosymbionts to her offspring.

**Callose:** A plant polysaccharide, composed of glucose residues linked together. It is thought to be manufactured at the cell wall and produced in response to wounding or infection by pathogens.

**Effector molecule:** A small molecule that selectively binds to a protein and regulates its biological activity; i.e. can increase or decrease enzyme activity, gene expression or cell signaling. Effector molecules can also directly regulate the activity of some mRNA molecules.

**Endosymbiont:** Any organism that lives within the body or cells of another organism. These can be obligate, meaning the host cannot live without the symbiont, or facultative, meaning the host benefits, but can survive without it.

**Fecundity:** Equivalent to fertility, or the actual reproductive rate of an organism or population, measured by the number of gametes (eggs), seed set or asexual propagules.

**Phylloidy:** the abnormal development of floral parts into leafy structures. Phyllody causes the affected plant to become partially or entirely sterile, as it is unable to produce flowers normally.

**Phytoplasmas:** Specialized bacteria that are obligate parasites of plant phloem tissue and transmitting insects (vectors). They cannot be cultured and are characterized by their lack of a cell wall, multi and changing shapes and their very small genomes.

**Stylet:** A long, slender, hollow feeding structure of nematodes and some insects; straw-like.

**Turgor pressure:** Pressure caused by the osmotic flow of water from area of low solute concentration outside of the cell into the cell's vacuole, which has a higher solute concentration.

**Vascular tissue:** In plants, xylem tissue transports mainly water from the roots upward to the plant canopy; whereas the contents of phloem cells move in both directions transporting nutrients from the roots upward to the canopy and downward by transporting photosynthetic products (carbohydrates) produced by the leaves to the plant roots.

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### CRB JOB ANNOUNCEMENT

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DEVELOPMENT OF AN
ACP MANAGEMENT PLAN
FOR ORGANIC CITRUS

Jawwad A. Qureshi and Philip A. Stansly

BACKGROUND
The Asian citrus psyllid (ACP – Photo 1) vectors pathogens that cause huanglongbing (HLB) or citrus greening disease, which has spread throughout Florida and has been reported in limited areas of California, Texas and Louisiana. Management of this vector-disease complex in all habitats is critical to prolonging the life of the citrus trees and sustaining production. Vector control is essential to reduce disease spread among habitats, including organic citrus. However, there is little information on the efficacy of permitted products that could be used to develop effective plans to manage ACP in organic groves.

Management programs to suppress ACP need to consider the biology and ecology of this and other pests. Citrus trees go through periods of dormancy during cold or
dry weather, producing little or no new growth, which is required for ACP to develop and reproduce. Adult ACP living on these trees need to wait for new growth to mature and lay eggs. Insecticidal sprays made during winter before bud break in Florida are commonly known as dormant sprays. The aim is to reduce psyllid entry into spring flush and thus subsequent reproduction during the growing season. Generally, broad-spectrum pyrethroids and organophosphates are preferred in winter followed by selective chemistries, microbials and horticultural spray oil during the growing season.

It is important to integrate multiple control approaches. Sole reliance on insecticides may not be adequate to sufficiently reduce vector populations. Insecticide use may also generate problems with other pests and beneficials normally present in citrus orchards. Extensive use, particularly of insecticides with similar modes of action, will select rapidly for resistant populations of all pests. Pest populations that typically remain in check may also flare due to the suppression of natural enemies.

Biological control has always been an important component of citrus pest management. Generalist predators already present in the citrus groves contribute to pest reduction on a consistent basis. These include several species of lady beetles and green or brown lacewings that are common and contribute to mortality of ACP mainly by feeding on eggs and nymphs. Spiders are also abundant and attack immature and adult ACP, which also get trapped in spider webs. Parasitoids that specialize on the target pest are also needed. Tamarixia radiata is such a parasitoid of ACP and is being mass produced and released in Florida, California and Texas. Biological control, either by conservation or augmentation, can help reduce vector populations and ultimately the spread of HLB without populations of other pests flaring.

Our research is focused on evaluating different management programs for organic and conventional citrus, including biological control, through conservation of natural enemies or augmentation of Tamarixia radiata.

**RESEARCH OBJECTIVES**

1: Determine the effectiveness of the organic insecticide Pyganic® (natural pyrethrum) to suppress ACP during dormant winter months in comparison with Danitol® (synthetic pyrethroid used extensively for ACP control) as a conventional grower standard.

2: Evaluate rotations of organic products that are potentially effective against ACP for their impact on ACP and natural enemies during the growing season. Three organic programs and one conventional program are being compared to an untreated control.

3: Release and evaluate Tamarixia radiata to determine the feasibility of parasitoid use in conjunction with insecticides.

We are in the first year of pursuing these objectives. Findings from this applied research will be useful to develop management plans for ACP and HLB for organic citrus that will provide the level of control necessary on an area-wide basis. Conventional growers will benefit from better control in nearby organic citrus and from development of more selective control options for their own groves. Products suitable for organic citrus may also be useful for residential areas where synthetic chemicals are not always welcome.

**STUDY DESIGN, INSECTICIDAL SPRAYS AND SAMPLING FOR ACP AND NATURAL ENEMIES**

A 22-acre block of mature Valencia oranges in Hendry County, Florida, which had not been sprayed for ACP, was divided into 20 plots each with three to five rows and 50 trees. These were distributed among three organic programs, one conventional program and one untreated control in a randomized complete block design experiment with four replicates. Four treatment regimes included:

1: three conventional insecticides (Danitol, Closer and Mustang Max),

2: five organic insecticides (Pyganic 3 times, Entrust and Grandevo),

3: the organic insecticides in combination with two percent horticultural spray oil (435 oil), and

4: the organic insecticides in combination with two percent soybean oil (Citru-Soy™).

These were compared to an untreated check. Pyrethroids are typically used as (winter) dormant sprays because of their broad spectrum activity and sensitivity to heat. Pyganic contains natural pyrethrins, which are also broad spectrum and break down quickly in sunlight and, therefore, are more suited for use in winter. Horticultural spray oil is petroleum based and is approved for organic citrus. Soybean oil was included as a possible alternative to petroleum oil.
Psyllid populations during Fall 2013 were low in the Valencia block, so we added another replicated experiment in a block of younger Hamlin orange trees with higher psyllid populations to test the organic + 435 oil and conventional programs against an untreated check. Recommended rates of insecticides applied in 100 gallon per acre (gpa) water were sprayed by ground in both blocks using a Durand Wayland AF100-32 air blast speed sprayer (Photo 2); and the application on April 10 was made using a low volume Proptec sprayer (Photo 3) at 10 gpa.

The organic program during the dormant season compared three applications of Pyganic sprayed on November 2013, December 2013 and January 2014 to one application of Danitol made in January 2014. Pyganic was applied alone or in combination with the same oils used in the Valencia block or with the 435 oil as in the Hamlin block. Details of dormant and growing season treatments are provided in Table 1. In addition, a total of 40,339 and
Table 1. Product application rates used were 100 gallons per acre, except on April 10 when a low volume application at 10 gallons per acre was used.

<table>
<thead>
<tr>
<th>Treatment date</th>
<th>Organic insecticide*</th>
<th>Organic insecticide with 435 oil*</th>
<th>Organic insecticide with Citrusoy*</th>
<th>Synthetic insecticide*</th>
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<td>Nov 21, 2013</td>
<td>Pyrethrin Pyganic 5.0EC 48 oz MGK Corp.</td>
<td>Pyrethrin + Mineral oil Pyganic 5.0EC + 435 Oil 48 oz + 2 gallon MGK Corp., Drexel</td>
<td>Pyrethrin + Soybean oil Pyganic 5.0EC + Citrusoy 48 oz + 2 gallon MGK Corp., Drexel</td>
<td>None</td>
</tr>
<tr>
<td>Dec 18, 2013</td>
<td>Pyrethrin Pyganic 5.0EC 32 oz MGK Corp.</td>
<td>Pyrethrin + Mineral oil Pyganic 5.0EC + 435 Oil 32 oz + 2 gallon MGK Corp., Drexel</td>
<td>Pyrethrin + Soybean oil Pyganic 5.0EC + Citrusoy 32 oz + 2 gallon MGK Corp., Drexel</td>
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<td>Fenpropatrin Danitol 2.4 EC 16 oz Valent</td>
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<td>Mineral oil 435 Oil 2 gallons Drexel</td>
<td>Soybean oil Citruso® 2 gallons Drexel</td>
<td>Zeta-cypermethrin Mustang Max™ 1.5 EC 4.3 fl oz FMC Corporation</td>
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<th>Organic insecticide with Citrusoy*</th>
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<td>Not applicable</td>
<td>Fenpropatrin Danitol 2.4 EC 16 oz Valent USA Corporation</td>
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<tr>
<td>Mar 20, 2014</td>
<td>Not applicable</td>
<td>Spinosad + Mineral oil Entrust®80W + 435 Oil 10 oz + 2 gallon Dow, Drexel</td>
<td>Not applicable</td>
<td>Sulfoxaflor + Mineral oil Closer 240SC + 435 Oil 5 oz + 2 gallon Dow, Drexel</td>
</tr>
<tr>
<td>Apr 10, 2014</td>
<td>Not applicable</td>
<td>Chromobacterium substugae + Mineral oil Grandevo + 435 Oil 3 lbs + 2 gallons Marrone, Drexel</td>
<td>Not applicable</td>
<td>Zeta-cypermethrin Mustang Max™ 1.5 EC 4.3 fl oz FMC Corporation</td>
</tr>
</tbody>
</table>

*Product information is arranged as active ingredient followed by trade name, rate and manufacturer.
18,508 *Tamarixia radiata* wasps (Photo 4) were released in the Valencia and Hamlin blocks, respectively, and distributed equally among all plots.

