CELL CULTURE BASED PRODUCTION OF *HOMALODISCA COAGULATA VIRUS 01* (HOCV-01): TOWARDS A GLASSY-WINGED SHARPSHOOTER BIOLOGICAL CONTROL SYSTEM

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ABSTRACT

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive pest and important vector of *Xylella fastidiosa (Xf)*, xylem-limited bacteria that causes Pierce's disease in grapevine as well as other agricultural diseases. The primary method of managing the spread of *Xf* is controlling its insect vector populations. Methods such as chemical control are not insect specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. Identifying agents that can impact vector populations is the goal of a biological control strategy. In this study, we have extracted *HoCV-01* from populations of GWSS collected in Texas. GWSS primary cell cultures were produced then inoculated with the viral extract. The introduced virus killed all treated cell cultures within 5 days. Increase in virus titer in treated cell culture was monitored over time by virus-specific PCR. Increased amounts of *HoCV* infection may lead to weakened populations of GWSS.

LAYPERSON SUMMARY

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is the most economically important insect with respect to the transmission of *Xylella fastidiosa*, the causal agent of Pierce's disease. Therefore, control of this insect is of paramount importance to the management of the disease. While insecticides have been used successfully to reduce the economic impact of this disease system, alternate methods of population insect control are needed to continue management in the future. Biological control offers alternatives to chemical control that can be effective in negatively impacting insect population without harmful environmental effects or concern for insecticide resistance. In this work, we introduced the extracted virus to GWSS cell cultures as a tool for biological control. While this virus, at natural doses, does not shown to cause significant acute mortality in live insects, it may reduce the fitness of insects to a point where other control methods would be more effective.

INTRODUCTION

The glassy-winged sharpshooter (GWSS) is the major vector of *Xylella fastidiosa (Xf)* in the southern USA (Adlerz 1980; Blua et al., 1999). The plant pathogenic bacterium, *Xf*, has caused economic losses to several agricultural industries in North America and is associated with many plant diseases such as Pierce's disease (PD), and oleander leaf scorch. PD of grapevine has become a well recognized *Xylella*-related disease; the vector profile is well known and the epidemiology of the disease is well documented (Hopkins et al., 2002). The introduction of GWSS into new areas is directly related to increased occurrence of PD in vineyards (Perring et al., 2001). Therefore, the management of PD depends heavily on the ability to control its vectors, especially GWSS. Methods of vector manipulation such as chemical control with the use of insecticides are not target specific and lead to problems such as residue contamination, injury to non-target organisms, and insecticide resistance. The search for more benevolent pest management strategies has led to the use of biocontrol agents such as fungi and parasitoids. However, by utilizing viruses that currently reside in GWSS populations, a viral bio-control that is even more precise may be developed (Hunnicutt et al., 2006).

Homalodisca coagulata virus 01 (HoCV-01) is a member of the genus *Cripavirus* and family *Dicistroviridae (*Hunnicutt et al., 2006). It is a novel virus that harbors pathogenic potential with regard to GWSS. The focus of this study was to extract whole *HoCV-01* from GWSS then inoculate GWSS cell cultures with the extracted *HoCV-01* in hopes that the cell culture would act as a medium for amplification of the virus.

OBJECTIVES

1. Inoculate the cell culture with *HoCV-01*.
2. Use cell culture as a medium to produce large volumes of *HoCV-01* for extraction.

RESULTS AND DISCUSSION

Cell Culture of GWSS. The *Hv* cells were cultured from eggs which were ready to hatch, eyespots were visible. In a sterile hood, eggs were surface sterilized with 70% Ethanol for 15 min. The eggs were rinsed twice with syringe filtered (0.22 um) sterilized water. Then ~10 eggs were placed onto a sterile watch glass, along with 20 uL of medium. The eggs were then crushed with a single tap from a sterile, rounded tip, glass rod. More medium was added to suspend cells and tissues, which were then dispensed into a 24 well, multi-well tissue culture plate (Costar®, Corning, NY). Culture media containing

antibiotics Gentamicin (10,000 U/mL) (Invitrogen, Carlsbad, CA) was added to 1.5 ml total volume in each well and incubated at 23°C.

