

Citrus Industry Update

Working
To Keep You
Informed

Published by the University of Florida, Institute of Food and Agricultural Sciences, with the mission of keeping the Florida Citrus Industry informed of current research concerning canker and greening.

Genetic Engineering of *Murraya* as a Psyllid Control Tool

— Fred Gmitter

Murraya paniculata (orange jasmine) is a citrus relative and a common ornamental plant known for its attractiveness. Its utility and desirability by homeowners/landscapers is well known. However, it is the alternate host of Asian citrus psyllids. The rapid proliferation and spread of psyllids, and subsequently the spread of HLB in Florida, was aided by the transport and sale of *Murraya* plants through the nursery trade. Accurate, rapid, and robust methodologies for the detection and removal of HLB infected trees and better disease management strategies are required to slow the spread of HLB.

One strategy to manage psyllid populations may be by genetic engineering of *Murraya* with genes encoding products deadly upon ingestion by psyllids. Deployment of such plants in the urban landscape would satisfy homeowner's desires for the attractive ornamental, while diminishing the potential urban/suburban reservoir of vectors and the HLB pathogen. However, to develop a transformation system for *Murraya*, a previously defined tissue culture system for plant regeneration is a prerequisite.

To standardize the protocol for in vitro propagation of *Murraya*, seedling stem segments were used as explants and cultured onto various tissue culture media supplemented with different concentrations of cytokinins and auxins in combination or alone to induce regeneration. Nineteen different tissue culture media were tested of which only two media gave satisfactory regeneration efficiencies of around 62% and 75%. Work is being carried out to standardize the protocol for the genetic transformation of *Murraya* using marker genes. This standardization will

enable us to first develop a reliable protocol for genetic transformation of *Murraya*, and then test genes encoding compounds/products that may be deadly upon ingestion by psyllids to produce a potentially toxic trap plant.

For questions and further details, please contact Dr. Fred G. Gmitter Jr.: 863-956-1151, fgmitter@ufl.edu

Methods for Killing HLB Infected Trees and Abandoned Groves

— Gene Albrigo

We are still carrying out limited research on ways to kill HLB infected citrus trees without removal as we wait for possible additional funding from the FCPRAC grant process. With the recent citrus tree census reporting that over 120,000 acres of abandoned citrus trees exist in the state, viable procedures to eliminate these trees and individual affected trees in commercial groves is perhaps more important than ever. Of particular concern is the recent report of active movement of psyllids from abandoned groves to nearby commercial groves (Stelinski, CREC). For the abandoned tree issue, an aerial spray test is scheduled by the South Florida Water Management District that will be conducted on an area scheduled for reversion to natural habitat. We have nearly completed tests of trunk application of herbicides to HLB infected trees in a commercial grove. Spray applications were made over several shallow cuts into the wood. Some of the treatments appear successful, but many herbicides did not have any noticeable effect compared to direct canopy sprays. A newer fumigant (methyl iodide)

is also being tested, and preliminary results suggest this may be a viable alternative to herbicide use. In general, tests are planned for winter periods to see if chemicals have the same efficacy during cold temperatures as they do during warmer periods with active tree growth.

For more information, contact Dr. L. Gene Albrigo, albrigo@crec.ifas.ufl.edu

**Growth Regulator Use for Controlling Excess
Citrus Tree Flushing**
— Tim Spann

Because psyllids feed on and require new flush to reproduce, one horticultural management strategy for slowing the spread of HLB is to reduce or eliminate excess flush. This past summer, greenhouse trials were initiated to test the efficacy of the plant growth regulator Apogee (prohexadione calcium, BASF Corp.) to control citrus tree growth. Apogee is currently labeled for and routinely used to control the growth of apple trees in high density plantings. Apogee is considered to be a growth retardant and not a growth inhibitor, which means that treated plants will still produce new flush, but less of it.

