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Article

Establecimiento de novo de un cultivo *in vitro* de una línea celular derivada del intestino de moscas sierra (Hymenoptera: Diprionidae)

De novo establishment of an *in vitro* cell line culture derived from the intestine of sawflies (Hymenoptera: Diprionidae)

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Resumen
En México, los bosques de pino son afectados por plagas insectiles, dentro de las cuales destacan las avispas de la familia Diprionidae, conocidas como moscas sierra —insectos causantes de defoliaciones en grandes extensiones de pinos y *juniperus* a lo largo del territorio nacional—, por lo que existe la necesidad de implementar medidas de control. Una estrategia es el uso de baculovirus que ha mostrado efectividad en varios países, con problemas similares, en donde se ha demostrado que los *gamma-baculovirus* infectan a las larvas de estas avispas, aumentan su mortalidad y con ello regulan sus poblaciones. En México se carece de estudios sobre la identificación, aislamiento y cultivo *in vitro* de *gamma-baculovirus* específicos para moscas sierra. En el presente escrito se analiza el establecimiento de una línea celular del hospedero de los virus, las moscas sierra, en especial de células epiteliales del intestino de las larvas; ya que, según las fuentes bibliográficas, el virus no es capaz de replicarse en otro tipo de órganos. El desarrollo de una línea celular resulta ser un poco laborioso, en particular cuando no se tienen antecedentes; por ello, se desarrolló un protocolo de establecimiento celular, con el cual se obtuvieron tanto las células requeridas, como cultivos primarios de diferentes órganos del estadio larval de las moscas sierra.

Palabras clave: Control biológico, cultivo celular, cultivo *in vitro*, epitelio intestinal, líneas celulares, moscas sierra.

Abstract
Pines, cedars and oaks forests are affected by different arthropod pests, one of which are the wasps of the family Diprionidae commonly known as sawflies. Swarms of these organisms cause defoliation on large extensions of pines and oaks along the national territory, and there is an urgent need to implement measures for the control of sawflies. One successful measure applied in other countries with similar forest pest control issues is the use of specific baculoviruses for the control of Diprionidae wasps, which has proved to be effective in the biological control of sawflies by infecting the larval stage of theses wasps, and thus increasing their mortality. However, there are no current projects in México that evaluate the identification and isolation of *gamma-baculovirus* destined to the biological control of forest pests. Our study addresses the technical difficulties of obtaining an intestinal cell line from Diprionidae sawflies, and we report the isolation and in vitro culture of intestinal cells.

Keywords: Biological control, cellular culture, *in vitro* culture, intestinal epithelial, sawflies, Nuclear Polyhedrosis Virus.

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Introduction

The use of baculoviruses for the control of agricultural pests has spread commercially, and is currently being successfully applied in the biological control of corn, soybean, and cotton pests (Beas-Catena *et al.*, 2014). These cases justify the research on the use of baculoviruses to control different arthropods that act as forest pests and cause serious damage to pine, cedar, and oak forests in different regions of Mexico.

Such is the case of wasps of the Diprionidae family, better known as sawflies, which cause the defoliation of thousands of hectares of temperate forests in Mexico, resulting in substantial losses in timber resources (González *et al.*, 2014). In the last seven years, epidemic outbreaks have occurred in *Chihuahua*, *Durango, Guerrero*, *Jalisco*, *Oaxaca*, and *San Luis Potosí*, among other states (González and Sánchez, 2018).

The main hosts of sawflies are species of the Pinaceae and Cupresaseae families, their larvae cause defoliation of trees by feeding on needles, and, although the damage caused does not in itself produce the death of the host, it leaves the latter susceptible to other types of pests and diseases that may result in severe damage and even the death of the tree (González and Sánchez, 2018). The Diprionidae family has several genera, among which *Neodiprion* spp., *Monoctenus* spp., and *Zadiprion* spp have been reported as the main ones responsible for defoliations in the forests of Mexico (González *et al.*, 2014).