The medium is classified as modified Wayne Hunter-2, WH2, Honey bee cell culture medium (Hunter 2010). Schneider's Insect Medium (Sigma), 0.06 Histidine solution, Hanks' Salts, Medium CMRL 1066 (Invitrogen), Gentamicin, Insect medium supplement (Cat. No. 17267, 10X, 500 ml, Sigma), Fetal Bovine Serum (Invitrogen).

Within 48 hours cells were observed attached to the substrate. Both fibroblast and monolayers developed.

Whole Virus Extraction. Infected GWSS bodies were placed in a mortar and pestle and homogenized in 100mL of phosphate buffer containing 0.02mg DETCA. The homogenate was then transferred to 50mL centrifuge tubes and centrifuged at 1600rpm for 20 minutes in an Eppendorf 5804R Centrifuge (Eppendorf, Hamburg, Germany). The resulting supernatant was split into two ultra-centrifuge tubes, combined with more phosphate buffer with DETCA, vortexed, and ultra-centrifuged at 22,000rpm for 16 hours in a Sorvall® RC-5B Refrigerated Superspeed Centrifuge (DuPont Instruments, Wilmington, DE). Following ultra-centrifugation, the supernatant was discarded, and the pellet was dissolved with 5mL phosphate buffer with 0.4% Na-deoxycholic acid and 4% Brij 52. The resulting solution was centrifuged and 1600rpm for 15 minutes, passed through a 0.45µm filter, and collected into large Eppindorf tubes. The unrefined *HoCV-01* solution was placed in a dialysis membrane, placed in a large beaker containing a stir-bar and ddH20, and placed in a refrigerator at 4°C. The ddH20 was changed out ever five-six hours until a white precipitate could be seen in the dialysis membrane. The purified *HoCV-01* solution was collected into micro-centrifuge tubes and stored at -80°C.

Cell Culture Inoculation with HoCV-01. The *Hv* culture plates were labeled V (Virus), and C (Control). Plate V was inoculated with virus (5.0 uL/well). For the next five days samples (100 uL/day) were taken from each well of plates (C,V). After virus inoculation, the plates were examined under an inverted microscope (Olympus DP30BW, IX2-SP, IX71) at 40X everyday for five days (**Figure 2**).

RNA Isolation and PCR. Total RNA was isolated from the samples collected over the five day experiment (Plates C,V) Qiagen RNeasy kit (Qiagen™, Germantown, MD); according to manufacturer's protocol. The samples (2.0 uL) were analyzed on a ND-1000 Spectrophotometer (**Figure 1**).

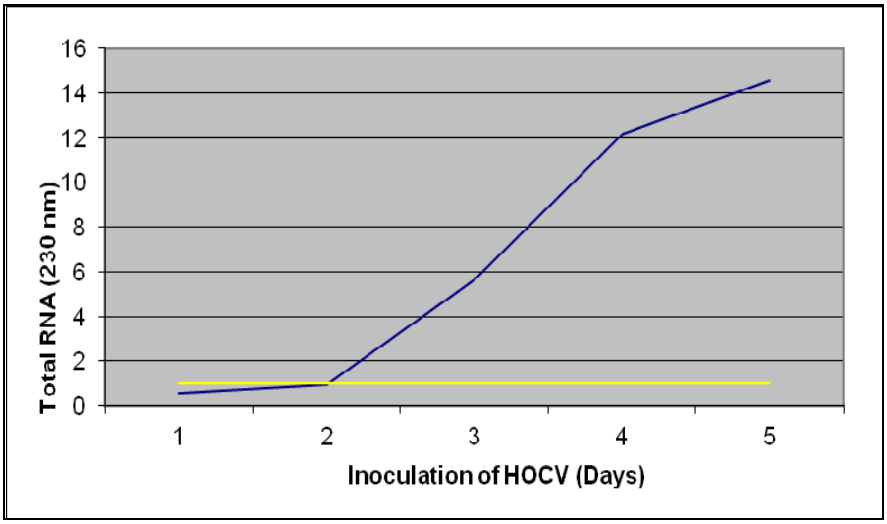

Figure 1. Total RNA increased over five days of viral inoculation (Blue). Total RNA remained the same over five days in the control plate (yellow).

