Title:	Rear and Release Psyllids as Biological Control Agents - An Economical and Feasible Mid-Term Solution for Huanglongbing (HLB) Disease

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Project Director

Thomas Turpen
469-371-2608
catp@citrusrdf.org

Recipient Organization

CITRUS RESEARCH AND DEVELOPMENT
700 EXPERIMENT STA RD
Lake Alfred, FL 338502243
DUNS No. 961745697

Performing Department

{NO DATA ENTERED}

Co-Project Directors

Burns, Jacqueline
Polek, Mary Lou
Browning, Harold
Patt, Joseph
Shatters, Robert
Pelz-Stelinski, Kirsten

Departments

Citrus Research&Education Ctr.
{NO DATA ENTERED}
Agricultural Research Service
Subtropical Insects Research

Non-Technical Summary

This proposal presents research targeting the elimination of Huanglongbing (HLB) as an economic threat to US citrus production by blocking the ability of the psyllid insect to move the causative agent of this disease between infected and healthy trees. The primary long term goal of this project is to interfere with the spread of HLB within citrus orchards where HLB disease is established and to interfere with the invasion of disease organism into areas where the insect that transmits the causal agent is established, but in which HLB has not been detected, by strategically releasing a nuPsyllid population that is incapable of moving the disease. A further goal is to ensure the necessary adoption of the method by the social system of growers, and understanding and acceptance by consumers and the general public. Once established, this novel system of biological control would be operationally transferred to the citrus industries of U.S states (Florida, California, Texas and Arizona). Other ongoing support, if necessary, will be provided by the stakeholder organizations. We believe current management practices are not sustainable, and in any event psyllid vector eradication has never been achieved, except on small islands. Alternative HLB-management approaches must be developed as a mid-term solution to the HLB problem. Without control measures in hand, citrus growers have no incentive to replace infected trees or to replant entire orchards. The uncertainties associated with HLB will undermine the stability of the industry in currently HLB-free areas. A mid-term solution is crucial to maintain a profitable industry until citrus varieties with resistance to HLB can be developed and released. Therefore, we present a novel and more environmentally friendly alternative strategy, which we will convey to growers and the public. Grower response to this disease has resulted in a mix of increased costs, modifications to long-successful production management systems and acceptance of at least short-term yield and/or quality reductions. Total orchard loss is increasing as HLB spreads. The current situation suggests that without development of an adequate control strategy, commercial citrus production will become economically unfeasible. This disease also is impacting the millions of citrus trees grown in homeowner yards.

Accomplishments

Major goals of the project

The primary long term goal of this project is to interfere with the spread of HLB within groves where HLB is endemic and to interfere with the invasion of CLas into areas where ACP is established but HLB has not been detected. Once released and established, the nuPsyllid population will naturally penetrate and displace the wtPysllid population. A further goal of this proposal is to ensure the necessary adoption of the method by the social system of growers, consumers and the general public in citrus states. To achieve these goals, we propose a three-fold approach: 1). Develop a psyllid management strategy based on the development of psyllid populations incapable of transmitting CLas (nuPsyllid) and strategically release the nuPsyllid population to displace current ACP populations that have invaded the US. 2). Provide optimized management strategies for integration of the proposed population displacement technique into current management practices: a. Southeast and Southern U.S. (FL and TX) where both the ACP and CLas are endemic. b.Western U.S. (CA, AZ) where ACP is present and spreading while there is currently no detection of HLB. 3). Integrate the management strategies with monitoring strategies to continually assess effectiveness and provide outreach education to the grower stakeholders and citizens about the control strategy. The feasibility of the approach proposed here is supported by the experience with HLB management in Florida through the creation of Citrus Health Management Areas (CHMAs). The CHMA is based on the recognition that HLB has an important edge effect. Although insecticide applications can control ACP populations within the grove, without effective ACP management in the surrounding areas, CLas-bearing ACP rapidly returns. A CHMA coordinates the insecticide sprays and other management activities over a large area, thus greatly reducing the edge effect of ACP re-invasion. Thus, we are proposing that most nuPsyllid releases will be focused at the periphery of CHMAs and other smaller management areas to displace the endemic population at the periphery. Vigorous ACP control measures would be continued temporarily in the interior of the management area but would be gradually tuned down to allow populations of CLas-transmission-deficient ACP populations to become established

What was accomplished under these goals?