In the Valencia block, growing season treatments included Entrust used in all three organic programs and Grandevo used in the organic program without oil rotation. Entrust and Grandevo treatments in the Hamlin block were used with 435 oil. Entrust contains spinosad, a naturally occurring compound found in the bacterial species *Saccharopolyspora spinosa* and is expected to act by contact and ingestion. Grandevo is a microbial product obtained from the bacterium *Chromobacterium substugae* with multiple compounds to create a complex mode of action. Closer (sulfoxaflor) was used in March in the conventional program, because it is recommended for use during bloom when bees and other beneficial insects are common. Closer was applied with 435 oil in the Hamlin block and without it in the Valencia block. Mustang Max, a restricted use pesticide toxic to bees, was used in April without any adjuvant. Both Closer and Mustang Max are known to have good activity against ACP.

Adult psyllids and predator insects were monitored using the stem tap sampling method. In this method, samples were collected onto sheets of 8.5” x 11” laminated white paper held on a clipboard. The clipboard was held horizontally under randomly selected branches at three to six feet above the ground in the outer tree canopy. The branches were struck sharply three times with a short length of PVC pipe (photo 5). The number of individual insects that fell onto the paper sheet were identified and counted. This method provides rapid and reproducible information on psyllid and other pests and beneficial insects important in making management decisions.

Four tap samples were taken on each of three trees at three randomly selected locations for a total of nine trees per plot. Overall, samples were collected from 36 trees of each treatment for a total of 144 samples per treatment. We used a threshold of 0.1 adults per tap sample (10 adults in 100 tap samples) to trigger a spray during the growing season. This economic threshold is based on research conducted in Florida under high HLB conditions and does not pertain to citrus grown elsewhere under different conditions. Obviously, no ACP is always the best scenario but impossible to achieve where ACP is well established.

We also took suction samples using a leaf blower operating in reverse (Photo 6), which collects more psyllids at low density as well as active and other predators such as green lacewings lady beetles, spiders and ants. *Pseudomyrmex* ants are very common in the Florida system. Small colonies nest in twigs, but ants forage individually, are highly predaceous and do not tend other insects such as aphids.

Fluss density was estimated by placing a one-foot-square quadrat frame made from PVC pipe at randomly chosen locations in the outer tree canopy and counting the number of shoots at the feather stage to recently expanded leaves. Depending upon availability, 15
randomly selected shoots were collected from each plot and examined in the laboratory using a stereoscopic microscope to determine the percentage of shoots infested with ACP nymphs. In the Valencia block, shoot samples were collected twice in December, three times in January and February, once in March and twice in April. Shoots were not available from the Hamlin block in March, but were collected three times in April.

Additional shoots containing three to five instar nymphs were collected in February and April from the Valencia block and in December, February, March and April from the Hamlin block. These were held under ventilated cylinders in the laboratory to allow for the emergence of adult psyllids or *Tamarixia radiata* to estimate the percentage of ACP nymphs parasitized.

**RESULTS: VALENCIA ORANGE**

During the dormant winter season, ACP adults averaged only 0.01 per tap sample before the start of dormant sprays and stayed below 0.1 per tap sample through mid-February (Figure 1). Treatment effects were only apparent after the third application of Pyganic and the first application of Danitol in January. Five days after treatment (DAT), we saw a 50 percent reduction in ACP adults on trees sprayed with any of the Pyganic treatments as compared to untreated trees; whereas no psyllids were found on Danitol treated trees. At 14 DAT, no ACP were observed in the samples from trees receiving Pyganic + 435 oil or Danitol, and 80 percent less in the Pyganic + Citru-Soy treatment. These were all significantly different compared to the untreated check, but not from each other.

This trend of reduction persisted through February 17 (27 DAT), although numbers were very low and treatment effects were not significant. On March 4 (42 DAT), ACP were reduced by 73 percent in the Danitol treatment and 61 percent in the Pyganic + 435 oil treatment. Only the Danitol treatment differed significantly from the untreated check. Two weeks later at 56 DAT, there was a 68 percent ACP reduction in the Pyganic + 435 oil treatment and 79 percent in the Danitol treatment. Both treatments were significantly different from the untreated check, but not from each other. Between February 17 and March 18, ACP populations increased two- to threefold in the plots of Pyganic alone or with Citru-Soy, suggesting the importance of including the 435 oil with Pyganic. ACP suppression by Pyganic + 435 oil or Danitol was apparent for almost two months after the January application.
During the growing season, Entrust 80 W + 435 oil or Citru-Soy and Closer 240 SC sprayed on March 20 provided significant reduction of ACP through April 8, averaging 78-98 percent in Closer, 74-84 percent in the Entrust + 435 oil and 42-53 percent in Entrust + Citru-Soy. Entrust applied alone did not result in significant reduction. The low volume application of Mustang Max, 435 oil, Citru-Soy and Grandevo in April provided significant reduction for about three weeks averaging 75-99 percent, 84-86 percent, 58-66 percent and 34-60 percent, respectively.

Treatment effects also were reflected in flush infestation with nymphs. Again, Danitol and Pyganic + 435 oil were the two effective treatments, and more reduction was observed in the Danitol treatment as compared to Pyganic + 435 oil (Figure 2). Very little flush was available in April and thus was not very indicative of the ACP population.

**HAMLIN ORANGE**

In the dormant winter season, the initial adult ACP population averaged 0.8 per tap sample, 80 times higher than in the Valencia block. The application of Pyganic + 435 oil made in November provided a 50-79 percent reduction in ACP adults compared to the untreated control (Figure 3). Suppression
after the second application increased to 75-98 percent and persisted at 66-98 percent as compared to 87-100 percent with the Danitol spray in January (Figure 3).

During the growing season, ACP population trends set during the dormant winter season with either organic or conventional insecticides continued. Entrust was sprayed on March 20 for the organic program and Closer for the conventional program because it carries no restrictions with regard to bloom. Both products were applied with 435 oil. These products provided significant reduction, although they were insufficient to bring the psyllid population down to the 0.1 adults per tap sample threshold. Therefore, follow-up applications were made with Grandevo + 435 oil for the organic program and Mustang Max for the conventional program. Repeat applications of pyrethroids or any other product during the year are not recommended, although Mustang Max is a popular choice in this timeframe because it is cheap and works reasonably well while it is still relatively cool. A 69-89 percent reduction of ACP was observed compared to the control for the organic option and 97-100 percent for the conventional regime, which held the psyllid population below the 0.1 ACP per tap threshold for about three weeks. These young Hamlins flushed heavier than the older Valencias (Figure 4), although infestation levels and treatment effects were similar in the two blocks. A large percentage of shoots were infested in all treatments later in the season; but this was of little consequence, as overall shoot density was quite low.
Lacewings, spiders, ants, and lady beetles were monitored in both the Valencia and Hamlin blocks (Photo 7). Lacewings were the most abundant predator in the Valencias, followed by ants and spiders, all of which may have contributed to reducing the ACP population (Figure 5).

In the Hamlin block, ants were the most common, followed by spiders and lacewings (Figure 6). Fewer predators were observed in the plots treated with conventional products, although ants seemed to be suppressed by the Pyganic + Citru-Soy treatment in the Valencia block. Lady beetles were greatly reduced by conventional insecticides in both blocks. These beetles used to be the most important predators of ACP in Florida, but populations have decreased due to the increase of insecticide applications over the past several years. Brown lacewings and convergent lady beetle (Photo 7-B and 7-E3), which are common in California, may enhance the natural mortality of ACP considering that both performed well on a psyllid diet in the laboratory.

