In Vitro Cell.Dev.Biol.—Animal (2009) 45:317–320
DOI 10.1007/s11626-009-9188-3

REPORT

Establishment of Asian citrus psyllid (*Diaphorina citri*) primary cultures

Mizuri Marutani-Hert · Wayne B. Hunter · David G. Hall

Received: 6 November 2008 / Accepted: 4 February 2009 / Published online: 14 May 2009 / Editor: J. Denry Sato

Abstract The Asian citrus psyllid (AsCP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is a highly competent vector of the phloem-inhabiting bacterium *Candidatus* Liberibacter asiaticus associated with the citrus disease huanglongbing (HLB). Commonly referred to as citrus greening disease in the USA, HLB causes reduced fruit yields, quality, and ultimately tree death and is considered the most serious citrus disease. HLB has become a major limiting factor to the production of citrus worldwide. Studies of HLB have been impeded by the fact that *C.* Liberibacter has not yet been cultured on artificial nutrient media. After being acquired by a psyllid, *C.* Liberibacter asiaticus is reported to replicate within the psyllid and is retained by the psyllid throughout its life span. We therefore hypothesized that *C.* Liberibacter asiaticus could be cultured in vitro using psyllid cell cultures as the medium and investigated the establishment of a pure culture for AsCP cells. Several commercially available insect cell culture media along with some media we developed were screened for viability to culture cells from AsCP embryos. Cells from psyllid tissues adhered to the plate and migration was observed within 24 h. Cells were maintained at 20°C. We successfully established primary psyllid cell cultures, referred to as DcHH-1, for *D. citri* Hert-Hunter-1, with a new media, Hert-Hunter-70.

M. Marutani-Hert (✉) · W. B. Hunter · D. G. Hall
USDA, ARS, US Horticultural Research Lab,
2001 South Rock Rd,
Ft. Pierce, FL 34945, USA
e-mail: Mizuri.Marutani-Hert@ARS.USDA.GOV

Keywords Asian citrus psyllid · Hemiptera · Cell culture · Huanglongbing · Liberibacter

Citrus greening disease, also known as huanglongbing disease (HLB), is a devastating citrus disease causing reduced fruit yields, quality, and tree death (Halbert and Manjunath 2004). Currently, there are no known cures or effective treatments for HLB. HLB has become a major limiting factor in the production of citrus worldwide. The causal agent of HLB is thought to be the bacterium *Candidatus* Liberibacter, of which there are three known species: L. asiaticus in Asia and the Americas; L. africanus in Africa; and L. americanus in South America (Brazil; Bové 2006). *C.* Liberibacter has never been successfully cultured in vitro on traditional media, and thus, pure cultures of *Candidatus* Liberibacter have not been available for research. The Asian citrus psyllid (AsCP), *Diaphorina citri* Kuwayama (Hemiptera: Psyllidae), is an important agricultural pest insect due to its ability to transmit HLB bacterial pathogens. The phloem-inhabiting bacterium is introduced by the vector into the plant during feeding (Hung et al. 2004). Reportedly, once acquired, *C.* Liberibacter replicates within the psyllid and is thus retained throughout the psyllid's lifespan (Hung et al. 2004). Since the bacterium replicates within a psyllid, we hypothesized that it might be possible to grow the HLB bacterium on a culture of psyllid cells. No information was available on developing cell cultures of AsCP, but it has been notoriously difficult to produce cell cultures of many hemipterans such as plant hoppers and aphids (Mitsuhashi 2002). Although developing psyllid cell cultures proved difficult, careful screening of different media produced fruitful results. In this study, several commercially available insect cell culture media in addition to some of our own preparations were screened for

viability to culture cells from AsCP embryos. This is the first report on the successful establishment of primary psyllid cell cultures.