Each 2µL *Hv* RNA sample was combined with 12.5µL 2X Reaction Mix (Invitrogen Molecular Probes™, Eugene, OR), 0.5µL forward (*HoCV-01* specific) primer, 0.5µL reverse (*HoCV-01* specific) primer, 0.5µL Platinum® Taq DNA Polymerase (Invitrogen Molecular Probes™, Eugene, OR), and 9.0µL DEPC H2O in solution. Samples were then subjected to a Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) using an iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The thermal profile used was: (A)pre-denaturation: 50ºC for 25 min, 94 ºC for 2 min. (B)PCR amplification: 40 cycles 94 ºC for 15 sec, 52 ºC for 30 sec, 72 ºC for 30 sec. (C) Final extension: .72 ºC for 7 min. After the PCR, 7.0µL of each *Hv* DNA sample was subjected to gel electrophoresis using 7.0µL ladder, 2µL loading dye per sample, and a 1% agarose gel containing 100mL TAE buffer and 1g agarose gel. Gels were subjected to 100V and 400A for 50 minutes and observed under Ultraviolet light in a Bio Doc-It Imaging System (Cole-Parmer™, Hanwell, London) (**Figure 2**).

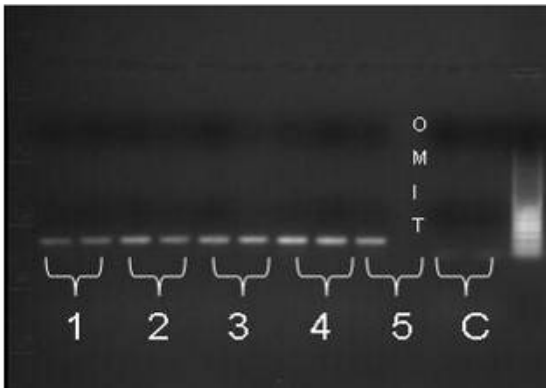

Figure 2. Increased concentration of *HoCV-01* produced from Hv cell cultures over five days.

The presence of *HoCV-01* was confirmed in the extract; since this is a general virus extraction protocol, it is possible that other known or unknown viral particles were extracted during the process. Previous studies found a new photoreovirus (Stenger et al. 2009) and phytoreovirus-like sequences (Katsar et al. 2007) present in GWSS. We hope to confirm all viral constituents that may be in our extract. We have successfully replicated *HoCV-01* through *Hv* cell culture. The concentration of *HoCV-01* increased throughout the experiment (**Figures 1 and 2**). In all trials, cell culture death was observed within five days **(Figure 3**). With these results we plan to scale up production of *HoCV-01*.

CONCLUSIONS

The presence of *HoCV-01* in populations of GWSS collected in Texas is vital in developing an ideal viral biocontrol and pest management strategy. We now have a method for mass *HoCV-01* production through the successful use of cell culture. In the future, we plan to extract the virus from the cell culture plates in hopes that in combination with other insecticides will increase the mortality rate of GWSS populations. The results of this experiment are crucial in further understanding the insect vector,GWSS. The management of PD depends heavily upon the ability to control its insect vectors.

