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REPORT

Medium for development of bee cell cultures (*Apis mellifera*: Hymenoptera: Apidae)

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Abstract A media for the production of cell cultures from hymenopteran species such as honey bee, *Apis mellifera* L. (Hymenoptera: Apidae) was developed. Multiple bee cell cultures were produced when using bee larvae and pupae as starting material and modified Hert–Hunter 70 media. Cell culture systems for bees solves an impasse that has hindered efforts to isolate and screen pathogens which may be influencing or causing colony collapse disorder of bees. Multiple life stages of maturing larvae to early pupae were used to successfully establish cell cultures from the tissues of the head, thorax, and abdomen. Multiple cell types were observed which included free-floating suspensions, fibroblast-like, and epithelia-like monolayers. The final culture medium, WH2, was originally developed for hemipterans, Asian citrus psyllid, *Diaphorina citri*, and leafhopper, *Homalodisca vitripennis* cell cultures but has been shown to work for a diverse range of insect species such as bees. Bee cell cultures had various doubling times at 21–23°C ranging from 9–15 d. Deformed wing virus was detected in the primary explanted tissues, which tested negative by rt-PCR for Israeli acute paralysis virus (IAPV), Kashmir bee virus, acute bee paralysis virus, and black queen cell virus. Culture inoculation with IAPV from an isolate from Florida field samples, was detectable in cell cultures after two subcultures. Cell culture from hymenoptera species, such as bees, greatly advances the approaches available to the field of study on colony collapse disorders.

Keywords Bee · Colony collapse disorder · CCD · Hymenoptera · Leafhopper · Psyllid · Virus

Colony collapse disorders (CCD) continues to be a serious problem in pollinating species of bees. One of the bottle necks in researching CCD has been the lack of bee cell cultures. Cell culture systems permit rapid evaluations of pathogen interactions and their impact on bee cell growth and survival. Many industries depend on mass production or survival of insects, such as for food for amphibian and reptile pet industries; parasitoids for biological control programs; and pollinators for food and forage crops, as with bees. However, when CCD was noticed in honey bees and other pollinating species of hymenoptera, there was great concern for the survival of apiculture which is tightly linked to national food security. Viral pathogens when causing the decline of insect pests in nature are considered potential biological control agents to reduce pests and disease transmission and have been documented (Hunnicutt et al. 2006; Hunter et al. 2006; Stenger et al. 2009). Colony collapse disorders have been observed in many other insect colonies, when being reared under artificial conditions for research or commercial purposes (*Homalodisca vitripennis*, leafhoppers: Hunter et al. 2006, *Solenopsis invicta*, fire

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ants: Valles et al. 2004, and *Tenebrio molitor* beetles: Dr. Jeff Lord, ARS, in prep). Typical viral infection symptoms include a thriving colony with many individuals at the pupae stage just prior to eclosion into adults; however, most of these newly forming adults never survive. Extensive research on honey bee genomics along with metagenomics approaches (Cox-Foster et al. 2007) lead to the identification of an emerging bee-infecting virus, Israeli acute paralysis virus (IAPV). Bees have been reported to be susceptible to at least 17 different bee viruses (Ellis and Munn 2005). They also are susceptible to infection by several microsporidia, as *Nosema* species causing special concern. However, bee pathology has been hindered by the lack of a cell culture system. Bee cell cultures permit researchers to address some of the most difficult questions concerning bee/virus and bee/fungi interactions along with their roles in CCD. Further applications of bee cell culture systems will solve this impasse which has hindered efforts to isolate and elucidate the pathogens specifically influencing or causing colony collapse disorder of honey bees (*Apis mellifera* L.).

Cells from Honey Bee Larvae and Pupae

A frame of capped brood was provided by a local bee producer. Larvae in open comb cells and pupae in capped cells were gently removed. These larvae were white with the head capsule just starting to form (Fig. 1). Pupae were removed from capped cells using fine-tipped forceps.

