4.11 Production of Asian Citrus Psyllid (*Diaphorina citri*) cell cultures

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We successfully developed Asian citrus psyllid cell cultures as a tool to subculture *Canidatus* Liberibacter asiaticus (Las), which is a fastidious bacterium that reportedly replicates within the psyllid host. This novel approach uses psyllid cell cultures as the medium to isolate and culture this bacterium to permit further research. The Asian Citrus Psyllid (AsCP), *Diaphorina citri*, (Hemiptera: Psyllidae) is a highly competent vector of the phloem-inhabiting bacterium (Las), associated with the disease Huanglongbing (HLB). World-wide HLB has become a major limiting factor to the production of citrus. A specific psyllid cell culture medium was developed during the screening of 12 commercially available insect cell culture media. Attempts were made using starter cells from embryos and midgut tissues. Cells and tissues could be maintained for up to 4 months. The developed psyllid culture medium, Hert-Hunter-70, provided the best growth rates with doubling times at ~ten days. Previous attempts to use a lepidopteran cell culture, *Spodoptera frugiperda* (Sf9), to culture Liberibacter were unsuccessful, suggesting further that a psyllid cell culture would be more permissive. Psyllid cell cultures are also being used to propagate and study psyllid viral pathogens.

Introduction

Citrus greening disease, also known as Huanglongbing disease, HLB, is a devastating disease on citrus causing reduced fruit yields, fruit quality, and ultimately tree death. The causal agent of HLB is thought to be Las, which has thus far remained elusive in the development of pure cultures. This study attempts a new approach to isolate and culture this bacterium in a medium of psyllid cell cultures. The Asian Citrus Psyllid, AsCP, *Diaphorina citri*, (Hemiptera: Psyllidae) is a highly competent vector of this phloem-inhabiting bacterium. World-wide citrus greening has become a major limiting factor to the production of citrus. First attempts to isolate the bacteria were made using a commercially available insect cell culture, Sf9 cell culture to isolate Las from infected citrus sap. However, the PCR product derived from Las dramatically decreased one day after inoculation (dai) compared to 0 dai. This result showed Las could not survive in Sf9 cells. There may be interaction requirements needed between Las and the AsCP, or its endosymbionts. To examine these potential interactions we created psyllid cell cultures. Several commercially available insect cell culture media were screened for viability to culture cells/tissues from AsCP embryos and midgut tissues (Figure. 1 and 2). We defined an insect culture medium, labeled Hert-Hunter-70, which permitted psyllid cell lines to be established. These primary AsCP cell cultures appear healthy, and growth rates average a doubling ~10 days.

Materials and Methods

Inoculation leaf sap in Sf9 cells: Sf9 cells were maintained with Grace's insect medium containing 10% fetal bovine serum (FBS). Cells were incubated at 27°C feeding cultures every 3 days. Citrus leaves which were Liberibacter positively detected by PCR analysis were collected from Picos Farm in Ft. Pierce, Florida. Leaves were processed in sterile-laminar hood, surface sterilized in 70% ethanol and washed with syringe filtered water three times (0.22µm).

Leaf midribs were cut and ground with water and filtrated by cotton mesh cloth. This filtrated leaf sap was transferred into Sf9 cells (0 d) and sampled daily over next three days.
PCR Analyses: PCR was conducted with Las primers(Shatters, USDA, ARS) to monitor presence of Las over time.
Cells from psyllid egg: Psyllid eggs were isolated from the tips of growing shoots and the crevices of unfolded 'feather-flush' leaves from citrus, collected at the USDA in Ft. Pierce, FL.
(1) Eggs were disinfected by submersion in 70% ethanol, 10 min, then rinsed 3 to 5 times by submersions in 0.05% sodium hypochlorite (= 1% chlorine bleach).
(2) Rinse 6 times with sterile distilled water.
(3) Transfer to tube and crush with glass rod.
(4) Add culture medium (Hert-Hunter-70) containing antibiotics (Pen-Strep).
(5) Culture at 25 °C.

The adult Psyllid alimentary tract
(1) Surface sterilized adult psyllids were immersing in 70% ethanol for 30 min.
(2) Rinse 5 times with sterile distilled water.
(3) Sterilized insects were fixed on a glass slide and circle with a PAP pen and the circle filled with PBS buffer (pH6.5).
Dissection was performed in the buffer under a dissecting microscope.
(4) Forceps and needles were used to remove the gut.
(5) The excised gut was placed into a tube and crushed with a glass rod.
(6) Add culture medium containing antibiotics (as above #4).
(7) Culture at 25 °C.

