Florida Citrus Advanced Technology Program

QUARTERLY & FINAL REPORTS: Control of Citrus Greening, Canker & Emerging Diseases of Citrus

Instructions Complete the fields based on your project specs. When finished, save the form to your local disk using a unique name. Then, go to **http://research.fcprac.com**, and log in with your user name and password using Researcher Login in the lower left. Find this project title and click on **Submit a Report**. Update your profile information if needed, then upload this report as directed.

2009-2010 REPORT	CATEGORY (drop-down)	TODAY'S DATE (m/d/yr)
○ *Quarterly Report* ◉ *Annual Report* ○ *Final*	HLB Pathology	3/11/10

WHAT IS THE "HEADLINE" FOR THIS REPORT (e.g. a one-sentence "newspaper headline" describing what you accomplished)

In vitro culture of LAS in association with insect feeder cells. Primo-cultures and improved cultivation.

TITLE and CONTACT INFORMATION

Proposal Title In vitro culture of the fastidious bacteria Candidatus Liberibacter asiaticus associated with Citrus Greening (Huanglongbing or HLB) Disease.

Principal Investigator DOLLET Michel	*PI Last Name* Dollet
Email michel.dollet@cirad.fr	*FDACS Contract Number* 48
Phone (33)4 67 59 39 22	*Project Duration (years)* 2 *Year of Project* 1
Organization CIRAD, Montpellier, France	*Total Direct Funds (current year)* $95,313.00

REPORT UPDATE (650 words; provide details about your headline)

We want to obtain a pure culture of Ca. Liberibacter asiaticus (LAS) by first co-culturing the bacteria with insect cells. The strategy consists of primocultures of the bacteria in insect cell cultures used as feeder cells. For this annual report our project objectives are described and discussed below.

Objective 1: LAS inoculum. LAS source materials are from infected symptomatic citrus trees from Vietnam. Transmissions from citrus to citrus or from citrus to periwinkles are performed via the insect psyllid Diaphorina citri and subsequent grafting. A maceration method was found to be the most appropriate way to release the bacteria in the insect cell cultures. Antibiotics are used to select for Gram negative bacteria.

Objective 2: Primo-cultures. Various insect cell cultures in various culture media were tested.

-We didn't detect any Las in Mamestra (hemocyte, ovarian cells) or Spodoptera (hemocyte cells) cell lines after inoculation.

-We detected LAS in two lines of drosophila cell cultures by direct PCR after inoculation. One line lost the detection after 6 subcultures. For the second line, positive PCR signal was obtained up to the 20th subculture, then LAS detection troubles occured due to high insect cell density and we detected the presence of another bacteria (Bradyrhizobium).

We started new LAS primo-cultures with much less drosophila cells and got LAS positive cultures for some combinations of cell line/culture medium, still positive after 5 weeks and successive dilutions (confirmed by sequencing).

-First attempt to co-culture LAS with an Aedes albopictus insect cell line failed. However, adding an antioxidant and osmoprotectant at the time of inoculation allowed us to get LAS positive Aedes cultures still positive after 9 weeks and successive dilutions (confirmed by sequencing).

We are currently adapting conditions of described LAS qPCR detection to quantify LAS in insect cell cultures. We can detect LAS bacteria by qPCR but more efforts are needed to quantify bacteria:insect cell ratio with a multiplexed qPCR approach.

Our objectives 3 and 4 are aiming to improve culture conditions to get a higher bacterial titer and to free the co-cultures of insect cells (axenization).

Objective 3: Axenization. LAS/Aedes primo-cultures were obtained at a low insect cell concentration. We are progressively diluting the concentration of insect cells through each new passage .

Objective 4: Medium optimization. To maintain the bacteria for a longer period of time and to reach higher bacterial concentration, we started complementing the primo-cultures with various sugars, vitamins described in citrus/periwinkle phloem. In parallel we analyzed metabolic pathways potentially encoded by the released Liberibacter genome sequences to define limiting factors and/or growth inhibitors. Of the complements tested we selected sodium pyruvate, proline and fructose for their positive effect on the bacteria detection and they now are systematically assayed on our new inoculations. We are analyzing sugar, amino-acids and minerals and trace elements variations in insect cell culture media over culture time to identify potential LAS growth limiting factors.

We are reaching our milestones for the first year of this project with significant progress on objectives 1, and 2 (inoculums, primo-cultures). For objective 2, we will test new insect cell lines. As at least two laboratories involved in the FCPRAC program established Diaphorina citri cell lines, we will request for these lines to trial our inoculation protocol and try to obtain LAS/ D. citri co-cultures. We are now fully involved in objectives 3 and 4 (axenization/medium improvement). If they succeed in time, we will attempt to inoculate cultivated bacteria through Diaphorina citri to Citrus trees (objective 5).