Parasitism by *Tamarixia radiata* was observed only in the untreated plots in the Valencia block reaching 25 percent and 12 percent in samples collected in February and April 2014, respectively. In the Hamlin block, four percent parasitism was observed in December 2013 in a sample from a plot treated with Pyganic + 435 oil and 15 percent from an untreated plot in January 2014. Parasitism of three percent was observed again in April in another plot treated with Pyganic + 435 oil in Hamlin block. Lower populations of nymphs and the use of insecticides probably contributed to the low rate of parasitism. We expect parasitism rates to increase with the higher nymphal populations now being observed.

**CONCLUSIONS**

The effectiveness of three applications of Pyganic + 435 oil was evident and long lasting under low ACP populations in the Valencia block. Even in the Hamlin block where ACP populations were much higher, all three applications of
Pyganic + 435 oil provided significant reduction in ACP (Figures 1 and 3). In contrast, Pyganic alone or with soybean oil did not work well. Thus, although not as effective as a single dormant spray of Danitol, three sprays of Pyganic with horticultural spray oil in winter may be a reasonable option for organic growers to achieve significant reduction of ACP populations. This winter suppression of ACP is critical to reduce HLB spread in individual groves and area-wide management where organic farms are intermixed with conventional farms. Effects observed in winter extended into the growing season, although it is yet too early to make judgments on continuous organic programs. Nevertheless, it is expected that the trend will continue for organic insecticides to provide relatively short residuals and, therefore, require more applications than conventional insecticides.

ACP suppression in winter plays a critical role later in the growing season in Florida. Hot dry conditions in California during the summer may provide another good opportunity to attack psyllids when there is little or no flush available for reproduction. ACP populations remained low during the growing season in the organic or conventional program where they were suppressed hard in winter. These results demonstrate the importance of the dormant spray even if psyllid populations are low, because they build up rapidly once flush is available in the spring.

Acknowledgements

We would like to thank the Citrus Research Board for funding this research.

Jawwad A. Qureshi, Ph.D., (jawwadq@ufl.edu) is a research associate professor of entomology; and Philip A. Stansly, Ph.D., is a professor of entomology. Both are with the University of Florida-IFAS at the Southwest Florida Research and Education Center at Immokalee, Florida.
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FROST FORECASTS

Weather forecasts of varying detail are generally available from a variety of sources, including the Internet, newspapers, radios, television, the National Weather Service and private forecasters. These forecasts are generally made in the morning, with an updated forecast in late afternoon. Minimum temperatures for historical key locations (often with a reference weather station) are given for that night.

The forecast may include a comment as to whether an inversion exists and the strength of the inversion (the increase in temperature with height from about 5 to 33 or 40 feet, which is commonly several degrees). The forecast may include an estimate of when damaging temperatures will be reached. The dew point temperature may also be given (to obtain the dew point, see fig. 15.2; to estimate the dew point at a given temperature and humidity, see table 15.4). Purchasing a battery-powered hand-held instrument to measure air and dew point temperatures is a wise investment. Many are available on the Internet and from local farm stores. The dew point is the temperature when the air reaches 100% relative humidity if the air is cooled without changing the water vapor content of the air. At the dew point temperature, water vapor will begin to condense as liquid water (dew) or ice (frost) on the surface of the trees and ground. With high dew points, the air temperature tends to drop slowly and steadily at night because water vapor intercepts upward long-wave radiation and partially reradiates the energy back downward, and because heat is released as the dew or frost forms. Under low–dew point conditions, a rapid temperature drop during the night is likely to be experienced.

For many years the citrus industry has made available district-by-district private weather forecasts. For an additional fee, a grower can obtain even more detailed on-site forecasts during frost nights. The forecasts are disseminated via a telephone recording at a phone number provided at the beginning of the frost season.

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Note: Select a relative humidity in the left column and an air temperature from the top row. Then, find the corresponding dew point in the table.

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FIELD MEASUREMENTS OF BIOCENIC TRACE GAS EMISSIONS FROM ORANGE TREES

John F. Karlik, Silvano Fares, Drew Gentner and Allen H. Goldstein

INTRODUCTION AND BACKGROUND

Volatile organic compounds (VOC) are small molecules containing carbon that evaporate easily and originate from both human activity (anthropogenic) and from natural sources, including green plants. Oxides of nitrogen (NOx) are produced mostly from internal combustion engines. VOC and NOx react in the presence of sunlight to form ozone and secondary compounds including aerosols. Ozone is the most important gas-phase air pollutant in California airsheds. To reduce ozone levels, the relative source strengths of VOC and NOx must be known. The VOC produced by green plants are known as biogenic VOC (BVOC), and these enter into photochemical reactions to produce ozone, but may also react with ozone to remove it from the atmosphere.

We were asked by the Citrus Research Board and California Citrus Mutual to investigate the role of citrus trees in producing BVOC, as well as in the destruction of ozone. This research will help the California citrus industry respond to air quality concerns, since we will better understand how citrus trees affect regional air quality.

To summarize our work, we present here the third of four articles to appear in Citrograph. In a previous issue (July-August 2010, pp. 40-43), we reported results for ozone destruction; and in the January-February 2012 issue (pp. 18-22), we discussed BVOC emissions. Both of those articles report work done in an enclosure system. In this article, we report on our investigation of BVOC emissions made in a field setting in Tulare County. We refer the reader to the previous articles for description of the BVOC of particular interest with regard to citrus, as well as other background information. In our fourth and final article, we will discuss ozone removal by a citrus orchard.
The experimental site was a citrus orchard owned by Jim and Milo Gorden about two miles west of the UC Lindcove Research and Experiment Station. The block of trees in which the tower and instruments were located was Valencia orange on trifoliate rootstock, with a planting date in the 1960s. A seatainer was used to house the analytical and meteorological instruments (Photos 1 and 2), and sampling lines were installed on the tower (Photo 3 and Figure 1) and connected to the field lab. A schematic diagram of instruments and inlet line heights is shown in Figure 2.

We used an eddy covariance method to measure real-time concentrations of BVOC and ozone, as well as water vapor and carbon dioxide (Figure 3). Eddy covariance is a technique that couples measurements of gas concentrations with direction of air movement. Wind velocity (direction and speed) was measured 10 times per second with a three-dimensional sonic anemometer. These data were used to correlate measured BVOC concentrations with air movement and whether the respective compounds were moving up or down in the orchard. Hourly mean values of temperature, vapor pressure deficit, photosynthetically active radiation (PAR) and turbulence could be separated into three well-defined periods: summer, flowering (day-of-year 116 to 145, i.e., April 27 – May 25 in our study) and fall-winter. To measure BVOC concentrations, we used a proton transfer reaction mass spectrometer (PTR-MS) for a one-year period; and for two selected time periods during the year, we also used a gas chromatograph mass spectrometer (GC-MS). For details about the
Near the conclusion of the study, we removed a Valencia citrus tree from within the study block to measure citrus leaf mass and leaf area. These data are critical for scaling measurements at the canopy level. The tree removed for measurement was surrounded on all sides by other citrus trees, so there was no edge effect from proximity to a road. Five fresh subsamples of leaves were measured for leaf area and then oven-dried to obtain leaf mass, so the ratio of leaf mass to leaf area could be calculated; and the mean specific leaf area (SLA) of citrus leaves was 85.4 cm² g⁻¹. All leaves from the citrus tree were removed and placed in paper bags to dry in a vacant greenhouse and later checked against bags that were oven-dried. The sum of dry mass values for all leaves from the citrus tree was 14,830 g. Leaf area was calculated to be 127 m² for the tree. Field measurements, as
well as counting trees using GoogleEarth, gave 96 trees per acre (237 plants per ha), so the corresponding planar area per tree was 42.2 m² and corresponding leaf area index (LAI) for the orchard was 3.00. (LAI is a standard measurement that expresses how many layers of leaves are above the ground and is used to compare different sorts of tree canopies.)

**RESULTS AND DISCUSSION**

**RESULTS FROM PTR-MS MEASUREMENTS**

We focused our observations on the most abundant BVOC we previously identified using enclosures of plants grown in greenhouses under optimal conditions (see earlier Citrograph reports). The compounds reported in this field study can explain the seasonality of the major BVOC emitted from citrus, although they do not represent their totality.

Monoterpenes and BVOC containing oxygen (methanol, acetaldehyde and acetone) were the major BVOC emissions we observed from citrus trees in the field (Figure 4). We found gradients in concentrations from the soil to above the canopy, especially at night, when the atmospheric boundary layer is low and vertical turbulence is minimized. The daily dynamic of measured fluxes showed maximum upward emission in the central hours of the day with minima at night (Figure 5).

