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Iridovirus infection of cell cultures from the *Diaprepes* root weevil, *Diaprepes abbreviatus*

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Abstract

We here report the development and viral infection of a *Diaprepes* root weevil cell culture. Embryonic tissues of the root weevil were used to establish cell cultures for use in screening viral pathogens as potential biological control agents. Tissues were seeded into a prepared solution of insect medium and kept at a temperature of 24° C. The cell culture had primarily fibroblast-like morphology with some epithelial monolayers. Root weevil cells were successfully infected *in vitro* with a known insect virus, Invertebrate Iridescent Virus 6. Potential uses of insect cell cultures and insect viruses are discussed.

Keywords: Citrus, *Diaprepes abbreviatus*, Insect, Lethal-Male Delivery System, Pathogen, Suppression, Virus, Weevil

Abbreviation:
IIV-6 Invertebrate Iridescent Virus 6

Introduction

The Diaprepes root weevil, *Diaprepes abbreviatus* (L.) [Coleoptera: Curculionidae], was first reported in the mainland U.S. at Apopka, FL in 1964 (Woodruff 1964) and has become a key pest of citrus, crops, and ornamental plants throughout the state. The larvae of this weevil burrow through the soil feeding on progressively larger roots as they grow. Girdling of structural roots or the root crown can kill mature citrus trees. In addition to direct damage, larval feeding provides infection sites for plant pathogens, particularly *Phytophthora* spp., that contribute to tree mortality and reductions in yield (Rogers *et al.*, 1996). This pest has become a major concern of citrus growers in Florida due to the difficulty of detecting larvae in the soil, and the fact that few effective and environmentally appropriate control options are currently available to growers for controlling subterranean pests (Lapointe and Shapiro 1999). To meet this challenge, we undertook to develop *in vitro* cultivation of *Diaprepes* root weevil cells that may provide materials for studies of cell biology, weevil physiology, and genetics. Our interests are in producing a method to screen for entomopathogenic organisms that may be used as biological control agents against the root weevil.

Materials and Methods

Source of Diaprepes root weevil

Insects were obtained from a laboratory colony maintained on artificial diet at the U.S. Horticultural Laboratory of the USDA-ARS at Orlando, FL as described by Lapointe and Shapiro (1999). Eggs were collected from caged adults on wax paper strips provided as oviposition substrates. Eggs were incubated at 26° C and hatched in approximately 7 days. Eggs were collected in 1.5 ml plastic vials and prepared as described below within 24- 48 h of hatching (Fig. 1).

Cell Culture Medium Components

The medium used for the culturing of weevil cells was a mix of Kimura's modified medium (Kimura, 1984) with an addition of 20% Excell 401 medium (JRH Biosciences, www.jrhbio.com). Components were mixed as follows: 100 ml of Schneider's *Drosophila* Medium, liquid 1X, with L-glutamine, (Gibco BRL, #11720-034, www.lifetech.com) plus 100 ml of L-histidine hydrochloride, monohydrate, pH 6.2 (1.3g/ 100 ml dH_2O) (Gibco BRL, #11062-015), 10 ml of Medium 199, 10X, with Hank's salts (Gibco BRL, #11181-039), and 5 ml of CMRL Medium-1066, 1X, (Gibco BRL, #11530-037). Penicillin-streptomycin solution (10,000 U/ml) was added 2.5 ml/ 250 ml medium, prior to filtration (Gibco BRL, #15140-122). After these components were mixed together, the pH was adjusted to 6.35 with 1 M NaOH or 1 M HCl. The medium was then filter sterilized by passing through a 0.2 µm membrane. To this sterile medium, 50 ml of sterile Excell 401 medium (JRH Biosciences), and 30 ml of sterile fetal bovine serum (Gibco BRL, #26140-079, www.sigmaaldrich.com) were added. Cells were subcultured using Cell Dissociation Solution, 1X (Sigma