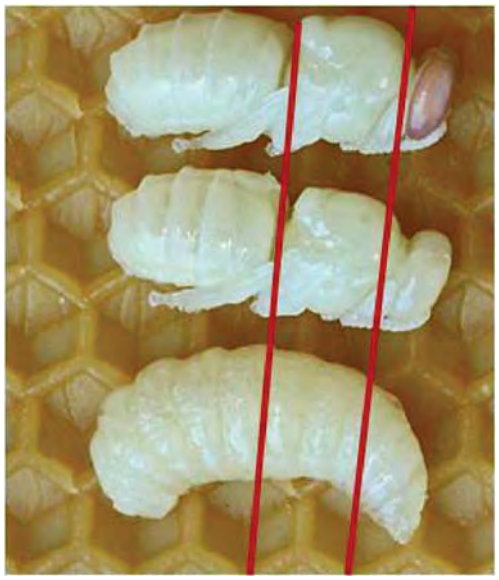

Figure 1. Honey bee, *A. mellifera*, larvae and pupae shown were dissected accordingly into parts representing head, thorax, and abdomen. Approximate age of bees based on developmental time tables is 8–11 d old. Larva: white-eyed pupae, brown-eyed pupae.

Table 1. Honey bee, *A. mellifera*, cell medium WH2 composition

Schneider's Insect Medium	150 ml
0.06 M L-histidine solution (pH 6.35)	200 ml
Fetal Bovine Serum (heat inactivated)[a]	50 ml
CMRL 1066	15 ml
Hanks' Salts	5 ml
Insect medium supplement (×10)	2 ml
Gentamicin, units/1 µl/ml	1 µl/ml

Total volume of medium was ~422 ml; pH adjusted to 6.3–6.5 with 2 N HCl or NaOH

[a] FBS heat treatment at 56°C for 30 min

Early stage pupae, where the eyes were white with fully formed head capsule to eyes with tan color and older, where the eye color was darker brown (Fig. 1) were used. In a sterile, laminar flow hood, bees were surface sterilized with 70% ethanol, 10 min with light agitation. Samples were then rinsed twice with syringe filtered water, 0.22 µm (millipore), 2 min each. Sterile forceps were used to separate the head, thorax, and abdomen. Tissues were torn apart in WH2 medium and dispersed across multi-well plates, 24 wells (Costar®, Corning, NY) at 22°C room temperature. Less tissue per well appeared to work better, and abdomen tissue was dispersed across four separate plates. After cells displayed sustained growth, usually within 4–10 d, one third of media was changed at intervals of 7–12 d. Floating cells and debris were transferred to 25 cm^2 tissue culture flasks, where new cell growth was observed within 48 h.

Media and Supplements

The bee cell culture medium formulation WH2 was produced from a slight modification of psyllid cell culture medium HH-70 (Marutani-Hert et al. 2009). Modifications include insect media supplement (×10; Cat. No. 17267, Sigma, Ronkonkoma, NY) substituted for Media 199 (×10, 500 ml, Sigma). Explanted tissues would show attachment and growth within 24 h in WH2 medium, regardless of whether L-glutamine (Cat. No. 25030, Gibco, Invitrogen, Carlsbad, CA) was added or not; so, this was not incorporated into WH2 medium. Components for WH2 are listed in Table 1 and include: Schneider's Insect Medium (Cat. No. S0146, 500 ml, Sigma), insect medium supplement (Cat. No. 17267, ×10, 500 ml, Sigma), Hanks' Balanced Salt Solution (Cat. No. H9394, 500 ml, Sigma), CMRL Medium 1066 (Gibco 11530-037, 500 ml, ×1, Invitrogen), Fetal Bovine Serum (FBS; Cat. No. F-0643,