At present, different methods are used to control this type of pests. One of them is chemical; although efficient, it causes severe damage to the environment and affects other organisms, wherefore the use of biological control has been sought (García-Robles *et al.*, 2001). In this regard, there are studies in which entomopathogenic fungi or bacteria are used, but they affect sawflies as well as other species; for this reason, their use is not recommended for the biocontrol of these insects (González and Sánchez, 2018). Baculoviruses, on the contrary, are

specific and efficient for the control of sawflies in Canadian forests (Moreau *et al.*, 2005).

This type of virus belongs to the Baculoviridae family, which has a double-stranded circular DNA genome ranging in size from 80 to 180 kpb. Baculoviruses exhibit two virion phenotypes: occluded viruses (ODV) and budding virions (BV) corresponding to their infective form (Wang and Hu, 2019). Those that infect sawflies belong to the genus *gamma-baculovirus*, which are characterized by forming occlusion bodies (OB) in the nucleus of the cells of the intestinal epithelium of the larvae. These occlusions, also called polyhedral, are aggregates of a viral protein called polyhedrin in nuclear polyhedrosis viruses (NPV), and granulin in granulosis viruses (GV); both protein matrices provide resistance and stability to environmental conditions.

The insect ingests the OBs, and after ingestion, the matrix surrounding them is digested in the larva's intestine due to the alkaline conditions, releasing the BVs, which initiate the systemic infection of the insect that results in its death.

Baculoviruses are classified into four genera: *alpha-baculoviruses* (NPV) and *beta-baculoviruses* (GV) affecting Lepidoptera; *gamma-baculoviruses* (NPV) affecting Hymenoptera and *Deltabaculoviruses* (NPV) affecting Diptera (Rohrmann, 2019; King *et al.*, 2012). In Canada, a concentrated suspension of NPV called Abietiv®, whose active ingredient is the *Neodiprion abietis* nuclear polyhedrosis virus, was developed for aerial application in forest areas affected by these organisms. In balsam fir [*Abies balsamea* (L.) Mill.] forests, this product proved effective in the control of sawflies (Lucarotti *et al.*, 2012).

The protocol for obtaining the viral suspension consists of the concentration of the virus from infected larvae, a product that is generated in a cyclic manner. Once the larvae are found to have died from the viral infection in the forest region where it has spread, the corpses are collected, as they are considered to be infective material to be used in the preparation of a new batch of the viral suspension. This procedure is carried out successively each time the biopesticide needs to be applied; however, its production costs are high (Moreau *et al.*, 2005).

A solution for avoiding the procedure described above is the in vitro culture of baculoviruses. For this purpose, it is necessary to implement a cell culture capable of replicating the virus and obtaining considerable quantities of it (Rohrmann, 2019).

Currently, different cultures of *alpha-baculovirus* host cells have been established, but, given their high specificity, it is very difficult to use them to grow *gamma-baculovirus* (Lucarotti *et al.*, 2012). Therefore, it is necessary to develop cell lines from different genera of sawflies in order to obtain and propagate a *Gammabaculovirus* that will affect Mexican species of diprionid wasps and thus be useful for their biological control. These baculoviruses start the infective phase and their replication in the intestine of the larvae, which, according to the bibliographic sources, is the only organ susceptible to infection; for this reason, the cell culture has to be derived from epithelial intestinal cells (Lucarotti *et al.*, 2012).

The objective of the present work is to implement a technique for obtaining a cell culture of different species of sawflies from larvae of the genera *Neodiprion* spp. and *Monoctenus* spp. Cell culture techniques applied in other arthropods were used for this purpose (Cossio-Bayugar and Miranda-Miranda, 2007; Mosqueda *et al.*, 2008) in Lepidoptera and for *alpha-baculovirus* (Summers and Smith, 1987).

Materials and Methods

Sample collection and handling

Diprionid wasp larvae were provided by the Forestry and Agricultural Health Laboratory of the Pabellón Experimental Field in *Aguascalientes* (INIFAP-CIR North Center-Cepab), collected in pine and white cedar forests in *Hidalgo* and *Guerrero*, respectively, during activities of the project Conafor 2017 C02 No. 291304. Batches of approximately 50 larvae were received, which corresponded to two genera: *Neodiprion* sp. and *Monoctenus* sp. They were stored in plastic jars at 4 °C until use.