Our results confirm that many BVOC species (e.g., ocimene) are emitted in large amounts when citrus trees are flowering (Figure 5). The full year of measurements allowed us to compare the emissions during different seasons. We found that during flowering, the emissions were consistently higher than during other times of the year for most BVOC studied. This seasonality of BVOC emissions from crops should be considered in global and statewide emission models. The largest annual fraction of emissions from Central Valley crops that flower in springtime is likely to occur during that period. A brief description follows of some of the compounds measured within the Tulare County citrus grove.
METHANOL
Methanol is an oxygenated VOC (the molecule contains oxygen). It was the BVOC observed with the highest mixing ratio, with peak values up to 35 ppb during the spring-summer months suggesting high emissions from vegetation. This observation is consistent with previous results showing that increased emission occurs due to leaf expansion.

ACETALDEHYDE AND ACETONE
For these oxygenated BVOC, the good correlation of acetaldehyde with acetone confirms a similar origin of these compounds. Acetaldehyde and acetone were measured in concentrations up to 15 ppb during the flowering period with ambient concentrations of each of these compounds equal to about one half that of methanol. The orchard was a source of acetone and acetaldehyde during the summer, with a positive gradient in the early evening, but the orchard became neutral or a slight sink during winter.

Acetone is the most abundant ketone in the atmosphere, released during senescence and oxidative stress in plants (e.g., from ozone). Acetone is another oxygenated BVOC emitted directly by citrus leaves, but also forms in the atmosphere through oxidation processes. Flowering significantly increased acetone emission to a value about two times higher than the typical summer emissions, as shown by the enhanced atmospheric concentrations and the hourly fluxes. Our results agree with previous research that found rural areas can have significant sources of acetone.

Acetaldehyde is emitted by leaves in large quantities during and after abiotic stresses of many sorts, one being anoxic conditions that may be brought about by saturated soils. Acetaldehyde in particular has been shown to be emitted by citrus plants, especially during flowering, although this compound is also produced by atmospheric oxidation processes.

Figure 4. Hourly average fluxes of BVOC species measured by PTRMS hourly during the winter (black line), flowering period (red line) and summer period (blue line). The daily dynamic of measured fluxes showed peak emissions in the central hours of the day with the lowest emissions at night.

Figure 5. Hourly average fluxes of BVOC species measured by PTRMS hourly during the winter (black line), flowering period (red line) and summer period (blue line). The daily dynamic of measured fluxes showed peak emissions in the central hours of the day with the lowest emissions at night.
**ISOPRENE (C$_5$H$_8$)**

Isoprene and its oxidation products, methyl-vinyl-ketone (MVK) and methacrolein (MACR), were shown to be emitted in very small amounts in the late afternoon, and occasionally deposited at night (again in small amounts) based on observation of their vertical profiles. Isoprene was measured in relatively low concentrations, rarely above two ppb, except during the flowering season, when nocturnal peak concentrations increased to five ppb. Isoprene fluxes were negligible in all seasons in agreement with previous findings showing that orange is not a high isoprene emitter. During the winter period, the orchard was acting more as an isoprene sink based on our observations of the concentration gradients. We hypothesize that isoprene was carried to the orchard in air from a source far away from our measuring footprint, likely oak trees in the foothills. Similar to isoprene, MVK+MACR follow the same pattern during the winter and in summer.

**MONOTERPENES (C$_{10}$H$_{16}$)**

Both in winter and in summer, a positive gradient from the ground to above the canopy was detected, although fluxes were quite small. Soil and litter may significantly contribute to monoterpene emissions, in part due to the organic matter degradation processes in soils, and in part due to biomass wounding and decay following harvesting or pruning operations.

In our study, both summer and winter fluxes of monoterpenes were quite low. Much higher monoterpane fluxes were observed during the flowering period, when the obvious smell of terpenes permeated the whole region, consistent with previously reported findings in greenhouse-based studies with plant enclosures. The monoterpane content in leaves and the cuticular wax thickness may explain the lack of large seasonal change in monoterpane emissions between winter and summer. Terpene accumulation within leaves was highest in summer, followed by spring, with the lowest levels in fall and winter.

Leaf waxes, showed the same seasonal pattern. During flowering, ambient concentrations of monoterpenes reached their maximum with nocturnal values up to 10 ppb, and fluxes reached the maximum annual levels in agreement with previous research. It has been previously shown that certain monoterpane species (e.g. E-B-ocimene) are emitted in large amounts during flowering to attract pollinators and also due to insect damage. The absence of a gradient from the ground to above the canopy during winter suggests that no significant emission took place during the cold period, but rather a strong deposition occurred in the afternoon at about 3:00 pm, so that we can hypothesize transport of emissions from remote sources to the site.

**SESQUITERPENES (C$_{15}$H$_{24}$)**

Sesquiterpenes are very reactive with ozone and, therefore, have very short atmospheric lifetimes. β-caryophyllene is the main sesquiterpene emitted from citrus. We estimated an atmospheric lifetime of about 30-80 seconds when ozone concentrations are between 40 and 100 ppb. Peak ozone was often measured at 70 to almost 120 ppb in summer at the orchard during the study. We tried to minimize the residence time of the air in the sampling line (about 2.2 seconds), but the high reactivity with ozone, the poor transmission efficiency of β-caryophyllene in the PTRMS, and losses likely in our sampling lines resulted in very low concentration measurements for these compounds (0.05 ppb during summer mid-day), and precluded us from providing a quantitative analysis of these emissions.

Our results, therefore, suggest that models that predict sesquiterpene emissions using measurements taken from leaf/branch enclosures from the controlled greenhouse experiments may be a better and more quantitative approach than what we could achieve in the field where oxidant (ozone) levels are high and sesquiterpene lifetimes are very short.

**SIX-CARBON COMPOUNDS**

In this study, we also measured atmospheric concentrations of other important volatile compounds that may have a biogenic source. This was the case of six-carbon compounds and carbon compounds containing a benzene ring that showed a positive concentration gradient from the ground to above the canopy, suggesting that orchards can emit a small amount of these compounds. Although these minor emissions may not be relevant for the chemistry of the atmosphere in comparison with anthropogenic emissions, they may be important for pollinator attraction and other ecosystem interactions.

**RESULTS FROM GC-MS MEASUREMENTS MADE DURING INTENSIVE STUDY PERIODS**

An additional analytical instrument, a gas chromatograph-mass spectrometer (GC-MS), was deployed for two periods, once in spring during flowering and once in summer to correspond to warmer temperatures and higher levels of ambient ozone.
**SPRING FLOWERING MEASUREMENTS**

During the spring measurement campaign, which spanned April 15 to May 6, a broad array of VOC was measured in ambient air, with more than 100 compounds identified. In addition to the BVOC, a suite of anthropogenic compounds was clearly present, such as toluene, ethylbenzene and xylene. Most of the anthropogenic VOC are associated with known sources in the Central Valley, such as automobile and diesel engine emissions.

There was a dramatic increase in both the magnitude and diversity of BVOC emitted during the flowering process. Due to strong nocturnal inversions, many of the BVOC were measured at ppb-level concentrations at night owing to their build-up in the shallow boundary layer where ozone had been scavenged to concentrations below 10 ppb. Perhaps of more interest is that daytime concentrations averaged above 10 ppt for most BVOC, a time when their emissions are most relevant to photochemistry. Additionally, several of the most prominent BVOC had daytime concentrations that regularly exceeded one ppb.

There were high concentrations of a wide variety of BVOC during the flowering period that had strong diurnal patterns. For example, cis-3-hexenyl-acetate, a well-known plant-wounding compound, was present in high concentrations despite no harvest or pruning activity, and it correlated well with other flowering compounds, suggesting it might be released as part of the flowering process. Total concentrations of monoterpenes were about three times greater during the spring flowering period as compared to summer non-flowering conditions.

There were several sesquiterpenes observed at the site during flowering, but the concentrations measured were considerably lower than many of the other terpenoids measured. Given the high reactivity of sesquiterpenes, the lower magnitude of concentrations does not necessarily imply lower emissions, but could also be a result of sesquiterpene compounds reacting at more rapid rates than other terpenoid compounds. The concentrations of sesquiterpenes during flowering were higher than previous work done in a ponderosa pine forest, where concentrations of individual sesquiterpenes were on the order of 10 ppt, but there are very few ambient air measurements of sesquiterpenes published with which to compare our observations.

**SUMMER MEASUREMENTS**

While we measured many fewer BVOC during the summer campaign, we still observed a variety of monoterpenes in ambient air; but we did not observe many of the compounds that appeared to be associated with flowering.

We observed similar diurnal patterns in the summer as in the winter due to boundary layer effects, with ambient ozone still falling below 10 ppb. There is a similar distribution and diversity of monoterpenes between the two seasons, with the exception of compounds associated with flowering. Concentrations of total monoterpenes during the summer were similar to those observed at a California ponderosa pine forest in warm temperatures (26°C daytime mean), but the distribution of monoterpenes was significantly different; there was much more limonene and less α- and β-pinene compared to the pine forest. Limonene was the most prevalent monoterpenes observed in the summer. The relatively comparable concentrations of several monoterpenes during the two measurement periods in the orange orchard imply similar emission rates during those two periods.