Our initial studies showed that Apogee reduced the growth of Swingle seedlings under greenhouse conditions similar to moderate drought stress. One application at 200 ppm was effective for approximately 6 weeks. Beginning in mid-August, three field trials using Apogee were initiated in a hedging trial in a commercial block of 'Hamlin' trees in Lake Alfred. Based on the greenhouse studies, Apogee was again applied at 200 ppm. The results of these field studies have been mixed. Apogee effectively reduced growth on both the north and south side of trees in one study, had no effect in another study, and only reduced growth on the south side of trees in the third study. These results may be due to the greater vigor of the field-grown mature trees compared to the greenhouse seedlings, suggesting that higher rates may be needed in the field. They may also point to a difference in sensitivity to Apogee between Swingle and Hamlin. Additional trials

with Apogee are planned for both the greenhouse and the field next season.

Additional trials are planned for next season using other plant growth regulators which are classified as growth inhibitors, meaning that they prevent vegetative growth for some duration of time. One of these products, NAA (naphthaleneacetic acid), has been used in citrus in the past to prevent trunk sprouts under the trade name "Tre-Hold." NAA-based products are still labeled for use in citrus if they prove to be effective at controlling growth in the canopy. Other products, such as dikegulac (Atrimmec, PBI/Gordon Corp.) or paclobutrazol (Bonzi, Syngenta), are routinely used to control vegetative growth of landscape trees and shrubs and greenhouse ornamentals. However, if such products are effective on citrus, considerable research will need to be conducted to test their safety on a food crop before they can be labeled for use.

For more information, contact Dr. Tim Spann, spann@ufl.edu

**Ongoing Developments on Exploiting Psyllid
Chemical Ecology for Improved HLB
Management**
— Lukasz Stelinski

Investigations of psyllid behavior and chemical ecology continue in Dr. Stelinski's laboratory. Following the discovery that a female-produced volatile sex attractant pheromone is produced by the Asian citrus psyllid, work has continued on its chemical identification. Postdoctoral researcher, Ebenezer Onagbola, in Dr. Stelinski's lab is hot on the trail of this pheromone, having already identified multiple components that show behavioral activity. The exact blend and ratio of the components have not yet been determined. Dr. Onagbola has also recently identified two components of the pheromone of the psyllid's main parasitoid species, *Tamarixia radiata*. Research is on-going to confirm whether this is the complete pheromone. Furthermore, citrus volatiles have been found to attract both sexes of Asian citrus psyllid, which has moved research towards discovery of plant-based

attractants. A recent breakthrough headed by Dr. Russ Rouseff, chemist at the CREC, in collaboration with Dr. Stelinski's lab has been the discovery of specific sulfur compounds that likely explain guava's repellent effects against the psyllid. Dr. Onagbola is finding that guava volatiles are both repellent and in some cases toxic to the psyllid. Candidate attractants and repellents are being tested that may be developed into practical pest control applications in the near future. The pheromone of the parasitic wasp *Tamarixia* will be used to monitor the establishment of this natural enemy in groves throughout Florida and to perhaps recruit these beneficial insects into groves to improve biological control.

For more information contact Dr. Lukasz Stelinski (stelinski@ufl.edu).

The "Hidden" Benefits of Systemically Applied Imidacloprid

— Lukasz Stelinski

Postdoctoral researcher, Raj Boina, is an insect toxicologist working in Dr. Stelinski's lab. Dr. Boina has recently uncovered many subtle, but very important physiological and behavioral effects of systemically applied imidacloprid at sublethal concentrations that are not high enough to kill psyllids. Such concentrations are commonly found in citrus trees after application of imidacloprid because of a) the variation in the spatial and temporal patterns of imidacloprid uptake and systemic distribution within trees; b) a time lag between application and the attainment of lethal concentrations within trees; and c) metabolic degradation of the pesticide over time within trees. However, Dr. Boina has found that the sublethal concentrations of imidacloprid negatively affect the survival, developmental time, longevity, and reproductive capability of Asian citrus psyllid that likely contributes to population reductions over time. Also, at the highest sublethal concentrations, Dr. Boina has found that imidacloprid substantially reduces psyllid feeding. These results are very encouraging and point to other potential positive impacts of soil applied imidacloprid at sublethal concentrations in addition to its

direct action of killing psyllids at lethal concentrations.