Sample washing and dissection

Five larvae of each genus were selected and washed with different disinfectant solutions, namely, a 100 % Benzal solution. Subsequently, they were placed in a 15 mL conical tube with 5 mL of Benzal and left in agitation for 10 min. Afterwards, the Benzal was discarded, and 5 mL of 70 % ethanol were added and again stirred for 10 minutes. Finally, the samples were washed with agitation for 30 min in 5 mL of ABAM (Gibco® antibiotic-antimycotic; final concentration Penicillin 100 U mL^{-1}, streptomycin U mL^{-1}, and Fungizone (amphotericin B) 0.25 μg mL^{-1})(Cossio-Bayugar and Miranda-Miranda, 2007). The organs were dissected with a sterile scalpel blade. Two cuts were made: one in the posterior abdomen, and the other, in the anterior abdomen; this cut caused the exposure of the intestine, which was taken with forceps in the middle and placed in 1500 μL microtubes with ABAM. For the identification of the larval gut, a comparison was made with photographs of *Neodiprion abietis* (Lucarotti *et al.*, 2011). Once the intestinal tract and accessory organs were identified, the protocol for obtaining cell lines was continued as described below.

Salivary glands and cellular interstitial network

These accessory organs of the intestine were placed separately in a 1.5 mL Eppendorf tube and washed with 500 µL of ABAM for 10 min under agitation; ABAM 1X was then decanted, and 500 µL of Hank's balanced solution were added (HBSS, ThermoFisher Scientific) 1X.; then, they were centrifuged at 2 000 rpm for two minutes; Finally, the HBSS 1X solution was removed, and 500 µL of a culture medium prepared with equal parts of minimal essential medium were added (MEM; ThermoFisher Scientific). Leibovitz's L-15 medium (ThermoFisher Scientific), supplemented with 20 % of fetal bovine serum (FBS; Gibco™, ThermoFisher

Scientific), 10 % triptycasein soy broth (TSB; Sigma), an antifungal antibiotic solution (ABAM, Gibco™,ThermoFisher Scientific, a final concentration of 100 U mL^{-1} penicillin, 100 U mL^{-1} streptomycin, and B 0.25 ug mL^{-1} amphotericin) (Cossio-Bayugar and Miranda-Miranda, 2007).

The organs were suspended in the medium and added to 12-well culture plates (Corning), each containing 2.5 mL of fresh supplemented MEM. The final culture volume was 3 mL in each well of a 12-well plate; the plate was maintained at a temperature of 28 °C and in an atmosphere of 5 % CO_2 (Mosqueda *et al.*, 2008; Cossio-Bayugar *et al.*, 2011).

Intestine

The intestines obtained from the dissection were placed in a 1.5 mL Eppendorf tube with 500 µL of ABAM 1X for 10 min; at the end of the incubation time, the intestines were centrifuged at 2 000 rpm for two min to sediment the intestines; the ABAM 1X was decanted, and 500 µL of HBSS were added for washing, the centrifugation was repeated, and the HBSS 1X was also decanted. Once the intestines were clean, 500 µL of culture medium were added and mechanical disintegration was performed with a 1 000 L micropipette tip, using light pressure on the intestines. Finally, the disaggregated cells were suspended in 2.5 mL of supplemented MEM, which was in 12-well cell culture boxes. The total culture volume was 3mL per well, and the cultures were maintained at 28 °C in an atmosphere of 5 % of CO_2 (Mosqueda *et al.*, 2008; Cossio-Bayugar *et al.*, 2011; Lucarotti *et al.*, 2011).

The cultures were checked daily under a Zeiss Axiovert 40 inverted microscope.

Results

Sawfly larval gut morphology

Different organs of sawflies from the thoracic-abdominal area were identified, including the intestine, the salivary glands, and the interstitial network of cells (Figure 1a).