**IMPLICATIONS FOR CALIFORNIA’S BIOGENIC EMISSION MODELING**

Plants in the citrus genus are among the most widely cultivated crops in California, and our greenhouse enclosure measurements highlighted orange trees as one of the higher BVOC emitters among crop species, particularly during flowering. To accurately model biogenic emissions from agriculture and air quality in the San Joaquin Valley, the seasonal events need to be taken into account.

The GC-MS measurements taken at the site yielded information on large emissions of previously unobserved aromatic BVOC that were associated with flowering in this region. This burst of emissions could have an effect on the biogenic emission inventory for the region if the emissions are extrapolated across all the citrus of the valley during the periods of flowering. These compounds should be included

**UNIT ABBREVIATIONS**

- **g** – gram
- **cm** or **cm²** – centimeter or square centimeter
- **m** or **m²** – meter or square meter
- **ppm** – parts per million
- **ppb** – parts per billion
- **ppt** – parts per trillion
- **cm² g⁻¹** – square centimeters per gram
in emission models, since their emissions during flowering were on the same order as all the terpenoids observed. Further study will likely be necessary to determine their potential contributions to ozone and secondary organic aerosol formation, and to assess flowering emissions from other major crops grown in California.

Acknowledgments
This project was supported by the Citrus Research Board, the California Air Resources Board, and the EU program IOF-PEOPLE 2008. The authors thank the Gorden Ranch for hosting the study and the director and staff at the UC Lindcove Research and Experiment Station for their help and support.

References


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CRB Project 5100-137
REACH COMMERCIAL CITRUS GROWERS IN CALIFORNIA, ARIZONA AND BEYOND

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In 2012, the Citrus Research Board provided funding to the University of California at Riverside to set up a mass-rearing program of Tamarixia radiata for the control of the Asian citrus psyllid (ACP). The primary goal in rearing T. radiata is to preserve a genetically diverse source population from which we can supply insectaries and other mass rearing programs. In biological control programs, natural enemies are often reared for a long time under laboratory conditions. Due to the benign environment, there is relaxed selection for several traits, including host finding, mate selection and adaptation to environmental conditions (temperature, humidity, etc.). Over time, wasps adapt to laboratory rearing, thus making them less adaptable to field conditions.

At UC-Riverside, the rearing of wasps is done in such a way as to maintain genetic variability and hybrid vigor by keeping 16 genetically distinct small populations of Tamarixia. These wasps were collected from different regions of Pakistan during multiple collecting trips over a period of two and a half years. The reason for maintaining several genetically unique lines instead of one large interbreeding colony is that it prevents the loss of genetic variants that may prove essential to the establishment of the wasps in the field. Prior to release, individuals from the 16 distinct populations are placed into a “mixing cage” and allowed to mate randomly. The hybrids that result from this mass cross-mating are released and should carry with them high levels of genetic variation.
HOST PLANT PRODUCTION

To produce large numbers of *Tamarixia*, successful rearing of healthy host plants and psyllids is vital. All ACP are reared on *Murraya koenigii* (curry leaf), which is found to consistently produce optimal numbers of nymphs (Figure 1). *Citrus volkameriana* and *Murraya paniculata* (orange jessamine) were previously used as ACP host plants; however, higher nymph mortality was observed, along with fewer plants found to be infested. Curry leaf host plants are started from seed, placed individually in Ray Leach Cone-tainers (Stuewe & Sons) in a soil mixture of 60 percent peat moss, 10 percent organic material, 10 percent coarse silica, 10 percent plaster sand and 10 percent vermiculite. This soil mixture provides optimal moisture and oxygen levels to the plant leading to a high root density and overall root health. Root health was found to be essential to increasing ACP production numbers per plant.

When plants are between two and four months old (approximately three inches in height), they are transferred to 3.75-inch square pots. Primary greenhouse pests of curry leaf include soft scale, mealybugs and aphids. Pests are controlled mechanically (removed via shop vac and blasting with water), and biologically (*Metaphycus* spp.). In greenhouses where pests cannot be controlled with mechanical and biological methods, chemical control is employed. Pyganic® and Purespray Green (Petro-Canada) are the main chemicals used to treat pests. Plants treated with pesticides are not used for ACP production for at least two months or longer depending on the type of pesticide used.

Figure 1: Curry leaf production for use in ACP rearing.
REARING OF ACP FOR Tamarixia Production

Because Tamarixia are host specific, meaning they only feed on ACP, it is necessary to maintain a healthy colony of ACP as a food supply. Weekly, 200 curry leaf plants are transferred to the insect-rearing facilities to use for ACP nymph production. Plants are selected from the greenhouse with three main characteristics: 1) pest free; 2) good root structure (determined by examining the underside of the pot and looking for white roots); and 3) age structure (plants must be at least six months old). This is done to ensure optimal plant health, which in turn supports large numbers of ACP nymphs per plant.

Plants are pruned two weeks prior to inoculation of ACP adults in order to produce feather flush that is necessary for ACP to lay eggs upon (Figure 2). All ACP rearing takes place at 80-82°F with a relative humidity of 30 percent (humidity within actual cages is likely higher). Each insect-rearing cage is provided with 15 plants (with optimal flush) and 20 ACP adults per plant for a total of 300 adults per cage. ACP adults are allowed to lay eggs and feed on plants for five days before being removed. Thirty-two plants with varying ages of ACP (early-instar nymphs, late-instar nymphs and adults) are produced for research purposes, depending on the needs of the experiment. Weekly, 122 plants are supplied to maintain line production and mass rearing of Tamarixia. The preferred age of ACP nymphs for parasitism by Tamarixia is 10-11 days old (3rd-4th instar) (Figure 3).

Figure 2: Feather flush on a curry leaf plant that is optimal for ACP egg laying.
REARING OF *TAMARIXIA RADIATA*
LINE PRODUCTION FOR THE SOURCE COLONY

Thirty-two cages (16 lines in total, two cages per line) containing genetically unique *Tamarixia* are maintained. Weekly, 16 new cages are established in order to have a consistent supply of source population parasitoids. Each new cage set-up contains one curry leaf plant with 150-200 nymphs. Ten females and five male *Tamarixia* are added to each plant. Parasitoids from these lines are collected daily and used to maintain lines and parasitize psyllids contained in mass production cages. Extra wasps collected from the line colonies are mixed (to ensure hybrid female offspring) and sent out for field releases.

MASS PRODUCTION FOR FIELD RELEASES AND RESEARCH

To ensure a genetically diverse population for release in the field, parasitoids are collected from every line cage, mixed for cross-mating between lines, and then added to the cages containing ACP-infested plants that are used for mass production. Five mass-sting cages are set up weekly with each cage containing 15 curry leaf plants infested with a total of 1,800-2,000 ACP nymphs. Parasitoid wasps are added at a ratio of one female for every 30 ACP nymphs (approximately 60-65 females per sting). The number of males added ranges from 10-15 per sting cage. We have found that female wasps that first feed on several ACP nymphs produce more offspring; therefore, two days before introducing *Tamarixia* into the

**Figure 3:** Late instar nymphs on a curry leaf plant ready for inoculation of *Tamarixia radiata* wasps.
mass-sting cages, adult wasps are allowed to host-feed in 6x6x12-inch clear plastic ventilated cages containing one curry plant with 80-100 ACP nymphs (Figure 4). Host-fed Tamarixia wasps are found to produce significantly higher rates of parasitism than non-host fed wasps (Figure 5).

A total of 15,500 Tamarixia wasps were produced in 2012 and 160,000 in 2013 (Figure 6). Highest monthly totals occurred from August until November 2013 when 5,900 Tamarixia per week were produced on average. From August through December 2013, 1,000-2,000 parasitoids per week were supplied to the USDA APHIS and CRB cooperative-rearing program to inoculate field insectary cages. Presently, 1000 wasps are supplied weekly to FAR Insectary, and 1,500 wasps per week are supplied to the CDFA to use as a source colony in their mass sting production.

Figure 4: Cages used to host feed Tamarixia wasps prior to inoculation on ACP-infested plants.

Figure 5: Percent parasitism of host fed vs. non-host fed wasps. Star indicates a significant difference (P ≤0.05) between treatments.

Figure 6: Monthly Tamarixia radiata production numbers from July 2012 through November 2013.
All wasps collected from mass-sting cages are provided to researchers or supplied to the CDFA for release in the field.