For more information contact Dr. Lukasz Stelinski (stelinski@ufl.edu)

Bridge between Research and Management of Citrus Greening

— Nian Wang

In 2007 and 2008, multiple projects were conducted in the Wang Lab to understand the citrus greening pathogen and the potential implication in controlling citrus greening. Consequently, five papers were produced from those studies on aspects related but not limited to the following areas: early diagnosis, bacterial diversity associated with greening diseased trees, distribution of the pathogen in citrus, and host response to the greening pathogen infection (One published, one in press, three submitted). Here the connection between the research and its potential use in citrus greening management is discussed.

Reliable and robust diagnosis is necessary. The current management strategy of greening is to remove infected citrus trees and reduce psyllid populations with insecticides. This strategy requires sensitive and reliable diagnostic methods for early detection. Symptoms have been the most convenient means of diagnosis and are widely used in the field. However, symptom based diagnosis could be confusing. A *Phytoplasma* sp. was reported to cause greening-like symptoms in citrus in Brazil. Some greening-like symptoms were also observed in Polk County without detection of the potential causal agent: *Candidatus Liberibacter asiaticus*. Economically, it makes sense by confirming the greening disease by other means including PCR and Quantitative PCR besides symptoms, rather than just cutting the suspect which might not be greening at all. Losing productive trees with some greening-like symptoms without confirmation is far more costly than a simple confirmation.

Early diagnosis is necessary and possible. Our results indicated that a minimum bacterial concentration was required for greening

symptom development in studying the population of the greening pathogen in symptomatic and asymptomatic leaves.

A speculation has existed between the threshold of the greening pathogen population and successful psyllid transmission. Thus, early diagnosis will provide means to limit the spread of the greening pathogen by psyllids by cutting greening diseased trees before the greening pathogen reaches certain population in the phloem. We have optimized detection sensitivity of the greening pathogen with different PCR based methods with primers/probe targeting 16S rDNA, beta-operon, or 16S rRNA. Preliminary data indicated that we could detect the greening pathogen up to 6 months before showing any symptoms.

What pathogens are associated with citrus trees showing greening symptoms? Our results revealed that citrus leaf midribs can support a diversity of microbes by a comprehensive study of two citrus groves in Florida. However, our data clearly indicated the dominance of the greening pathogen *Ca. Liberibacter asiaticus* in symptomatic leaves. These data strongly implicated *Ca. Liberibacter asiaticus* as the pathogen responsible for greening disease in Florida.

Where are the citrus greening pathogens? We found that '*Ca. Liberibacter asiaticus*' was distributed in bark tissue, leaf midrib, roots, and different floral and fruit parts, but not in endosperm and embryo, of infected citrus trees. Consequently, we recommend digging the whole tree out to prevent new flush from the cut greening diseased trees in the future.

New means to control citrus greening. It is challenging to control any disease whose pathogen lives in the phloem. Our previous data indicated that accumulation of callose and up-regulation of phloem proteins was associated with greening disease symptom development. Currently, we are investigating the possibility to reduce greening disease by manipulating callose and phloem proteins.