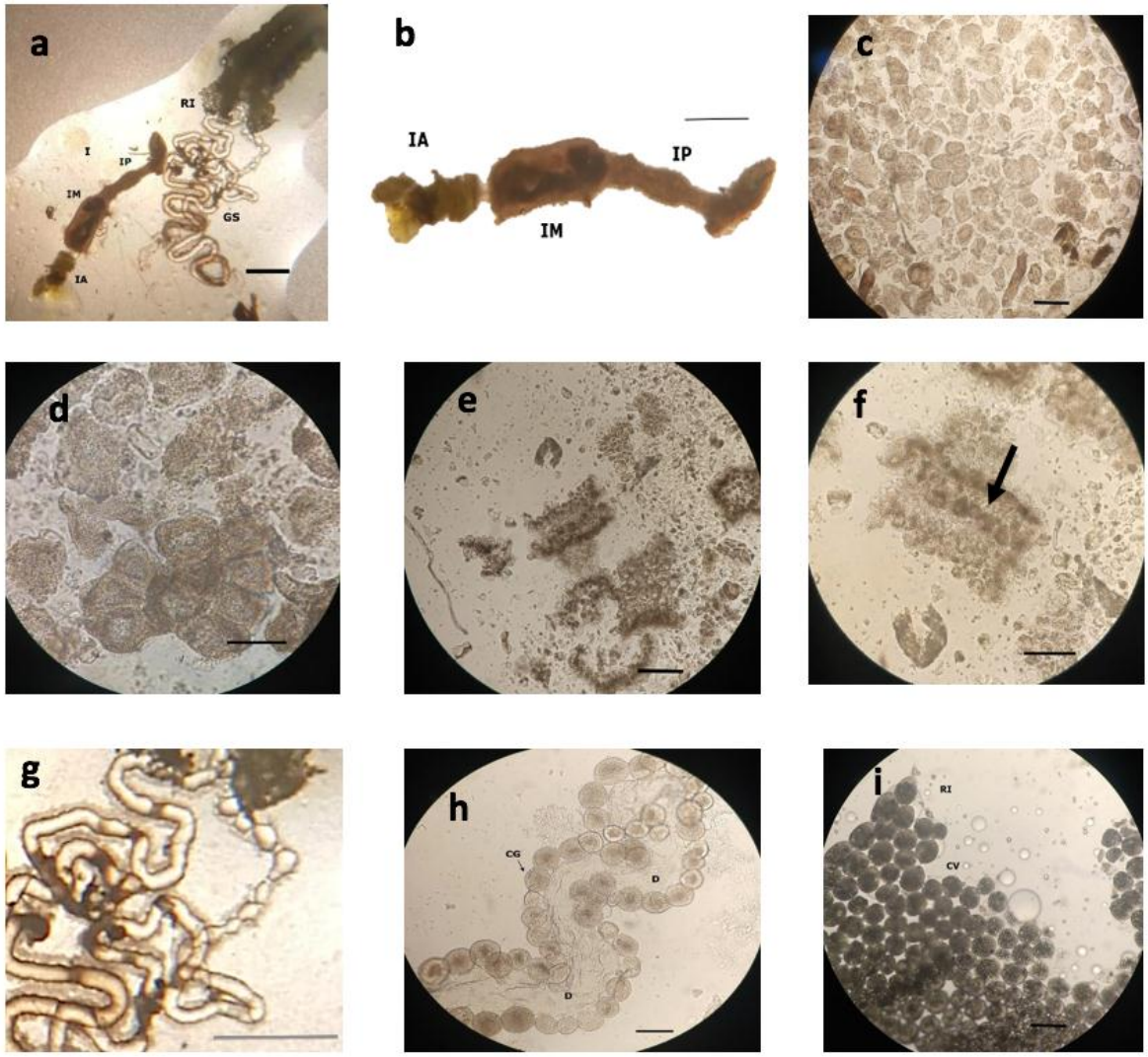

Figure 1. a. Appearance of the intestine of a *Monoctenus* sp. larva, larval intestine or alimentary canal (I), foregut (FG), midgut (MG), hindgut (HG), salivary glands (SG), cellular interstitial network (IN), bar scale 2mm. b. Intestine of *Monoctenus* sp. larvae, foregut (FG), midgut (MG) and hindgut (HG). The arrows indicate the junction that delimits each section of the intestine. In the midgut, the larval cud is

