CITRUS ADVANCED TECHNOLOGY PROGRAM

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

▶ INSTRUCTIONS

○ *Quarterly Report* ○ *Annual Report* ● *Final*

Required: What is the "headline" for this report (e.g. a one-sentence "newspaper headline" describing what you accomplished)

In vitro culture of Candidatus Liberibacter asiaticus

Proposal Title

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

Today's Date	Sponsoring Organization (drop-down)	Category (drop down)
5/25/12	Citrus Research and Development Foundation	Culture, Molecular Biology, Koch's Postulates

REPORT UPDATE (500 words; summarize your accomplishments)

The aim of this project was the attempt to in vitro culture the bacterium associated with the Citrus Greening disease: Candidatus Liberibacter asiaticus (LAS). The strategy was anchored on the use of insect cells cultures as feeder cells for the primocultures of LAS.

The infected plant material originated from Vietnam and maintained in greenhouse by grafting and by Diaphorina citri transmission. This LAS strain was identical to the LAS strain occuring in Florida according to its 16s rDNA. Another aspect of the strategy was the use of LAS infected periwinkles in which LAS was transferred by D. Citri and maintained by grafting. The highest concentrations of LAS were obtained in periwinkles.
We used several cell lines from Lepidoptera (Mamestra brassicae, Spodoptera littoralis, S. frugiperda, Lymantria dispar), Diptera (Aedes albopictus, Drosophila melanogaster) and Hemiptera (D. citri). For Spodoptera and Drosophila, "commercial lines" adapted for recombinant protein production and "laboratory lines" were used. The main culture media were: Grace insect cell medium, LM 15 and Drosophila medium.
The first step concerned the inoculum. This was obtained from Citrus or periwinkle after surface sterilization with ethanol and 1% sodium hypochlorite, and addition of proline (10mM) or/and sodium pyruvate according to the cell line used and 5 fluorocytosine. The best plant for primocultures depended of each cell line (e.g. periwinkle for A. albopictus).
Lepidoptera cells did not provide transferable cultures. The same for D. citri cells (no more than 3 transfers).
Diptera cells allowed the best results. LAS could be maintained for 4 to 8 passages (every 7-10 days) in "commercial" Drosophila cells, but LAS was lost, overgrown by the fast growth of the insect cells. "Laboratory line" D-S2 allowed much better results: up to 20 transfers. From 10 to 19 transfers of LAS could be obtained when using Aedes cells for primocultures. After successive dilutions we could get rid of of insect cells without losing LAS detection. To verify that the LAS culture was axenic, we checked by PCR if LAS was the only bacterium. Other bacteria were detected in several cultures when using Aedes/periwinkle system (mainly, actinobacteria and Delftia acidivorans). Strong antibiotic selection was applied to remove the contaminants but this resulted in the loss of LAS signal. No contaminants were identified when using Drosophila/Citrus system and we conclude that we obtained a true axenic culture of LAS. Our strategy based on feeder cells proved to be very reproducible.
We tried to freeze the obtained cultures with addition of glycerol or DMSO. Unfortunately it has been impossible so far to freeze and thaw the cultures.
Recently, in order to fulfil the Koch's postulates, we inoculated healthy Citrus plantlets with LAS from "axenic cultures" (cultures from the Drosophila system at a stage when no other bacteria was detected) and with culture orginating from Aedes cells system even if we knew there was a contaminant. Two protocols were used: mechanical inoculation despite the fact that never a phloem-restricted microorganism has been re-introduced mechanically in the sieve tubes of phloem by this way; or by D. citri, after acquisition through membrane. These inoculated plants are under observation for HLB symptoms in the greenhouse and will be checked by PCR from time to time.

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Organization CIRAD

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Total Funds (current year) $88.60

SUBMIT REPORT

CITRUS RESEARCH AND DEVELOPMENT FOUNDATION, INC.