For questions and further details or paper requests, please contact Dr. Nian Wang: 863-956-1151, nianwang@ufl.edu

Economics Projects Evaluating Citrus Greening

—Ron Muraro

Proposals for four projects dealing with the economic implications of citrus greening have been submitted to the Florida citrus production research advisory council for funding. These are: (1) "Survey to Estimate the Rate of Greening Infection in Florida Citrus Groves"; (2) "Long-Run Processed Orange Production and Price Impacts Associated with Citrus Greening in Florida and Sao Paulo, Brazil"; (3) "Economic Evaluation of Alternative Replanting Production Systems." and (4) "Long-Run Structural Implications of Citrus Greening in Florida." Researchers working on one or more of these projects include Tom Spreen, professor, Food and Resource Economics Department, UF/IFAS; Ron Muraro, professor and farm management economist, CREC; Allen Morris, associate extension scientist and agribusiness economist, CREC; Fritz Roka, associate professor of Food and Resource Economics, SWFREC; Mark Brown, research economist, and Robert Norberg, deputy executive director of research and operations, FDOC; Richard Gaskalla, director and Mark Estes, environmental administrator, Division of Plant Industry, FDACS; Jim Ewing, deputy director, and Candi Eric, agricultural statistics administrator, Florida Agricultural Statistics, National Agricultural Statistics Service.

The first project is a survey of Florida citrus groves conducted as part of the FDACS Division of Plant Industry's CHRP plan survey of greening and canker management practices. The objective of this (IFAS) part of the project is to develop reliable estimates of the degree that greening currently infects Florida citrus groves (as measured by symptomatic trees), the rate it is spreading, and the extent that growers are following management practices to slow its spread. Data gathered from this joint FDACS-IFAS survey will be statistically expanded to provide estimates for each of the five citrus

growing regions and state totals by the National Agricultural Statistics Service. Results from this study will provide the basis for long-run production and price forecasts for processed citrus and evaluation of long-run structural impacts of citrus greening on the Florida citrus industry. These estimates should also be useful to other research that seeks to determine various future impacts of greening on the Florida citrus industry. Allen Morris is the principal investigator. Collaborators include Tom Spreen, Ron Muraro, Richard Gaskalla, Mark Estes, Jim Ewing, and Candi Eric.

The second project will utilize results from this greening survey in Florida and estimates of the incidence of greening in Sao Paulo from surveys conducted in Brazil to identify plausible scenarios of production impacts from citrus greening and incorporate these impacts into a model of the world orange juice market. Orange juice supply regions in the model will be Sao Paulo, Brazil and Florida, and demand regions will be the United States, Canada, Europe, and Japan. Scenarios to be developed will consider alternative tree survival rates, various demand growth assumptions, and the possible impact of greening on new tree planting. Expected output from the study includes projections of orange production and processed orange prices in the two supply regions and consumer impacts in the demand regions. The principal investigator is Tom Spreen. Collaborators are Mark Brown, Ron Muraro, and Allen Morris.

The third project evaluates replanting groves to higher tree densities as a strategy to offset increased tree attrition and thus increase the economic life of future groves infected with greening. Two alternative high-density planting production systems will be analyzed: (1) tree densities of 33% to 100% higher than traditional tree densities using microsprinkler irrigation along with traditional management programs and (2) an open hydroponic system at more than double traditional tree densities where nutrients and water are precisely managed through a drip irrigation system. Capital requirements, operating costs, fruit yields and production, additional costs required

for citrus greening management, increased tree loss and removal due to greening, revenues, and other variables will be analyzed for each production system. Break-even prices, time required to break even, and internal rates of return on investment will be evaluated and compared to a traditional lower-density planting system also experiencing tree attrition from greening and under a greening management program. The principal investigator is Ron Muraro. Collaborators are Allen Morris and Fritz Roka.

The fourth project will integrate the results from these other three studies and consider the consequences of endemic citrus greening on the infrastructure of the Florida citrus industry including citrus processing capacity, fresh packing capacity, associated input supply industries, and the economic impact on communities located in citrus producing regions of Florida. The expected results of this project address a fundamental issue confronting the citrus industry: what is the long-run impact of endemic citrus greening on the Florida citrus industry and its size and economic performance in Florida? The principal investigator is Tom Spreen. Collaborators are Ron Muraro, Allen Morris, and Bob Norberg.