**CONCLUSION**

It is especially important to introduce sufficient genetic variation in the initial releases for classical biological control. Initial genetic variation is best done by maintaining populations with little genetic variation, such as 16 lines, and mixing them back together prior to release in the field. Once the population has established in the field and selection has taken place, mass rearing can be done with field-selected individuals, but it is recommended not to exceed ten generations in the lab. Laboratory experiments to better understand factors affecting parasitoid egg production (host feeding) and subsequent parasitism rates have helped to increase monthly totals of wasp production. Additionally, lab experiments currently are being conducted to determine if other sources of protein have an effect on *Tamarixia* egg load and percent parasitism.

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**ACKNOWLEDGEMENTS**

This project is funded by the California Citrus Pest & Disease Prevention Program through the Citrus Research Board.

CRB Project Number 5500-196
Bacterial diseases pose a great threat to the citrus industry. Three disease-causing gram-negative bacteria of major concern are:

1. Candidatus Liberibacter asiaticus (CLas), causing citrus greening or huanglongbing (HLB)
2. Xanthomonas axonopodis pv. citri [formerly X. campestris pv. Citri] (Xcc), causing citrus canker
3. Xylella fastidiosa (Xf), causing citrus variegated chlorosis (CVC)

Of the three, HLB is the most devastating disease, affecting industries in the major citrus producing countries. In the United States, HLB most severely affects Florida. The threat of HLB is also looming large in California and Texas, where Asian citrus psyllids (ACP) carrying Liberibacter have been found. It is not a question of if, but when HLB will appear in California and Texas.

In addition to HLB protection, we also need protection against citrus canker and CVC. It was only a few decades ago that citrus canker was widespread in Miami, Florida. Even after an extensive eradication effort, new cases of citrus canker continue to be reported in Florida. Although, CVC has, so far, been endemic only to Argentina and Brazil, it will be worthwhile to be prepared in advance with a protection strategy against CVC in the U.S. Protection against these three diseases is crucial to the survival of citrus industries worldwide. In particular, protection against HLB is the highest priority in the United States.
HLB, like CVC, is a vector-borne disease. Since highly robust naturally occurring or transgenic HLB-resistant citrus is not yet available, first attempts to manage HLB have involved psyllid control, which limits the spread but does not clear or eliminate the causative Liberibacter. Total clearance and elimination of the causative Liberibacter is the most desirable strategy for HLB control; i.e., if there is no Liberibacter in citrus, there is no development of disease, nor is there any Liberibacter transmission by psyllids to other citrus trees.

Our project is aimed at developing strategies that clear the HLB-causing Liberibacter from the phloem, the site of Liberibacter colonization in citrus. The strategy is based upon a simple concept, i.e., making citrus better equipped to clear Liberibacter thereby not allowing citrus to become a permissive host for HLB development. This is achieved by engineering a novel innate immune defense in citrus.

Innate immunity in citrus is the first line of defense against invading pathogens. This innate immune defense consists of three steps: (1) recognition of pathogen signals by citrus, (2) processing of the signal in response to pathogen recognition, and (3) release of the effector molecules for pathogen clearance (or lysis) as a result of the signal processing. These three steps occur in sequence in citrus (or in any other hosts that are under pathogen attack).

Our strategy is to combine two of these steps, namely pathogen recognition and lysis, in parallel to generate more robust innate immune defense in the host. About ten years ago, we introduced this strategy for clearing bacteria from the infected human, animal and plant hosts. This approach involves design and delivery of a protein chimera at the site of bacterial infection. A chimera, as shown in Figure 1, consists of two protein domains: one for recognition and the other for lysis of the targeted bacterium. We chose two types of recognition domains. One is a protease that cleaves a conserved protein on the bacterial membrane, whereas the other is a lipid binding peptide that binds to a conserved moiety on the bacterial membrane. The lysis domain is an antimicrobial peptide (AMP), which creates a pore in the bacterial membrane. The synergy of the recognition and lysis domains ensures rapid clearance of the bacterium.

This approach was successfully applied to suppress Pierce’s disease development in grapes by Xf. For this, transgenic grape lines were constructed by the introduction of the anti-Xf chimera gene in the grape genome. These transgenic grape lines expressed a protein chimera (consisting of a human protease and

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**Glossary**

(CLas) *Candidatus Liberibacter asiaticus*: bacteria that has been associated with huanglongbing (HLB) in the U.S.

*Xf*: *Xylella fastidiosa*, bacteria that cause citrus variegated chlorosis (CVC)

*Xcc*: *Xanthomonas axonopodis* pv. *citri*, bacteria that cause citrus canker

**Outer-membrane proteins (OMPs)**: conserved (have not changed over thousands of years) proteins on CLas, Xcc, and Xf

**Plant Proteases**: enzymes that cleave OMPs

**Lipid A**: conserved region of the lipopolysaccharide (LPS) moiety on the membrane of CLas, Xcc, and Xf

**Lipid Binding Proteins** (*e.g.*, LBP1): bind to Lipid A in CLas, Xcc, and Xf

**Anti-microbial peptides (AMP)**: lyse bacteria such as CLas, Xcc, and Xf (examples – SynCec, D4E1, thionin)

**Chimera**: a protein consisting of two different functional domains (molecules) such as a protease and an AMP

**Nanoparticles**: particles consisting of lipid shells with encapsulated proteins (*e.g.*, anti-bacterial chimeras) for in planta delivery

**Baculovirus-insect cell expression system**: used for making a protein (*e.g.*, an anti-bacterial chimera) in the laboratory

**Transgene**: a foreign gene (*e.g.*, a chimera gene) introduced in the plant genome
insect-derived cecropin) in the xylem. This anti-Xf chimera successfully cleared Xf and blocked Pierce’s disease. However, it may be noted that the design and delivery of the anti-Xf chimera composed of human protease and insect-derived cecropin was just a proof-of-concept. Although, this protein chimera appeared to show no toxicity in grape, the delivery of human and insect protein components in plants is unlikely to receive consumer acceptance. Hence, we proceeded to design protein chimeras of plant origin for the protection of citrus against CLas, Xcc and Xf.

**SELECTION OF THE PROTEIN CHIMERAS**

As shown in Figure 2, the primary recognition targets are the outer-membrane proteins (OMPs) and core region (lipid A) of the lipopolysaccharide (LPS) moiety on the Liberibacter membrane. We have chosen recognition proteins from the plant repertoire. The recognition proteins fall into two categories: one that cleaves OMPs and the other that binds to the hydrophobic lipid A. The lysis domains are antimicrobial peptides either derived from plants or shown to be non-toxic to plants. Three candidate recognition domains are: a plant thionin, a lipid binding peptide (called LBP1), and a plant serine protease with potential cleavage activity against OMPs.

Thionins are compactly folded plant peptides with four disulfide bridges. There are different classes of thionins. Most of them are anti-fungal while a few of them are anti-bacterial. We have chosen a thionin candidate that possesses activity against gram-negative bacteria (such as HLB-causing Liberibacter). This thionin contains both hydrophobic (water-avoiding) and hydrophilic (water-attracting) faces. The hydrophobic face binds to the hydrophobic lipid A, whereas the positively charged hydrophilic face inserts into the negatively charged head groups of LPS. Thus, thionin has both recognition and lysis properties. The candidate lysis domains are linear antimicrobial peptides: D4E1 and a synthetic cecropin. D4E1 was discovered by Ed Stover, USDA-ARS, Fort Pierce, Florida. It is a synthetically designed AMP with activity against Liberibacter homologs i.e., *Agrobacterium tumefaciens* and *Sinorhizobium meliottii*, which are a proteobacteria species that are culturable and are closely related phylogenetically to CLas, CLam, and CLaf. Also D4E1 does not show toxicity in a hemolytic assay. The synthetic cecropin (hereafter referred to as SynCec) was designed by substituting a few amino acids in the original insect-derived cecropin. These amino acid substitutions reduce toxicity of SynCec to plants without the loss of activity. The recognition and lysis domains are joined by appropriate linkers to create four different chimeras as shown in Table 1. The linkers are chosen based upon their length and amino acid sequence so that they retain the functions of the recognition and lysis domains and confer synergy. Type I (with seven amino acids for chimeras 1-3) and Type II (with four amino acids for chimera 4) linkers were chosen.
**PREDICTION OF STRUCTURE: ACTIVITY CORRELATION OF THE CHIMERA 1**

We carried out in-depth studies on Chimera 1 consisting of plant thionin and D4E1 AMP. Since we have chosen a specific plant thionin that has demonstrated bactericidal activity against gram-negative bacteria, we expect that the chosen thionin will also be active against gram-negative CLas, Xcc, and Xf. Ed Stover’s laboratory has shown that D4E1 is active against Liberibacter homologs *Agrobacterium tumefaciens* and *Sinorhizobium meliottii*. Note that, to date, Liberibacter species have not been cultured in the laboratory. For the reasons described above, we postulated that joining the thionin and D4E1 would result in a chimera that would be highly active against CLas, Xcc and Xf. Our structural analysis predicted a specific seven amino acid long linker. Chimeras of different lengths are chosen to determine how short a chimera can be and still be active against CLas, Xcc and Xf. Also, we believe a short and compact chimera may be more suitable for in planta delivery via nanoparticles than larger chimeras.