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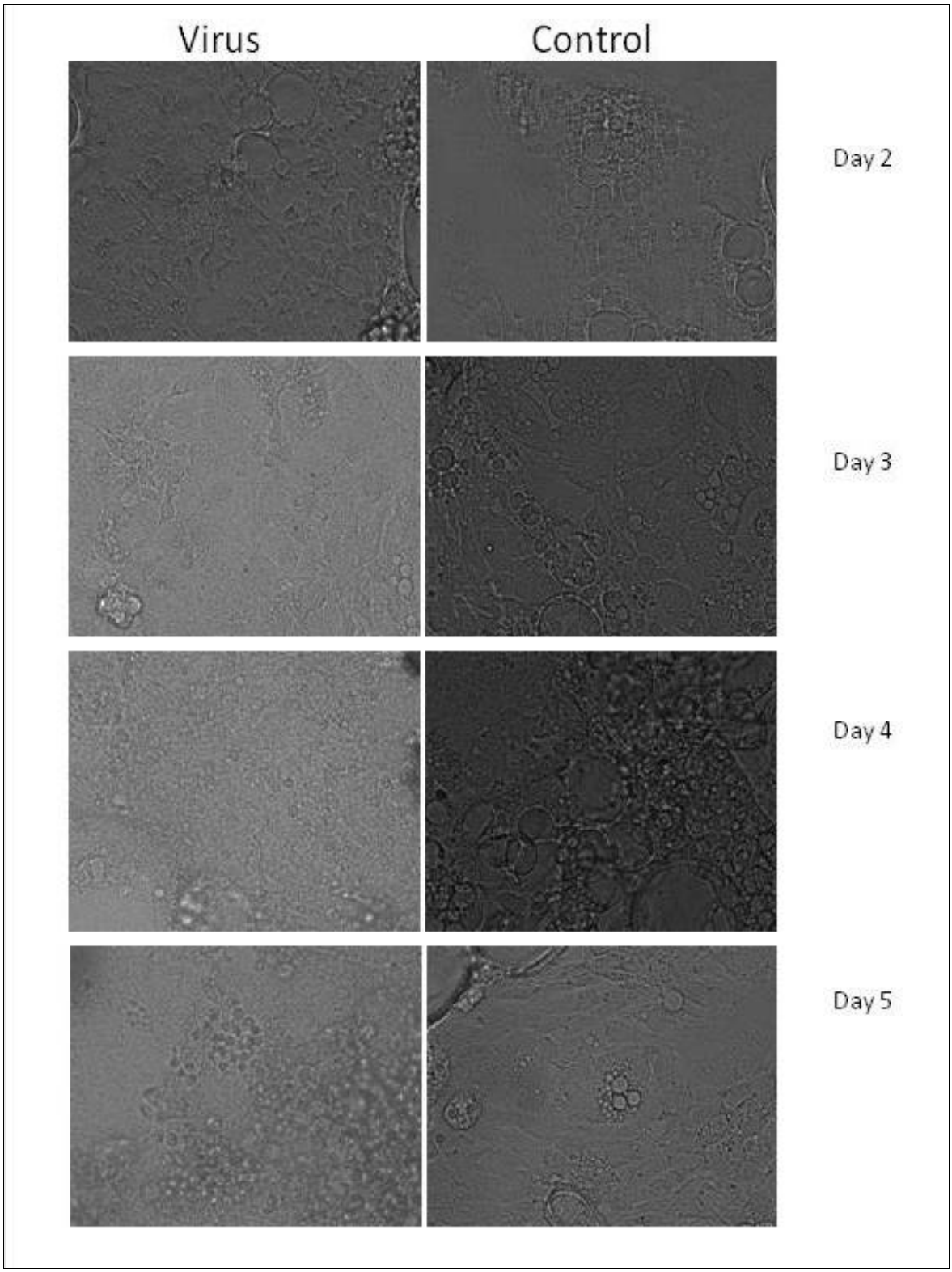

Figure 3. Shows the comparison of cells over the five day experiment assuming that day 1 was similar to day 2 of the control. Day 1 – all cells look healthy after initial inoculation (C,V). Day 2 – both plates look fairly similar, healthy (C,V). Day 3 – Low amounts of movement in the medium and within cells (V). Cells are healthy (C). Day 4 – Medium has turned a light shade of pink, high movement throughout the medium and cells, cells are looking unhealthy in appearance (V). Cells are healthy (C). Day 5 – Medium has tuned bright pink, cells are very unhealthy in appearance, assumed dead (V). Cells are healthy (C).
Note: Gentamicin was used in the medium allowing for little to no bacterial contamination.