differentiated, bar scale 2 mm. c. Epithelial cells of the intestine of larvae of the genus *Neodiprion* sp. are cells of cylindrical morphology with a well-defined nucleus, scale bar 100 µm. d. Cluster of intestinal epithelial cells of *Neodiprion* sp. larvae, distinct cell nucleus (arrow), scale bar 50 µm. e. Epithelial cells of the intestine of larvae of the genus *Monoctenus* sp. are cells of cubic morphology with a well-defined nucleus, scale bar 100 µm. f. Cluster of epithelial cells from the intestine of larvae of the genus *Monoctenus* sp., well-defined cell nucleus (arrow), scale bar 25 µm. g. Salivary glands (SG) of larvae of the genus *Monoctenus* sp. bar scale 2 mm. h. Cell culture of salivary glands of *Monoctenus* sp., granulated cell (GC), elongated ducts (D). Cells surround the transparent ducts along all cell glands, bar scale 100 µm. i. *Monoctenus* sp. interstitial network cell culture, interstitial network formed by the junction of cells (IN), granulated vacuolated cell (VC), bar scale 100 µm. Images taken under the optical microscope using a 40X objective.

Intestines

The intestine is a tube sectioned into three parts: foregut, midgut and hindgut (Figure 1b). The sections are differentiated by the narrowness of the beginning of each portion. The anterior part of the intestine connects to the oral cavity, followed by the midgut which is the widest portion of the intestine and where the largest amount of food bolus is found, and therefore, is attributed the function of digesting food, and finally, the posterior region, which is the thinnest duct compared to the other sections (Figure 1b).

Intestinal cell culture of the genus *Neodiprion* sp.

Intestinal epithelial cells have very distinctive characteristics common to the cellular structure of the intestinal epithelium of arthropods; they possess a defined nucleus and have a cubic, flat, or cylindrical morphology (Lucarotti *et al.*, 2011). The cell cultures obtained were shown to have these characteristics (Figures 1c and 1d); the cells were checked every third day, and no cell proliferation and growth were observed. The approximate duration for this culture was one month; since no proliferation or growth was recorded, it was considered a primary culture of larval intestine. The culture showed good adaptation to the MEM medium and to the established culture conditions. The formation of clusters (Figure 1d) of cells and cell debris in the culture is due to the type of disaggregation applied.

Intestinal cell culture of the genus *Monoctenus* sp.

The cultures showed the same morphological characteristics as the epithelial cells; however, in comparison to the cells from *Neodiprion* sp., they are smaller and more elongated (Figure 1f). Also, they highlighted clusters of small groups of cells that did not disintegrate properly, cell culture time approached one month.

Salivary glands

Salivary glands (Figure 1g) were observed in a large proportion, flanking the larval alimentary canal; they are a pair of elongated transparent ducts that are surrounded by a large number of granulated cells.

Salivary gland cell culture from the salivary glands of *Neodiprion* sp.

It is an organ resistant to manipulation which exhibited excellent tolerance to the culture medium. It was considered as a primary culture since it did not show cell proliferation; the duration of the culture was above 3 months, and the cells did not adhere to the culture plates. Due to its longevity in vitro, it is considered ideal for infection assays (Figure 1h).

Interstitial cell network

It is formed by a group of granulated cells joined together. It surrounds the intestine and the salivary glands, and its function could be fundamental in the change of molt of the larva (Figure 1a).

Cell culture of interstitial network of *Neodiprion* sp.

The cell culture was maintained for about 1 month with culture medium. There was no cell adhesion to the culture plate, and the cells did not exhibit any type of proliferation or growth because it is a primary culture (Figure 1i).

Discussion

Sawflies are hymenopterans of the Diprionidae family that cause defoliation of up to 60,000 ha per year (González and Sánchez, 2018). This fact has led to the search for efficient and safe biological control methods that will not cause damage to the

environment or to endemic species that are not subject to control. The use of baculovirus has proved efficient to treat this type of pests. There are numerous examples of commercial production of alpha baculoviruses for agricultural pest control (Grzywcz *et al.,* 2014; Grzywcz and Moore, 2017.), but only a few examples of the production of viral suspensions for the control of sawflies. An example is the use of *Gammabaculovirus* NeabNPV, which was purified and sequenced by Canadian researchers, increasing the mortality of *Neodiprion abietis* and reducing the endemic levels of involvement (Moreau *et al.*, 2005).