Cells from Psyllid Eggs A culture of AsCP was established at the USDA in Ft. Pierce, FL during 2004 using field-collected adults. The psyllids have been maintained on the host plants Madam vinous sweet orange [*Citrus sinensis* (L.) Osbeck] in an insectary glass house. Embryos at the blastokinetic stage of development (Mitsuhashi 1976; Hunter and Polston 2001) were used as the source for psyllid cell cultures. This stage can be recognized by the appearance of red eye spots. Adult psyllids were transferred to new cages which contained leaves with new flush to facilitate collection of eggs of the same stage. After a week, eggs were collected using an insect pin (size 2) under a microscope. Approximately 100 eggs were collected in 1.5-mL micro-centrifuge tubes and were disinfected by submersion in 70% ethanol for 10 min. After rinsing five times with 1× Hank's Salt™ sol (Sigma, St. Louis, MO), eggs were crushed with a glass rod. One milliliter of culture medium containing the antibiotics penicillin (10,000 U/mL) and streptomycin (10 mg/ml; Sigma) was added to the crushed eggs, and the eggs were then incubated in 24-well plates (Costar®, Corning, NY) at 20°C. The media was changed at intervals of 7 to 10 d.

Media and Supplements The following insect cell culture media and media additives were tested for their ability to establish primary cell cultures: medium 199 (Sigma), Grace's insect medium (Invitrogen, Carlsbad, CA), Kimura's medium (Kimura 1984), medium CMRL 1066 (Invitrogen), Sf-900™ III SFM (Invitrogen, Carlsbad, CA), EX-cell™ 405 (SAFC Biosciences, Lenexa, KS), Schneider's insect medium (Sigma), TNM-FH insect medium (Sigma), TC100 insect medium (Sigma), Shields and Sang M3 insect medium (Sigma), IPL-41 insect medium (Sigma), fetal bovine serum (Invitrogen), and L-glutamine solution 200 mM (Invitrogen).

Table 1. Asian citrus psyllid cell culture medium, Hert-Hunter-70 (HH-70), and Hert-Hunter-50 (HH-50) compositions

	HH-70 (mL)	HH-50 (mL)
Schneider's insect medium	350	250
Sf900 III SFM	150	250
Medium 199 (10×), with Hanks' Salts	50	50
Medium CMRL 1066	25	25
Fetal bovine serum[a] (heat-inactivated)	150	150
0.06 M histidine solution (pH 6.5)	500	500
200 mM L-Glutamine	12.25	12.25

Total volume of medium was about 1,225 mL, pH was adjusted to 6.5–6.6 with 2 N HCl.

[a] Heat treatment at 56°C for 30 min.

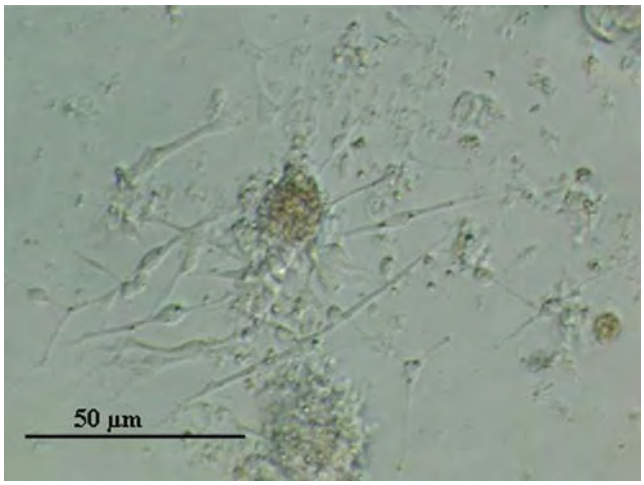

Figure 1. Asian citrus psyllid, *D. citri*, primary cell growth in Hert-Hunter-70 medium, 8 d post-processing. Newly formed cells migrated out of initial tissue masses.