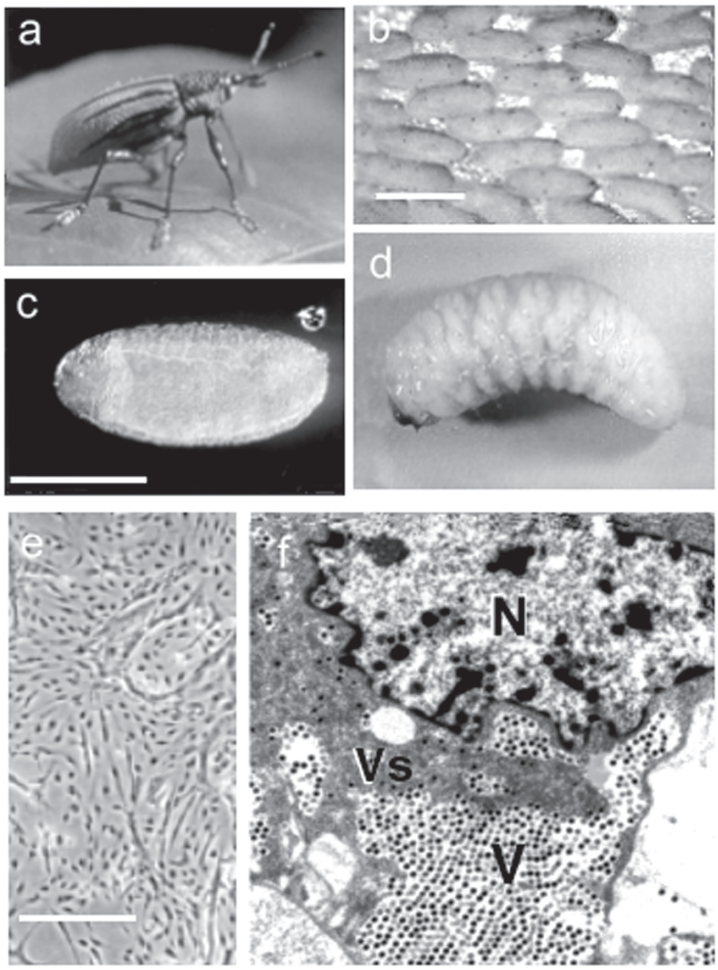

Figure 1. (a). Adult female *Diaprepes abbreviatus* (L.) on citrus leaf. Adult is approx. 15 mm long. (b). Light micrograph of root weevil, *D. abbreviatus* egg mass (bar = 2 mm). (c). Light micrograph of mature embryo ready to emerge. Head capsule is visible at left end of egg (bar = 1 mm). (d). Neonate *D. abbreviatus*, 1 d old. (e). Phase contrast light micrograph of cell cultures from *D. abbreviatus* (bar = 100 µm). (f). Transmission electron micrograph of IIV-6 virus in *D. abbreviatus* cell showing virogenic stroma (Vs) and virus (V) outside of cell nucleus (N) (bar = ~ 1200 nm).

Co., #C-1544).

Embryonic cells were taken from fully developed embryos (Fig. 1). Approximately 100 eggs prior to hatching were surface sterilized with 70% EtOH for 20-30 min then centrifuged for 1 min at 325 x g (Eppendorf 5415C centrifuge, www.eppendorf.com). After centrifugation the EtOH was replaced with fresh 70% EtOH. Under aseptic conditions the eggs were transferred to a sterile 15 ml centrifuge tube filled with 70% ETOH and centrifuged at 600x g for 3 min (IEC Centra CLA2 clinical centrifuge, www.labcentrifuge.com). The EtOH was drawn off and the eggs were rinsed 3 times with 10 ml sterile water, then transferred to a 1.5 m. centrifuge tube and centrifuged for 3 min at 1,320 x g in the CLA2 centrifuge. The eggs were rinsed with L- histidine solution (L-histidine, monohydrate 13 g/liter, pH = 6.2), and centrifuged for 3 min, at 1,320 x g in the 541C centrifuge. The histidine solution was replaced with 200 µl of insect medium and the eggs were crushed in the 1.5 ml tube using a sterilized glass rod with a smooth rounded end using 5-6 strokes. Insect medium was added to a total volume of 1 ml, transferred to a sterile 15 ml centrifuge tube and the volume of medium brought up to 4 ml. The tissues were then dispensed into wells of a 48 well tissue culture plate using a sterilized Pasteur pipette (~100 µl/well). Additional medium was added as needed to approximately 500 µl. Plates and lids were sealed with Parafilm® to prevent vapor loss. Explanted tissues were examined daily using an inverted microscope. For wells that contained excessive debris, three-fourths of the medium was replaced with fresh medium the day following transfer. Medium was refreshed by exchanging half the medium in each well with fresh medium at 7-10 day intervals.

Virus source and Inoculum

Virus was obtained from Dr. Joel Funk, at the USDA, ARS, Western Cotton Research Laboratory, Phoenix, AZ, courtesy of Dr. J. Kalmakoff, University of Otago, Dunedin, New Zealand. The virus was amplified in larvae of *Trichoplusia ni*, and was purified using differential centrifugation (Marina *et al.*, 1999). The virus solution was filter sterilized (0.45 µm) and used to inoculate the cell cultures (200 µl virus solution / ml / flask). Virus was stored at - 40° C. At 48 h post inoculation cells were dissociated from the substrate, pelleted, fixed in 3 % glutaraldehyde, 2 % paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2, overnight at 4° C, then postfixed (3 h in 1% OsO_4), embedded in Spurr's resin using standard methods (Dawes 1979), and observed with transmission electron microscopy.