<table>
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<tr>
<th>Chimera</th>
<th>Recognition Domain</th>
<th>Linker</th>
<th>Lysis Domain</th>
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<tbody>
<tr>
<td>1</td>
<td>Thionin</td>
<td>Type I</td>
<td>D4E1</td>
</tr>
<tr>
<td>2</td>
<td>Thionin</td>
<td>Type I</td>
<td>SynCec</td>
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<tr>
<td>3</td>
<td>LBP1</td>
<td>Type I</td>
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<tr>
<td>4</td>
<td>Serine Protease</td>
<td>Type II</td>
<td>SynCec</td>
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As shown in Figure 3, molecular modeling of Protein Chimera 1 predicted two low-energy structures. The lower the energy, the higher the stability and activity of the chimera. For both, D4E1 forms a helix, which can be oriented in such a way that it can insert into the LPS layer on the outer-membrane with the thionin hydrophobic face interacting with the lipid A (LPS core), and the thionin hydrophilic face inserting into the LPS outer-layer. The synergy of the thionin and D4E1 domains are expected to make the chimera a better bactericidal than the individual domains acting alone. The two structures of Chimera 1 differ in the position of the thionin helix relative to the D4E1 helix. At this point, we are not sure whether one structure would be more active than the other. We have begun to examine the in planta activity of Chimera 1 by constructing transgenic citrus (see below).

**IN VITRO EXPRESSION OF THE PROTEIN CHIMERAS**

In parallel with the expression of the chimeras in transgenic plants, we are planning to use nanoparticles to deliver the chimeras in planta at the site of infection. Nanoparticles are special systems for phloem-specific delivery of the protein chimeras. For our project, we proposed to design virus-like nanoparticles of about 100-nanometer radius. They will have lipid shells coated with proteins that enable easy movement across the plant and specific phloem targeting. These particles will encapsulate the anti-CLas chimeras and will release the chimeras near the phloem.

For nanoparticle-mediated delivery, we developed protocols for in vitro production of the protein chimeras 1, 3 and 4. For this, we considered baculovirus-insect expression systems. These systems appear to be suitable for chimera expression in our hands. We also noted that the protein chimeras are more stable and active when they are released from the insect cells, i.e., expressed in the extracellular space. Therefore, we added a signal sequence upstream of the chimera gene to ensure expression of the protein chimera in the extracellular space.

The chimera gene was then cloned into a pBacPAK8 baculovirus vector. The chimeric gene inserted into pBacPAK8 was co-transfected with BacPAK6 viral DNA into insect (Sf21) cells. Recombinant viruses formed by homologous recombination were amplified, and the protein expression was optimized in insect High Five cells, derived from *Trichoplusia ni* egg cell homogenates. About 35-50 percent of the expressed chimeric protein was secreted into the supernatant. The supernatant was collected and purified.

**CONSTRUCTION OF TRANSGENIC CITRUS EXPRESSING THE CHIMERA**

We completed *Agrobacterium*-mediated transformation of citrus Carrizo (and Hamlin) with the transgene of Chimera 1 in collaboration with Ed Stover, USDA-ARS, Fort Pierce, Florida. In addition to the gene encoding the active protein, the transgene contains the secretion
signal and a constitutive promoter and a downstream terminator sequence. Figure 4 shows different elements in the Chimera 1 transgene and their respective roles. The putative transgenic plantlets were examined by polymerase chain reaction (PCR) for the presence of the Chimera 1 transgene. The efficacy of the transgenic citrus in suppressing citrus diseases such as canker and HLB are being tested. The initial results appear to be promising. Once efficacy studies on HLB and citrus canker are completed, we will start our collaboration with Dr. Machado of Brazil on efficacy testing against CVC.

CONCLUSION

Once the efficacy studies with the Chimera 1 transgenic citrus are complete, we will have an estimate as to how much chimera is needed in planta (near phloem) for optimum anti-CLas activity. This information will guide the design of appropriate nanoparticles for in planta delivery given that we have already standardized the protocols for in vitro production of the chimeras as shown in Table 1.

Goutam Goupta, Ph.D., is in the Biosciences Division of Los Alamos National Laboratory, Los Alamos, New Mexico.

This report is prepared for the project funded to Goutam Gupta (PI); Ed Stover, Ph.D. (collaborator), research horticulturist at the U.S. Horticultural Research Laboratory, Ft. Pierce, Florida; and Marcos Machado, Ph.D. (collaborator), Centro de Citracultura Moreira, IAC, Laboratory of Biotechnology, Brazil.

References


REQUEST FOR QUOTE FOR MASS-PRODUCED *TAMARIXIA RADIATA* (WATERSTON) (HYMENOPTERA: EULOPHIDAE) FOR CONTROL OF THE ASIAN CITRUS PSYLLID

Quotes are due by October 1, 2014. The following is a summary. Please refer to entire Request for Quote at www.citrusresearch.org/Tamarixia-RFQ.

The California Citrus Research Board is requesting quotes for the mass-production of *Tamarixia radiata* for use to control Asian citrus psyllid (ACP) as part of the California ACP Cooperative Control Program.

Quotes are requested to provide between 250,000 to 2,000,000 parasitoids over the course of one year. A range of *Tamarixia radiata* production levels are requested to allow potential vendors to develop a proposal based on their determination of production costs at a number that can be sustainably produced that will fit within project budgets.

Vendors will supply documentation of their ability to perform and evidence of previous successful mass production of high-quality hymenopteran parasitoids, free of debris and other pests. Successful bids will include a production plan, which will demonstrate the amount of space and infrastructure that the vendor has available or planned that will be sufficient to support the targeted production. This will include a description of dedicated rearing space, measures taken to ensure adequate containment and biosecurity against releases of ACP, planned plant production or arrangements to purchase plants, and equipment needed to store and handle wasps.

The successful vendor(s) will establish a joint technology development project with scientists from the Citrus Research Board and USDA currently working on the ACP Control Program to develop mass-rearing methods for *Tamarixia radiata*.

For the objectives of this work, please see www.citrusresearch.org/Tamarixia-RFQ.

Although the development of high-efficiency rearing methods of high quality *Tamarixia* is one of the goals of this project, the wasps received under this agreement will meet minimum standards of quality assurance, which will include defined storage conditions, acceptable levels of mortality and adult longevity and accuracy of counting methods. These standards will be defined by project scientists and will be included in contract specifications that may be modified as rearing methods are improved. Starter material for these colonies will be supplied by the University of California so that the genetic diversity of the original Pakistani collections is maintained.

For production and delivery specifications, please see www.citrusresearch.org/Tamarixia-RFQ.

**Other requirements and conditions**

The production of *Tamarixia radiata* and ACP hosts will be regulated by Cdfa plant pest permit or, if production occurs outside of California, by a Federal APHIS-PPQ plant pest permit. One of the restrictions of this requirement is that production within California must occur within an ACP generally infested area and an area not under HLB quarantine. Production in areas outside of California cannot be from an area under HLB quarantine. Permitted insectaries shall be operating under a permit issued to an ACP Cooperative Program official. No commercial sales of *Tamarixia radiata* wasps will be permitted under the terms of any agreement resulting from this RFQ, during the life of such agreement. Movement of any plants for production purposes outside of or through HLB/ACP-regulated areas will be governed by compliance agreements with Cdfa and the California counties where the plants are produced. Meeting the requirements of this plant movement will be the responsibility of the vendor. Please contact us for any questions regarding technical, permitting or administrative questions.

**Quotes are due by October 1, 2014. Please send responsive quotes to brian@citrusresearch.org**

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Spiroplasma citri causes citrus stubborn disease in California. The disease stunts tree growth and inhibits fruit production. Early detection of the pathogen is critical in stubborn disease management. Current PCR detection of S. citri utilizes PCR primers from DNA sequences of low copy number genes. To improve PCR detection, this research made use of the recent biological information that S. citri cells often harbor a high number of bacteriophages or viruses. By targeting the bacteriophages, a technique over a thousand times more sensitive in detecting S. citri was developed. The technique can be used to enhance current stubborn disease control programs such as variety registration and nursery surveys.
Spiroplasma citri causes stubborn disease (Photo 1) in citrus and occurs in the Coachella Valley and interior valleys of central and southern California. Although the disease, in general, does not kill citrus trees, it stunts tree growth and inhibits fruit production. Since hot and dry weather favors the development and spread of S. citri, stubborn disease is a problem in the San Joaquin and desert valleys. Management of stubborn disease depends on disease prevention and reduction of pathogen spread. A key step is early detection of the pathogen, which requires a highly sensitive detection system.