If funded, these studies can have wide-ranging implications for all participants in the industry including research agencies such as ARS-USDA and UF/IFAS, organizations such as FDOC and Citrus Mutual, legislators and policy makers, input supply companies, lenders, and processing and packing companies that may be facing significant decisions regarding product supply. Estimation of the true economic cost of citrus greening also has implications with respect to the allocation of research dollars to confront it.

For more information, contact Ron Muraro, rpm@ufl.edu

Progress towards Incorporation of Antimicrobial Peptides for the Development of HLB/Canker Resistance in Commercial Citrus

—Manjul Dutt, Ahmad Omar, Vladimir Orbovic, Gary Barthe, and Jude Grosser

What are antimicrobial peptides?

Antimicrobial peptides are usually small proteins, usually 12 and 50 amino acids long. They form the first line of host defense against pathogenic infections and are a key component of the innate immune system. Antimicrobial peptides are involved in the antimicrobial defense system among all classes of life.

Why use antimicrobial peptides? Antimicrobial peptides have broad spectrum antimicrobial activity. Antimicrobial peptides act by disrupting the structure or function of microbial cell membranes. Antimicrobial peptides are usually bacteriocidal (bacteria killer) instead of bacteriostatic (bacteria growth inhibitor).

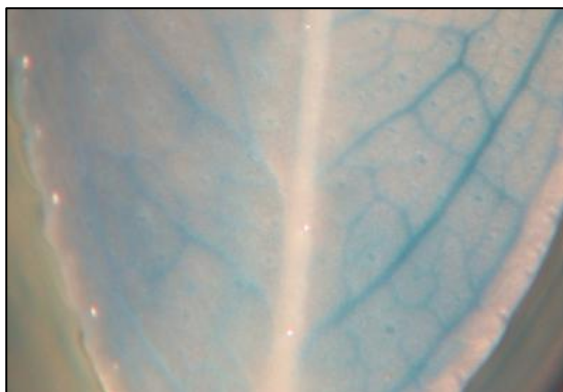
Our research objectives: (1) Design codon optimized antimicrobial peptide genes for citrus to combat HLB and Canker; (2) Target trans-protein in phloem tissue where HLB resides; (3) Produce a large number of transgenic lines using Agrobacterium and protoplast mediated transformation; and (4) Challenge plants with disease causing HLB and canker bacteria to evaluate resistance.

Antimicrobial peptides and HLB/canker: Both diseases are caused by gram negative bacterium. No natural resistance exists in any known commercially cultivated sweet orange, grapefruit, or tangerine. Antimicrobial peptides have been shown to provide resistance to bacterial diseases. The LIMA gene has been shown to control *Xylella fastidiosa*, the causal organism for Pierce's disease in grapes (Dennis Gray, MREC, UF/IFAS). This same gene can theoretically be used to combat both HLB and canker. Several other promising constructs are also being tested.

Transgenic Citrus Plants Produced Containing Antibacterial Constructs (All Are Being Propagated by Micrografting for HLB and Canker Disease Challenge Assays)

Cultivar	Gene	No. of plants in soil
Duncan	AttacinE	27
Hamlin	AttacinE	15
Misc Grapefruit	LIMA	43
Valencia, Hamlin, OLL-8	LIMA	56
Carrizo	LIMA	8
Misc Grapefruit	PTA	12
Valencia, Hamlin, OLL-8	CEMA	20
Carrizo	CEMA	20
Key Lime	CEMA	6
Misc Grapefruit	CEME	14
Hamlin	CEME	6
Valencia	CEAD	6
Carrizo	CEAD	12
Carrizo	LIMA under AtSuc2 promoter	25
Valencia, Hamlin, OLL-8	LIMA under AtSuc2 promoter	23
Key Lime	LIMA under AtSuc2 promoter	17
Misc Grapefruit	LIMA under AtSuc2 promoter	12

Use of phloem specific promoters to target foreign gene expression in the phloem tissue, where HLB resides: HLB resides in the phloem. Targeting the trans-protein in the phloem can minimize issues regarding the subsequent presence of the protein in the fruit and juice, thus facilitating FDA approval. Two phloem specific promoters are currently under evaluation and working well in citrus: (1) *Arabidopsis* (mustard) Sucrose synthase promoter; and (2) Rice Sucrose synthase promoter.