The purpose of this NIFA-CAPS is to create options for management of HLB by replacing the wild type insect vector (ACP) with a population that is unable to transmit the bacterial causative agent (CLas). Achieving this outcome will require progress in the following three areas of emphasis – An Effector Mechanism, A Driver System, and Diffusion.

Effector Mechanism – Initial assessments have not identified the required variation in CLas transmission to occur naturally in ACP populations. However the prospects for engineering a mechanism to achieve the desired phenotype are under active investigation. The effector is the content of the phenotypic change we aim to introduce. Candidate effectors are being identified through multiple parallel methods of investigation including bioinformatics, proteomics, yeast two-hybrid (Y2H), peptide-ligand and scFV-ligand libraries. An Effector Workshop with the participation of Team Leaders Bob Shatters and Judy Brown, Project Directors and additional collaborating scientists was held in Ft. Pierce, FL in Dec. 2013.

- There is a growing list of candidate effectors generated from bioinformatics (proteomic and transcriptomic), genetic (yeast two-hybrid) and physical methods (Far-Westerns). In the case of the yeast two-hybrid method numerous candidates are being tested reciprocally to confirm results. This workflow of the Effector team has already generated more high quality targets than can be analyzed in bioassays. In many cases loss of gene expression through RNAi is highly toxic to psyllids. We have only conceived of two tools to use to disrupt the Effector Mechanism, RNAi and competitive protein ligand inhibitors. Secondary metabolites or RNA aptamers are potential additional options. In order to use an Effector for insect replacement, we need to disrupt these interactions while maintaining psyllid fitness.
- The extensive transcriptome data set (the Transcriptome Computational Workbench) has been created from whole adults and nymphs as well as dissected salivary glands and guts of insects infected or not infected with CLas is now available to the research community at www.sohomopter.org/ACPPOP.
- Using the yeast two-hybrid method, gut and salivary gland library matings (24/ea) have been performed and the results reinforce the likely role of several complexes and individual proteins identified in the level 2 biological processes of the Gene Ontology categories of Adhesion, Nutrition, Invasion and Immune functions. Using RNAi knockdowns at least 5 of these targets show a significant reduction in CLas transmission in a functional transmission bioassay.
- A system for screening peptide libraries for protein ligand inhibitors was established based on the elements listed below. These results led to the decision to use 4-amino acid peptide libraries for the screening program.
- Several peptides have been identified from multiple experiments that bind specifically to digestive tract membranes at submicromolar concentrations. These results were corroborated with live adult and nymphs fed on artificial diet or leaves containing the biotinylated test peptides.
- scFv antibody to CLas surface antigens, FlhA and OmpA have been purified in quantities that will enable tests for effects on CLas transmission in a functional bioassay.
- Citrus flush takes up peptides in xylem and phloem when leaf petioles are placed in solutions containing the peptides. About 20% of the psyllids (nymphs or adults) that have fed on these leaves take up these peptides and these peptides can be detected throughout the alimentary canal. Previous work on Asian citrus psyllid feeding has shown that about 20% of the time they feed on the xylem. Peptides introduced by this method are found primarily in the xylem.
- A new diet feeding strategy allows nymph development from early instars through to adult emergence. This will enable

transmission blocking studies with a system that reflects the most efficient acquisition of CLas in the natural system.

Driver System – A new trait will not spread efficiently upon release within an existing population without a genetic bias of some kind. The driver is the medium of spread of the introduced phenotype--lack of CLas transmission. The drivers under investigation are viral, endosymbiont and chromosomal.