Culturing in artificial nutrient media is a standard technique for bacterial isolation to facilitate detection and identification. S. citri can be cultured in vitro, but complex media are required. The cultivation process is highly technical, arduous to achieve and time-consuming. For these reasons, polymerase chain reaction (PCR) procedures have been developed for the detection of S. citri.

Glossary

Bacteriophages: Bacterial viruses. One bacterial cell can have many viruses of the same type.

Conserved genes: Genes that have remained essentially unchanged throughout evolution.

Copy number: The number of the same DNA in a bacterial cell.

Cycle threshold (Ct): A value generated from a real-time PCR experiment. The lower the Ct value, the greater the amount of DNA in the sample.

in silico: an expression meaning “performed on computer or via computer simulation.”

in vitro: in an artificial environment such as bacteria in a test tube, rather than in the normal biological context such as within bacteria or in plant tissues.

Polymerase Chain Reaction (PCR): A biochemical technology used to amplify a single or a few pieces of DNA and generate thousands to millions of copies of a particular DNA sequence.

SYBR green-based real-time PCR: A type of PCR procedure employing a fluorescent compound called SYBR green to monitor the increase of DNA amplification.

16S rRNA: A type of RNA molecule involved in protein synthesis in a bacterial cell. The gene sequence coding for 16S rRNA in a bacterial species has changed very little over time; therefore, it is used to classify bacteria species.
Current PCR procedures utilize bacterial chromosomal DNA sequences of conserved genes. Unique DNA sequence patterns in these genes are important for pathogen description and identification. However, the copy number of these conserved genes is usually small, resulting in fewer DNA templates for PCR and lower detection sensitivity. For example, the 16S rRNA gene is typically used for bacterial species identification, and there is only a single copy of that gene per *S. citri* cell. To increase PCR detection sensitivity, it is desirable to target the DNA sequences of genes that have multiple copies.

Bacteriophage or phages are viruses that infect bacteria. They multiply inside a bacterial cell and, therefore, accumulate in high numbers (Figure 1). Phages were reported when *S. citri* was first described. Recent genome sequence analyses further confirmed the presence of multiple copies of phage DNA incorporated within the *S. citri* genome. Phages can multiply and accumulate in levels of tens to hundreds of phage genome copies per bacterial cell. These high levels often kill their bacterial hosts to release the phage particles. The number of phage particles at the point of bacterial cell death is called the burst size. For example, a phage called T4 in *Escherichia coli* has a burst size of approximately 100-150 viral particles per infected cell. Therefore, detection of the bacterium by targeting a phage gene could be 100-150 times more sensitive than targeting a single copy gene in the bacterial genome. Based on this principle, we initiated a project to develop a phage-based PCR detection system for the sensitive detection and identification of *S. citri*.

Another important requirement for bacterial detection is specificity. Phages are known to infect bacteria at different taxonomical levels or categories (genus, species, subspecies and strains). For example, if a species-specific gene is found, this gene can be used for specific species detection. If a strain-specific gene is found, it can be used as a target to identify and differentiate bacterial strains. Strain recognition is important in pathogen detection, because strain phage typing profiles can be used to track down sources of origin, identify certain biological traits (such as virulence) and determine the distribution of the bacterial population. Extensive characterization of a phage or a phage genome sequence is key to assuring appropriate PCR primer design and application.

Several *S. citri* phages have been reported. One of the better-known phages is designated SpV1-R8A2 B and its complete genome sequence is available. SpV1-type phages were first discovered as rod-shaped particles of *S. citri*. Many *S. citri* isolates, obtained either from plants or from insects, are naturally infected with SpV1-type phages. This information suggested the feasibility of using phage gene DNA sequences as targets for *S. citri* detection. In this study, two phage-based primer sets were developed and evaluated. The project involved three steps: 1) *in silico* analyses of *S. citri* phage gene sequences; 2) evaluation of phage primers with DNA samples from pure *S. citri* cultures; and 3) evaluation of phage DNA sequence primers with DNA samples from *S. citri*-infected citrus trees in field orchards.

![Figure 1. Illustration of the comparative number of PCR DNA targets based on a single gene in a bacterial chromosome (big circle on the left) and multiple (10) phage genomes (small circles on the right) in a bacterial cell. The phage gene has ten times more DNA in a single bacterial cell available for detection by PCR than the chromosomal gene.](image-url)
In silico analyses showed that Php-orf1 primer sequences had 13 copies, and Php-orf3 primer sequences had 11 copies in the whole genome sequence of \textit{S. citri} strain GII3-3X. In contrast, only a single copy was found for each of the spiralin and P58 primer set sequences. In regard to specificity, sequences of both Php-orf1 and Php-orf3 only matched with those of \textit{S. citri} from the GenBank DNA sequence database where thousands of bacterial genome sequences have been deposited. The same level of specificity was found with primer sets of spiralin and P58.

Evaluation with pure culture DNA

SYBR Green-based real-time PCR was further performed to evaluate phage primer set sensitivity and specificity. DNA was extracted from cultures of 18 strains of \textit{S. citri} originally obtained from a variety of hosts including citrus, carrot, peach, broccoli, horseradish and the beet leafhopper. In addition, DNA from two cultures of \textit{Xylella fastidiosa} (the pathogen causing Pierce’s disease of grapes and almond leaf scorch disease in California), and DNA from citrus infected with \textit{‘Candidatus Liberibacter asiaticus’} and \textit{‘Candidatus Liberibacter solanacearum’} were added to test primer-PCR specificity. Cycle threshold (Ct) values generated from the PCR analyses were compared. It should be noted that for PCR evaluation, lower Ct values equate to higher PCR sensitivity. As shown in Table 1, when compared with spiralin- and P58-gene primer sets, primer set Php-orf1 reduced Ct values by 3.02±0.62 and 1.76±0.51, (equivalent to 100 to 1,000x greater sensitivity), respectively. Similarly, primer set

<table>
<thead>
<tr>
<th>Strain</th>
<th>Host</th>
<th>Origin</th>
<th>Chromosome DNA primer set</th>
<th>Phage DNA primer set</th>
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<tr>
<td>\textit{Spiroplasma citri} Ca1</td>
<td>Sweet orange</td>
<td>Tulare Co., CA</td>
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<td>Fresno Co., CA</td>
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</tr>
<tr>
<td>\textit{Spiroplasma citri} CB1</td>
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<tr>
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</table>
Php-orf3 reduced the Ct values by 4.91±0.49 and 3.65±0.62, (equivalent to 3,000 to 5,000x greater sensitivity), respectively. No PCR amplification of DNA was detected from non-S. citri bacteria.

EVALUATION WITH FIELD SAMPLES

A total of 252 DNA samples from leaves in citrus trees showing stubborn disease symptoms in two orchards over a period of four years were analyzed. S. citri was detected in 240 (95.24 percent) samples by the spiralin primer set, and in 243 (96.43 percent) samples by the P58 primer set. A significant increase of S. citri positive samples was observed with primer set Php-orf1 (250 or 99.21 percent of samples), and primer set Php-orf3 (249 or 98.81 percent of samples).

It should be pointed out that the two citrus stubborn disease test sites had different citrus stubborn disease incidence; 22.3 percent in one orchard and 3.8 percent in the other orchard. The advantage of phage-based detection was more pronounced in the low (3.8 percent) disease incidence orchard. As shown in Figure 2, the S. citri level in tree samples of this orchard were low (Ct values > 30) in 2008 and 2009 by PCR using the primers for spiralin (also with the P58 primers, not shown in Figure 2 for clarity purposes). During these two years, the results would have indicated the trees as negative for S. citri. Generally speaking in our system, Ct values of 30 or more are not reliable for interpreting a sample as PCR positive. The inclusion of primer set SpV1-orf1, showing Ct values of 27 or lower, greatly increased the level of confidence in the detection of S. citri and reduced the chances of obtaining false positive or false negatives results.

CONCLUSIONS

In conclusion, available literature and recent genomic research data indicate a strong association between phages and S. citri cells. Utilizing this biological information, this research developed two phage-based primer sets and demonstrated their improved sensitivity and specificity for detecting S. citri. The improved detection systems will enhance current disease control efforts by more efficient and sensitive identification, particularly for asymptomatic plants and new insect vectors for their potential to transmit S. citri to citrus trees. Programs such as the California Citrus Clonal Program may consider using this technique in variety registration and nursery surveys.
Lastly, studies on *S. citri* not only directly address the stubborn disease problem in California, but also provide a model system for future research on “*Candidatus Liberibacter asiaticus*,” a phloem-limited (like *S. citri*) and unculturable bacterium associated with citrus huanglongbing (HLB).

**Referenced Literature**


**ACKNOWLEDGEMENTS**

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