GUS expression in citrus leaf phloem tissue using the Rice Sucrose Synthase promoter – shows targeted foreign gene expression only in the phloem.

Transgenic plant challenge via grafting with HLB infected budwood: Transgenic plants are being graft challenged with HLB infected sweet orange budwood. Transgenic plants are evaluated for HLB symptoms and infection that is verified by qRT-PCR. The first Duncan grapefruit plants containing the LIMA construct were challenged over 14 months ago, and some are showing no symptoms and remain PCR-negative, whereas the inoculum budstick remains PCR-positive (see photo top right). Some of the same plants are also showing resistance to citrus canker. Plans are underway to test these and all the other mentioned transgenic plants in field tests under heavy HLB and canker pressure, as necessary to validate any resistance.



Transgenic Duncan grapefruit containing the LIMA construct showing putative resistance to HLB 14 months after inoculation with HLB.

THANKS!

This research is supported by a block grant from FCPRAC provided to the UF Citrus Improvement Team (Fred G. Gmitter, Jr., Jude W. Grosser, William S. Castle, and Gloria A. Moore) entitled: SURVIVING HLB AND CANKER: GENETIC STRATEGIES FOR IMPROVED SCION AND ROOTSTOCK VARIETIES. The authors also thank Ron Brlansky, Jim Graham, and Marta Francis for assistance with disease screening; and the UF Core Citrus Transformation Facility for producing some of the transgenic plants.

For more information, contact Dr. Jude Grosser, jgrosser@ufl.edu

Identification of Metabolite Profile Changes in ‘Valencia’ Orange Leaves Induced by HLB

— Dr. José Reyes-De-Corcuera

Over the past 14 months Dr. Reyes-De-Corcuera’s team has worked on the determination of changes in metabolite profiles in ‘Valencia’ orange leaves caused by HLB using high performance liquid chromatography-mass spectrometry (HPLC-MS), capillary electrophoresis (CE), and gas chromatography-mass spectrometry (GC-MS). HPLC-MS untargeted analysis revealed about 190 compounds that differ in concentration between HLB-infected and healthy orange leaves. These compounds were grouped by

mass-to-charge ratio into three major groups. Two of these groups showed significant increase in overall concentration while the other showed significant decrease. The extent of change was correlated with an arbitrary scale of intensity of symptoms. CE experiments under optimized extraction and separation conditions consistently revealed significant differences in 6 of 24 compounds. GC-MS experiments revealed changes in several sugars and sugar alcohols, amino acids, phenols, and organic acids. Dr. Reyes-De-Corcuera's team is now starting the development of an amperometric enzyme biosensor that targets one of these metabolites while validating the correlation between its presence in HLB infected trees and not produced under other plant stresses. CE results were recently submitted for publication to "Electrophoresis." Most of the research results have been presented at the 2009 annual meeting of the Florida State Horticultural Society and at the 59th Citrus Processor's and Subtropical Technology Conference.

For more information, contact Dr. José Reyes-De-Corcuera, jireyes@ufl.edu

EVENT UPDATE

Florida Ag Expo

November 5, 2008

UF/IFAS Gulf Coast Research and Education Center, Balm, Florida 33503

For more information and registration, visit

<http://www.floridagrower.net/flaevents/index.html>

International Research Conference on HLB: Reaching Beyond Boundaries

December 1-5, 2008, Orlando

For more information and registration, visit

http://www.doacs.state.fl.us/pi/hlb_conference/