- Three viruses of ACP were discovered by deep sequencing and bioinformatic analysis of small RNA libraries from diverse ACP collections from 18 locations around the world. These viruses include a Reovirus (dsRNA), two Iflavirus-like isolates (+sense, ssRNA) and a Densovirus (ssDNA). Because the Ifla-like viruses are most readily manipulated as a gene vector, efforts are now focused on obtaining the full genome sequence.
- To date, RNAs have been extracted from 33 different ACP populations collected from 9 locations around the world.
- The Ifla-like viruses discovered in this work may be members of a new genus because parts of the genome show highly significant similarity to known members of the genus Iflavirus whereas other regions are more similar in genome organization to the genus Dicistrovirus.
- Over 95% of the genome appears to be cloned and efforts are now focused on identifying and confirming the 5' termini of each of the genomic RNAs to enable a recombinant vector system to be implemented for reverse genetics.
- There are significant regional differences in Wolbachia infection levels and sequence types among Florida psyllid populations assessed using multi locus sequence typing (MLST). All investigated D. citri populations harbor wDi from supergroup A.
- A qPCR assay was developed for ACP Wolbachia using a primer pair for the wsp gene.
- Artificial feeding and microinjection systems have been developed to introduce Wolbachia into ACP for the purpose of screening psyllids for vector and host competence. Inoculation of ACP with new Wolbachia infections must occur during the nymphal stage in order for the infection to establish and be maintained in the insect.
- Cell cultures of a range of Wolbachia strains have been established and novel Wolbachia strains have been introduced into ACP at the nymphal stage. Infected adults will be mated so that progeny can be evaluated for subsequent transmission, CI, and fitness assays. Using this system, foreign Wolbachia infections in ACP have been confirmed in adult ACP via PCR.
- Proof of concept has been established in a model system for chromosomal-based gene drive systems for population replacement in the psyllid. However, the challenge with single or two-locus underdominance is to fully rescue the siblings that should be protected by the antidote in an engineered toxin-antidote system.
- A third drive mechanism, engineered translocations has been constructed and implemented in a model system and future work will focus on this relatively high threshold system that will feature genetic containment and likely public acceptance advantages.
- This system shows great potential for ACP-HLB control because it should be readily transferrable once ACP can be transformed and is robust to mutations anticipated to inactivate drive while genes of interest can be easily linked to the translocation breakpoint.

Diffusion – Once a nuPsyllid population is developed, its successful use will depend on series of factors based on the overall phenotype and fitness of the population in the environment and most importantly, will depend on human adoption, including the behavior of regulatory agencies, growers and consumers. All of these attributes must be modeled accurately for a nuPsyllid release to be used effectively. As for any other innovation, diffusion is the rate of change.

Several aspects of the technical and communication plan can be addressed most effectively only when an actual candidate nuPsyllid is available for release. The ability to rear, release and monitor psyllids has been initiated and is of immediate use in HLB disease management applications outside of this proposal.

What opportunities for training and professional development has the project provided?

Elements of this project are being conducted in University and USDA laboratories in a number of states. These sub-projects are providing considerable professional training to undergraduate and graduate students through direct involvement in the nuPsyllid project. In addition, the project employs a number of Post-Doctoral trainees in the labs, whose contributions to the research objectives serve also to provide them additional professional training. All involved in this project are being exposed to the approaches and mechanics of team research on a large scale, and team meetings involve shared experience on how component research objectives fit into the larger picture.

How have the results been disseminated to communities of interest?

Numerous team meetings of team leaders and various collaborators have occurred. Interim annual progress and project overviews were presented to the Scientific and Regulatory Advisors and to Tom Turpen, Project Director and Mary Lou Polek, Administrative Team Co-Director in a Team Leader Annual Meeting on May 12th and 13th in Dallas, TX. The team discussed revisions to the current technical plan based on the progress achieved, as well as budget to plan variances. Presentations on the project goals and objectives, as well as progress to date have occurred at meetings of the citrus growers in California, Texas and Florida numerous times during the year. Presentation and discussion of the nuPsyllid project has occurred during

telephonic meetings as well, notably There is a substantial effort to rear and release any type of nuPsyllid under development. In July this group organized conference call and discussed the following key topics:

- Florida, Texas, and California will each develop and maintain its own colony to provide nuPsyllids for initial greenhouse studies and pilot field releases within its borders. The decision as to where to house nuPsyllid colonies within each state will be likely have to be made at several administrative levels.
- Regulatory agencies will likely require that nuPsyllid colonies be housed in a controlled/quarantine facility. Potential sites in each state were identified.
- An estimated population size for a nuPsyllid required for testing cannot be provided until the driver mechanism is selected. The effector mechanism may have associated fitness costs, as well, and these will have to be figured into rearing effort estimates.
- The design of release and monitoring studies will also need to be postponed until the driver and effector mechanisms are selected. Modeling studies that will help predict the spread of nuPsyllid have been developed and are being refined and validated with historical datasets on HLB spread.