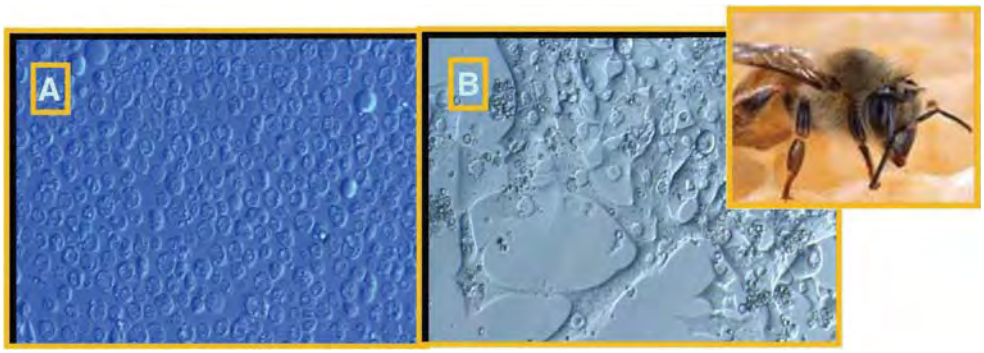

Figure 2. Honey bee, *A. mellifera*, cell cultures. *A*, Bee cell suspensions from abdominal tissues from white-eyed pupae. *B*, Bee epithelial-like monolayers of cells produced from all stages and body segments (both ~×60). Deformed wing virus (DWV) was detected in the primary explanted tissues, which tested negative by rt-PCR for Israeli acute paralysis virus, Kashmir bee virus (KBV), acute bee paralysis virus (ABPV), and black queen cell virus (BQCV).

500 ml, Sigma), L-histidine monohydrochloride monohydrate (Cat. No. H5659, 25 g, Sigma). Gentamicin was used as an antibiotic (Cat. No. G1397, 10 ml, Sigma). Primary screening of commercially available media consisted of using individual media as purchased, then examining cell attachment or growth over an 8-d period. Media was then evaluated when combined with 10% FBS: bee cells showed attachment and growth in: HH-70 psyllid culture medium (Marutani-Hert et al. 2009). Bee cells grew in Sf-900™III SFM (Gibco 12658, 500 ml, serum-free, ×1, Invitrogen) alone or supplemented with 10% FBS; however, cells showed slow growth, were translucent, and usually observed as individual cells. Cells in Ex-Cell 405 (×1, w/L-glutamine, Cat. No. 14405C-500 ml, SAFC Biosciences, Lenexa, KS) appeared alive, but cell attachment and growth was extremely slow and at very low frequency. The other cell culture media listed as components would provide an environment where bee cells appeared to remain alive but without additional attachment or growth observed.

Cell Line Characterization

Cell line characterization was checked using polymerase chain reaction (PCR) with *A. mellifera* specific primers to actin (Actin F5′-ATGAAGATCCTTACAGAAAG-3′, Actin R5′-TCTTGTTTAGAGATCCACAT-3′). Bee genomic DNA was extracted from cell culture and adult insects by AquaPure® Genomic DNA Kits (BIO-RAD, Hercules, CA). 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, and 72°C for 30 s; and 72°C for 5 min. As a negative control, leafhopper, *H. vitripennis* genomic DNA was used. Amplimer size and sequence from actin genes were the same for DNA from cell culture and adult bee but different when DNA was from leafhopper.

Bee virus evaluations were performed using rt-PCR protocols as in Maori et al. 2009. Only deformed wing virus was detected within the cultures (Fig. 2). Cells inoculated with IAPV tested positive over several subcultures. In bee cell culture medium, WH2 cells from larvae and pupae attached to the plate substrate and cell migration, and multiplication was observed in as early as 24 h. The monolayer cells were composed of two types of epithelial cells similar to descriptions from leafhopper Kimura (1984), Mitsuhashi (1976, 2002), Wayadande and Fletcher (1998). Cell cultures were labeled as “AmWH”. The spindle-shaped cells, 1–2-μm wide and 2.5–10-μm long also formed cell clusters. Suspension cultures resulted in round-shaped cells 1–2 μm in diameter. While previous media evaluated had 2 mM L-glutamine added, evaluation with and without L-glutamine showed no difference in adherence or growth (data not shown) (Lynn 2001, 2002). This trait of bee cells is different from lepidopteran cell lines developed from S*podoptera frugiperda* which reportedly would not grow in glutamine-deficient medium (Goodwin 1975).

Having media which supports cell cultures from *A. mellifera* now provides a greatly needed research tool which will be applied to address critical questions surrounding the causes and potential solutions to CCD in hymenopteran pollinators. Bee cell cultures also provide a highly controlled system for the study and propagation of bee-infecting viruses for further studies on bee virus pathology.

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