During the present study, several sawfly cell lines were established from the intestines and intestinal accessory organs, being regarded as an essential tool for the procurement, characterization and propagation of a *gamma-baculovirus utili* in the biological control and cellular metabolism studies of these forest pests in Mexico.

The production of a cell culture can take months or even years; today, there is no record of a cell line of sawflies of the family Diprionidae in Mexico. The results obtained reflect a breakthrough in the establishment of an intestinal epithelial cell line. This cell type is the only one that can incubate a virus for replication and propagation (Lucarotti *et al.*, 2012). It is therefore important to have a method for their cultivation and to define the appropriate conditions for the incubation of the cells.

There are cellular differences between the different genera of flies (*Neodiprion* and *Monoctenus*), especially in shape and size. *Monoctenus* cells are smaller and cubic in shape; while *Neodiprion* cells are cylindrical and larger. Both cell cultures lasted approximately one month. The conditions established were adequate for their maintenance, and although there was no cell proliferation or growth, they are good candidates for infection with baculovirus, since the viruses can multiply in the cell nuclei of intestinal epithelial cells. The above was observed in a remarkable way, as a defined nucleus encompassed a large part of the cell.

The clusters of cells formed in the cultures could be due to a deficiency in mechanical disaggregation; hence, the use of a chemical method for the separation of cells using enzymes such as collagenase, trypsin, elastase, papain or pronase is not ruled out (Beltrán and Gonzales, 2016). This type of enzymes may help with proper tissue disaggregation and may be used in conjunction with mechanical disaggregation. Due to the type of disaggregation used, the culture showed a large amount of cellular sediments; therefore, the implementation of the use of a filter that will help in the separation of the cells with the cellular sediments is suggested, as it would result in a cleaner culture, without sediments at the bottom of the culture plate.

Washing after organ dissection is an important step to prevent any contamination in the culture; although the cultures were carried out strictly and always under sterile conditions, the intestine cultures were contaminated by unidentified yeasts. Notably, the antifungal was at twice the concentration in the medium used. Contamination of gut cultures may be attributed to larval gut flora. This may be prevented by separating the the cells through filtration; thus, the organisms responsible for the contamination would not be cultured together with the intestinal epithelial cells. The medium used is for arthropod culture (Cossio-Bayugar and Miranda-Miranda, 2007).

The absence, in the present study, of results on the functioning of cultures obtained from salivary glands and the cellular interstitial network does not preclude their use in further research to understand biological issues of the larvae of the Diprionidae family. These cultures are easier to handle than gut cultures, as they are less likely to be contaminated. However, these tissues are not fixed but remain floating in the medium; therefore, we propose reducing the volume of MEM culture medium or the size of the culture plate.

Conclusions

The establishment of a cell culture of intestinal epithelial cells is possible following the protocol described in this work. Each genus of larvae can have cells with morphological differences; therefore, we propose the study of the three genera of flies existing in the Mexican Republic (*Monoctenus*, *Neodiprion*, and *Zadiprion*). The incubation conditions of the cells are suitable for the maintenance of the cultures: 28 °C temperature and atmosphere of 5 % of CO_2. MEM-Leibovitz L-15 (1:1) culture medium supplemented with 20 % SFB and 10 % trypticasein soy broth is suitable for cell maintenance and nutrition; cell exchange or aggregation is required to prolong the life of cell cultures. Established primary cultures are candidates for the identification, characterization, proliferation and maintenance of a *gamma-baculovirus* due to the morphological characteristics of the cells.

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Conflict of interests

The authors declare no conflict of interests.

Contribution by author

Tania Rocío Tapia-Uriza, Raquel Cossío-Bayúgar and Estefhan Miranda-Miranda: study design, experimental execution and manuscript elaboration; Ernesto González-Gaona, Karla Vanessa De Lira-Ramos and Yahaira Elizabeth Rodríguez-Cruz: sampling, review, analysis and discusión of the document.

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