The Outreach Team met and determined that 1) because the effector and driver systems are all progressing equally it will not be possible to eliminate one or more from the outreach efforts and 2) an educational program should focus on the context of genetic technologies in general so that the nuPsyllid option for disease management is contrasted for example with a genetically modified citrus host. Details of the nuPsyllid project have been made available for public consumption through inclusion in trade journal articles, through the nuPsyllid web page and through other mechanisms, including newsletters of the CRDF.

What do you plan to do during the next reporting period to accomplish the goals?

The proposal plan will continue to be followed, focusing on parallel development of effectors and drivers. Additional attention to areas which can be advanced through complementary funding also are being evaluated within the nuPsyllid Team, and Advisors. A comprehensive Annual meeting that will include all researchers associated with the project has been announced for a day just in advance of the upcoming HLB International conference in Orlando, Feb. 9-13, 2015. The priorities in the coming year are:

- select and prioritize effectors;
- obtain antibody reagents for top effector candidates;
- use the bioassay platform based on artificial nymph diet for comparative testing of the phenotypes in ACP, maximizing transmission blockage and minimizing fitness loss;
- accelerate development of a viral vector because this is likely to be the first tool for genetic manipulations and would be immediately useful for effector prioritization;
- analyze the phenotypes of non-native Wolbachia introduced into ACP;
- determine if Wolbachia transformation is a feasible goal;
- develop ACP transformation capacity at any level of efficiency;
- continue to ready the engineered translocation constructs;
- begin to model the logistics of rearing and releasing nuPsyllid around hypothetical specifications and explicit assumptions;
- engage the grower community in a broad educational outreach to raise awareness of the alternatives for genetic technologies in the management of HLB.

Participants

Actual FTEs for this Reporting Period

Role	Faculty and Non-Students	Students within Staffing Roles			Computed Total by Role
		Undergraduate	Graduate	Post-Doctorate	
Scientist	4.3	0	1	5.7	11
Professional	0.5	0	0	0	0.5
Technical	5.6	0	0	0	5.6
Administrative	0.3	0	0	0	0.3
Other	0	0	0	0	0
Computed Total	10.7	0	1	5.7	17.4

Target Audience

Target audiences include the primary benefactors of the research, the U.S. citrus growers. In addition, the target is the scientific community who is engaged in developmental research that has allowed this research project to be envisioned, and on whose progress we will continue to move forward. The general public is a target of our outreach. As consumers, they are interested and concerned about how research solutions are implemented to solve practical problems, and have shown interest in the foundations of this research project. Finally, policy-makers who often are involved in funding research for Florida citrus, need to be appraised of the project, its goals, and expectations that come from progress. Together with molecular biologist and extension expert, Peggy Lemaux, two grower seminars were developed and presented in Santa Paula, CA (June 27, 2014) and Exeter, CA (July 17, 2014) entitled "Food fights in the marketplace: Is there a way to use genetics to address HLB disease in citrus?" Based on the results of a questionnaire presented to this audience of over 100 growers, it appears that the California segment of the citrus industry represented by this sample, demonstrates strong acceptance of genetic solutions but remain concerned about public acceptance and government approvals. This audience was split on whether genetically modified trees or insects were preferable.

Products

{Nothing to report}

Other Products

Product Type

Other

Description

Novel Biopesticides - Technology assessments have suggested a near term application of this research for the protection of new plantings. This concept “Psyllid Shield” is being evaluated for field trials. While it is not full insect replacement, it is based in part on research progress in the search for Effectors. CRDF has supplemented funding to model and assess the minimum field trial plot size and time required to demonstrate efficacy in protecting new solid block plantings from HLB with RNAi.

Changes/Problems

Like many new projects of this magnitude communication and reporting between the 15 institutions involved in this project was not simple. The project meetings and progress reports indicate that, despite this complexity, the project is on target with its timeline of objectives. There are no major changes to the project in terms of approach and none are anticipated. CRDF, as the primary on this project, requires quarterly written progress reports on its funded projects, and we have included this term in all nuPsyllid project subcontracts, although it is not required under the NIFA terms and conditions. Consequently, we are receiving and posting these brief progress reports which are generated by each participant and coordinated through the team leaders up to a collective quarterly progress report which is submitted by the Project Director. We feel this keeps the team members focused on the goals and allows us to communicate regularly on progress.