Results

Diaprepes root weevil cell cultures (Fig. 1e), were successfully started using mature eggs as the source material. Initial cell growth was observed within 72 hrs. Continued growth was slow with a doubling time of 8 d. The first subculture was performed after four months in culture. The cells were firmly attached to the substrate and require either scraping or a cell dissociation solution for passage. Cells were passed every 10-15 d. Cells were maintained through 12 passages. The cell culture consists of cells with fibroblast-like morphology with some epithelial-like monolayers. Examination of root weevil cells after a 48 h exposure to purified IIV-6, by electron microscopy showed infected cells with virion accumulation just outside of cell nuclei, along with the presence of virogenic stroma, indicating virus replication (Fig. 1f). Virion size (~120 nm diameter), shape (icosahedral), and location of accumulation within the cells were comparable to other reports of typical IIV-6 infections (Funk, *et. al.,* 2001, Marina *et al.*, 1999).

Discussion

We describe methods for producing cell cultures of the *Diaprepes* root weevil, and on their subsequent infection with the Iridovirus, IIV-6. Previous studies support classifying IIV-6 as an entomopathogenic virus (Hall 1985, Hunter *et al*., 2003, Tesh and Andreadis 1992). Further evaluations of the interactions between IIV-6 and root weevils have also been reported (Hunter *et al*., 2003). However, host range of this virus in insects still needs to be completed before we fully understand its potential use as a biological control agent in an area-wide suppression program against the root

weevil.

Cell cultures represent an easy and safe method to screen new insect viruses for pathogenicity to the target organism without having to use live insects. Obtaining cell cultures from Coleoptera have also been quite successful. At least six cell lines have been produced from the cotton Boll weevil *Anthonomus grandis* (Barcenas *et al*., 1989, Stiles *et al*., 1992) and cell lines from *Heteronychus arator* (Crawford 1982), *Leptinotarsa decemlineata,* the Colorado potato beetle (Dubendorfer and Leibig 1991) and from *Diabrotica undecimpunctata* (Lynn and Stoppleworth 1984) have been reported. Boll weevil cell cultures have been used to study hormonal regulation in insect cells (Dhadialla and Tzertzinis 1997, Stiles *et al*., 1992) as have other insect cultures (Lynn *et al.*, 1987).

A major use of insect cell cultures is for the examination of virus/cell interactions in an environment wherein insect colonies are not necessary (Crawford, *et al*., 1984, Funk *et al*., 2001, Gopinadhan *et al*., 1990, Hunter and Polston 2001, Kimura 1984). There are other potential benefits to the development of new insect cell cultures. Since insect cell cultures are often grown without fetal bovine sera, relying on sera substitutes, the use of insect cell cultures have expanded to include many aspects of medicine. Researchers find that products made in insect cell cultures grown in media with sera substitutes are less likely to contain pathogens to vertebrates. Also, isolation of products is easier, less expensive, and specialty proteins can easily be produced in small or large quantities (Glaser 2002). For these reasons pharmaceutical companies have shown a growing interest in expanding their use of insect cells for the production of vaccines and other medicinal products (Glaser 2002). Each new insect cell culture may provide material for numerous types of future studies.

One of the best examples of using a virus to control a beetle pest is the control of the Palm rhinoceros beetle, *Oryctes rhinoceros* a pest of coconut and oil palms with a nonoccluded virus (Lacey *et al*., 2001). In this control strategy males are infected with a pathogenic insect virus and then set free to seek out and spread the virus to female beetles during mating (Zelazny 1973, Zelazny *et al*., 1992). We coined the term "Lethal-Male Delivery System" to represent this strategy and are conducting tests to determine an appropriate insect virus to be used in an area-wide suppression program to reduce the impact of the *Diaprepes* root weevil in citrus and other crops. Coleoptera in general have had several viruses identified from them. Iridoviruses have been reported to infect the Boll weevil, *Anthonomus grandis*, Boheman (McLaughlin *et al*., 1972) and have been identified from other scarab beetles such as the Black beetle, *Heteronychus arator* Say (Carey *et al*., 1978, Longworth *et al*., 1979) and *Sericesthis pruinosa* (Dalman) (Day and Mercer 1964). Nodaviruses have also been isolated from scarab beetles in New Zealand (Crawford *et al*., 1984) and a nuclear polyhedrosis virus has reportedly been isolated from the Boll weevil (Ryel and Cline 1970). As more insect viruses are discovered the possibility of developing an efficient, viral based, management system against the *Diaprepes* root weevil, comes closer to being realized.

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