Cell Line Characterization Cell line was confirmed using polymerase chain reaction (PCR) with AsCP primers to Cytochrome-c oxidase I (COX; COX5: 5′-GTAGTTGCC

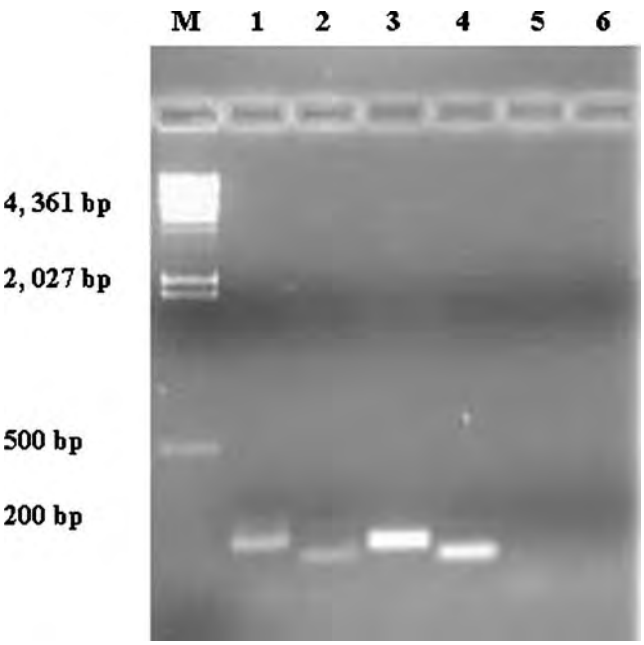

Figure 2. Asian citrus psyllid, *D. citri* cell culture genomic confirmation by PCR. PCR products with primers COX5 and COX3 (*lanes 1*, *3*, *5*), EF5, and EF3 (*lanes 2*, *4*, *6*). The genomic DNAs were isolated from homogenized cell culture (*lanes 1* and *2*) or adult psyllid (*lanes 3* and *4*) and aphid (*lanes 5* and *6*). *Lane M*: λ DNA/*Hin*dIII fragments marker (Invitrogen).

CACTTTCACTATGTGC-3′, COX3: 5′-CGTCGTGGCAT TCCTATAAGTCCT-3′), and a potential psyllid symbiont elongation factor (EF; EF5: 5′-TAGATCAAGGGCAAG CTGGT-3′, EF3: 5′-GCGACCTCCTTCATCTTTTG-3′). The genomic DNA was extracted from cell culture and adult insect by AquaPure genomic DNA kits (Bio-Rad, Hercules, CA). The PCR was performed using Platinum® PCR Supermix (Invitrogen) as follows: 95°C for 3 min; 30 cycles 95°C for 30 s; 60°C for 30 s; 72°C for 30 s; and 72°C for 5 min. As a negative control, brown citrus aphid genomic DNA was used.

We evaluated ten different commercially available media for suitability to culture psyllid cells. All the media evaluated had 2 mM L-glutamine added since past research demonstrated that a cell line from another insect, *Spodoptera frugiperda*, did not grow in glutamine-deficient media (Godwin 1975). Sf-900™ III SFM medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine showed that cells would remain viable for a significantly longer period of time but would not grow. Kimura (1984) successfully developed leafhopper cell lines whose main nutrient component was Schneider's insect medium. Since psyllids are hemipterans and closely related to leafhoppers, we decided to combine Schneider's and Sf-900™ III SFM media to make Hert-Hunter (HH) media (Table 1). Cell migration was observed in only four media (Fig. 1): Grace's with 20% FBS and 2 mM L-glutamine, Kimura's medium with 2 mM L-glutamine, Kimura's modified medium, Hert-Hunter-70, and Hert-Hunter-50 media. The most efficient medium for development of psyllid cell growth was Hert-Hunter-70 followed by Hert-Hunter-50, Kimura's medium with 2 mM L-glutamine, and then Grace's with 20% FBS and 2 mM L-glutamine, respectively. Media which did not support psyllid cells were easily determined as the cells shriveled and eventually disintegrated.

The blastokinetic stage embryos produced smaller cells with a distinct nucleus visible when crushed; the cells are tightly packed into tissue. Cells from the psyllid eggs attached to the plate substrate and cell migration was observed within 24 h. Monolayers of cells were composed of two types of cells. The majority was fibroblast-like cells, but a few round-shaped cells were present. Daily photos of cells provided direct counts of cell growth which demonstrated approximately 10 d to double. Cells grew from fragments of tissue and appeared to be connected and close together, with a few appearing to adhere to one another. We checked the cell viability in successful media using Trypan blue staining which indicated that the majority of cells were still viable after 2 mo. We have successfully passed cells once and observed that fibroblast-like cells and round-shaped cells continued to grow.