Media and supplements used in development and evaluations:

Medium 199 (Sigma)	Grace's (GIBCO)
Ex-cell 405 (Sigma)	Sf900 III SFM (GIBCO)
Schneider's Insect Medium (Sigma)	TNM-FH Insect Medium (Sigma)
TC100 Insect Medium (Sigma)	Shields & Sang M3 Insect Medium (Sigma)
IPL-41 Insect Medium (Sigma)	Gentamicin solution (Sigma)
0.05% sodium hypochlorite	100X Penicillin-Streptomycin (sigma)
Fetal Bovine Serum, not heat inactivated, FBS	L-Glutamine 200mM (GIBCO)

Results and Discussion
We have successfully produced several AsCP cell lines. Evaluation of different commercially available media for suitability to culture psyllid cells resulted in production of a medium suitable for psyllid cell growth. However, different combinations of supplements continue to be evaluated. The new medium labeled Hert-Hunter-70, supports psyllid cells in this media over three months. When psyllid eggs were used in which the eyespot could be seen, the resulting cells showed migration and adherence to the glass Petri dish. Successful cultures are being inoculated with citrus sap filtrates in an attempt to culture Las.

References
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Figure 1. ***Diaphorina citri*** egg and cells from crushed eggs, 3 days post treatment showing growth.

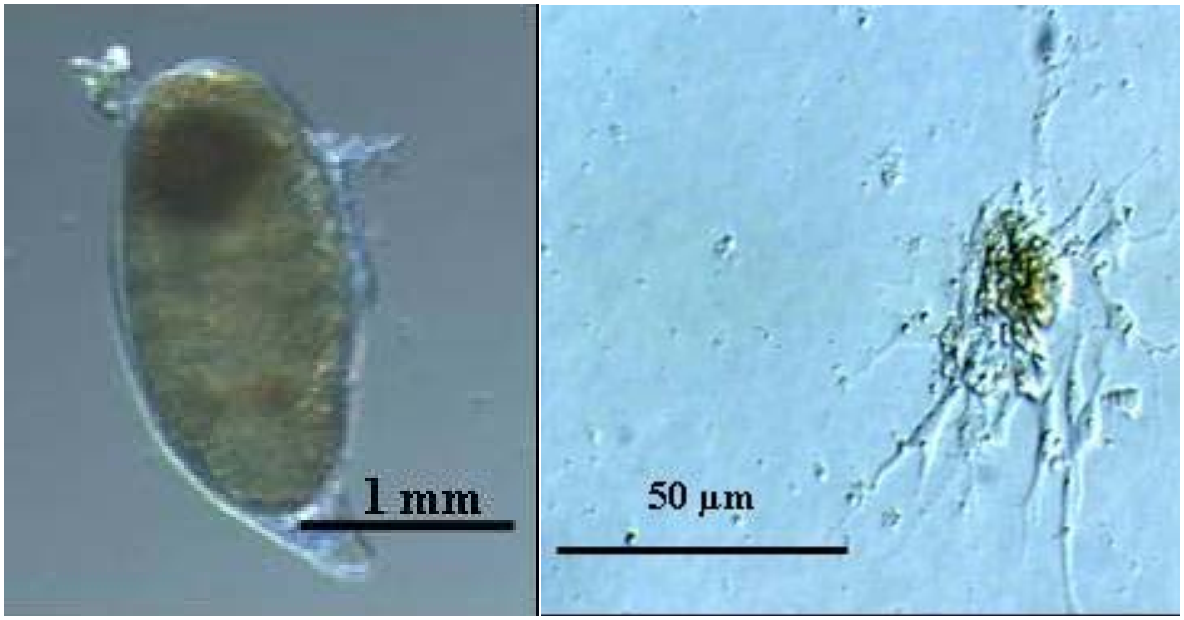
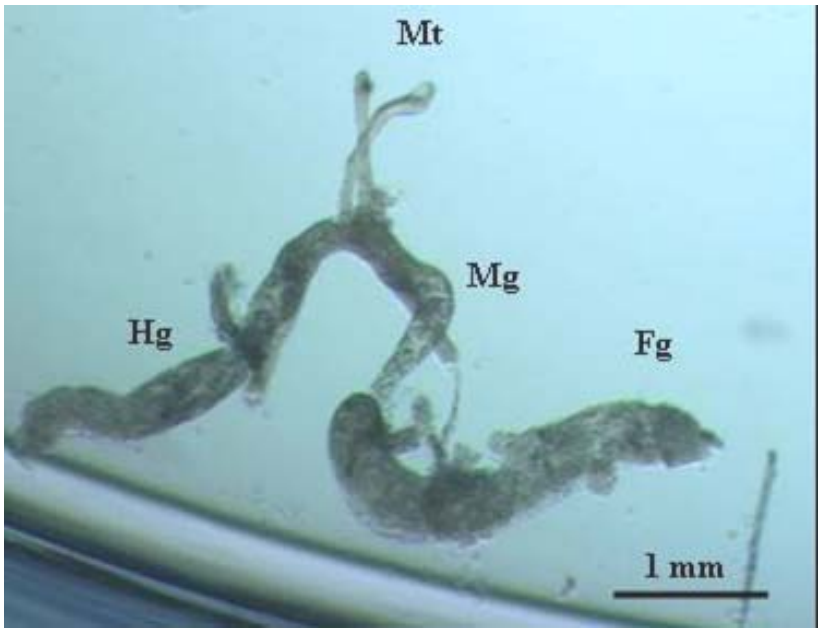
Figure 2. Asian citrus psyllid alimentary tract dissected from adult. Fg- foregut, Mg– midgut, Hg– hind gut, Mt– Malpighian tubules.