Cells were characterized as being AsCP by PCR with COX and a potential psyllids symbiont *EF* gene. PCR products of the size from COX and *EF* gene were the same for DNA from cell culture and adult psyllid (200 and 161 bp), but did not amplify a product in negative control, brown citrus aphid (Fig. 2).

Comparison of successful media was conducted to try to understand the important components necessary for maintain of the psyllid cells. We compared the list of components in each of media and determined which components were associated with media which successfully supported psyllid cell viability. Unfortunately, components of EX-cell™ 405 and Sf-900™ III SFM media were not published. However, other media components were published. Comparison of the successful media to others [Schneider's insect medium (Sigma), TNM-FH insect medium (Sigma), TC100 insect medium (Sigma), Shields and Sang M3 insect medium (Sigma), and IPL-41 insect medium (Sigma)] demonstrated that media which did not support psyllid cell growth were lacking or insufficient in one or more of the following: KCl, $NaHCO_3$, NaH_2PO_4, alanine, L-cystine, *p*-aminobenzioc acid, D-biotin, D-calcium pantothenate, folic acid, i-inositol, nicotic acid, pyridoxine, riboflavin, thiamine, fumaric acid, α-ketoglutaric acid, L-malic acid, and succinic acid. One or more of these components appeared to be necessary for psyllid cell migration. The sodium/potassium ratio of Grace's media and Kimura's media were approximately 0.31 and 3.28, a broad concentration range that suggested they did not impact the growth rate of psyllid cells. The AsCP cell line, DcHH-1, provides a greatly needed research tool which will now be applied to studies of Liberibacter–psyllid cell interactions.

The development of psyllid cell cultures increases the opportunity for in vitro research, including physiology, toxicology, and pathology.

Acknowledgments We thank Christine Lynch, Maria Gonzalez, Kathryn Moulton, Matthew Hentz, Biological Science Technicians, USDA, ARS, Ft. Pierce, FL, for providing technical assistance and for helpful comments during manuscript preparation. We thank Dr. Aaron P. Hert for helpful discussions and review of the manuscript. This research was supported in part by the Florida Citrus Production Research Advisory Council, 2007.

References

Bové J. M. Huanglongbing a destructive, newly-emerging, century-old disease of citrus. *J. Plant Pathol.* 88: 7–37; 2006.

Goodwin R. H. Insect cell culture improved media and methods for initiating attached cell lines from the Lepidoptera. *In Vitro Cell Dev. Biol.—Plant.* 11: 369–378; 1975. doi:10.1007/BF02616373.

Halbert S. E.; Manjunath K. L. Asian citrus psyllids (Sternorrhyncha: Psyllidae) and greening disease of citrus: A literature review and assessment of risk in Florida. *Florida Entomol.* 87: 7–37; 2004.

Hung T. H.; Hung S. C.; Chen C. N.; Hsu M. H.; Su H. J. Detection by PCR of *Candidatus* Liberibacter asiaticus, the bacterium

causing citrus huanglongbing in vector psyllids: application to the study of vector–pathogen relationships. *Plant Pathol.* 53: 96–102; 2004. doi:10.1111/j.1365-3059.2004.00948.x.

Hunter W. B.; Polston J. E. Development of a continuous whitefly cell line (Homoptera: Aleyrodidae: *Bemisia tabaci* (Genn.) for the study of Begomovirus. *J. Invertebr. Pathol.* 77: 33–36; 2001. doi:10.1006/jipa.2000.4993.

Kimura I. Establishment of new cell lines from leafhopper vector and inoculation of its cell monolayers with rice dwarf virus. *Proc. Jpn. Acad.* 60: 198–201; 1984. doi:10.2183/pjab.60.198.

Mitsuhashi J. Primary culture of leafhopper embryonic tissues. *J. Tissue Cult. Methods* 46122: 151–154; 1976.

Mitsuhashi J. Invertebrate tissue culture methods (Springer lab manual). Springer, Tokyo-Shoseki, Japan 2002.