Centro de Investigación en Alimentación y Desarrollo, A.C.

EFECTO DE LA INTERACCIÓN DE BACULOVIRUS DE *Spodoptera frugiperda* DURANTE EL PROCESO DE INFECCIÓN MÚLTIPLE

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Esta tesis la dedico **a Dios**, por darme la salud, fuerza y sabiduría para salir adelante

A **mis padres, mis hermanos y mis sobrinos**, por ser el pilar que me motiva a salir adelante, por su amor y el apoyo que me dan a cada instante.

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"Quien nunca ha cometido un error, nunca ha intentado nada nuevo".

Albert Einstein

CONTENIDO

RESUMEN

Los virus entomopatógenos, especialmente los de la familia Baculoviridae, son excelentes agentes de control biológico de insectos plaga, entre ellos, el gusano cogollero *Spodoptera frugiperda*, principal plaga del maíz en México y otros países. El objetivo del estudio fue caracterizar aislados nativos de Nucleopoliedrovirus y Granulovirus de *S. frugiperda* y evaluar el efecto de coinfecciones sobre la actividad insecticida de aislados de ambos géneros virales. Los Nucleopoliedrovirus y Granulovirus se caracterizaron; morfológicamente mediante microscopía electrónica de barrido (MEB) y de transmisión (MET), biológicamente determinando la dosis letal media (DL_{50}) y tiempo letal medio (TL_{50}) y molecularmente mediante enzimas de restricción. De los ocho Nucleopoliedrovirus (NPVs) evaluados, se seleccionó el aislado SfCH32-NPV y se probó en coinfección con el aislado SfCH13-GV de Granulovirus contra larvas de *S. frugiperda*, mediante bioensayos suministrando dosis virales letales en los tiempos cero, 12 y 24 h, después de haber suministrado el primer aislado viral. Las larvas infectadas por Nucleopoliedrovirus mostraron rompimiento de cutícula a partir de las 72 horas post-infección (hpi). Los aislados SfCH32-NPV y SfCH15-NPV fueron los más virulentos con dosis letales medias de 5.6×10^2 y 6.4×10^2 COs/larva, a los 14 dpi, respectivamente. Ambos aislados fueron diferentes, con un genoma de ~ 128 kb y 132 kb, respectivamente y sus cuerpos de oclusión (COs) mostraron formas y tamaños (1.02-2.24 µm) irregulares. El TL_{50} más bajo (114.5 h) se observó con el aislado SfCH15-NPV al usar la concentración más alta (2.5×10^6 COs/larva). Los Granulovirus SfGV-CH13 y SfGV-CH28 correspondieron al tipo I y mostraron los mismos perfiles de restricción de ADN con un genoma de aproximadamente 126 kb, sus COs fueron ovoides con un tamaño de 0.4 µm. Las primeras larvas muertas por los GVs se registraron a partir de los 12 dpi. Los valores de DL_{50} para SfGV-CH13 y SfGV-CH28 fueron de 5.4×10^2 y 1.1×10^3 COs/larva, a los 45 dpi, respectivamente. El TL_{50} osciló entre 17 y 24 días. En los bioensayos de coinfección, donde las dosis virales se suministraron al tiempo cero, el aislado de NPV no se vio influenciado. Sin embargo, se observó un efecto antagónico en la actividad insecticida del NPV al suministrarlo 12 o 24 h después de haber suministrado el GV. Se observó, un aumento en el tiempo de supervivencia (~ 13 días) en las larvas coinfectadas con la dosis más baja (DL_{10}) del NPV y más alta (DL_{90}) del GV.

Palabras clave: Baculovirus; Gusano cogollero; Control biológico; Interacción, Plaga.

ABSTRACT

Entomopathogenic viruses especially Baculoviridae family, are excellent biological control agents for insect pests, among them the fall armyworm (FAW) *Spodoptera frugiperda*, the main pest of corn in Mexico and other countries. The aim of the study was to characterize native isolates of *Spodoptera frugiperda* Nucleopolyhedrovirus and Granulovirus and to evaluate the effect of coinfections on the insecticidal activity of isolates of both viral genera. Nucleopolyhedrovirus and Granulovirus were characterized by scanning electron microscopy (SEM) and transmission (TEM), biologically by determining the median lethal dose (LD_{50}) and median lethal time (LT_{50}) and molecularly by restriction enzymes. Of the eight evaluated Nucleopolyhedroviruses (NPVs), the SfCH32-NPV isolate was selected and tested in coinfection with the SfCH13-GV Granulovirus isolate against *S. frugiperda* larvae, through bioassays supplied lethal viral doses at times zero, 12 and 24 h, after having supplied the first viral isolate. Infected larvae by Nucleopolyhedrovirus showed lysis of the integument at 72 hours post-infection (hpi). The most virulent isolates were SfCH32-NPV and SfCH15-NPV with median lethal doses of 5.6×10^2 and 6.4×10^2 OBs / larva, at 14 dpi, respectively. Both isolates were different, with a genome of ~ 128 kb and 132 kb, respectively, and their occlusion bodies (OBs) showed irregular shapes and sizes (1.02-2.24 µm). The lowest LT_{50} (114.5 hpi) was observed with the SfCH15 isolate at the highest concentration (2.5×10^6 OBs/larva). The SfGV-CH13 and SfGV-CH28 Granuloviruses belonged to type I, they showed the same DNA restriction profiles with a genome of approximately 126 kb and their OBs showed ovoid shapes with a size of 0.4 µm. The first larvae killed by the GVs were recorded from 12 dpi. The LD_{50} values for SfGV-CH13 and SfGV-CH28 isolates were 5.4×10^2 and 1.1×10^3 OBs/larva, at 45 dpi, respectively. The LT_{50} ranged from 17 to 24 days. In coinfection bioassays, where viral doses were supplied at zero time, the NPV isolate was not influenced. However, an antagonistic effect was observed on the insecticidal activity of NPV when it was supplied 12 or 24 h after having supplied the GV. An increase in survival time (~ 13 days) was observed in larvae coinfected with the lowest dose (LD_{10}) of NPV and the highest dose (LD_{90}) of GV.

Keywords: Baculoviruses; Fall armyworm; Biological control; Interaction; Pest.

1. SINOPSIS

1.1. Justificación

El gusano cogollero *Spodoptera frugiperda* (J. E. Smith; Lepidoptera: Noctuidae)*,* es considerada la principal plaga del maíz provocando cuantiosas pérdidas económicas en varios países, incluido México. Actualmente, el control de este insecto está basado en el uso de insecticidas químicos; sin embargo, dados los inconvenientes que estos generan a la salud humana y al ambiente, es necesario generar nuevas alternativas amigables con el ambiente e inocuas a los humanos. Una alternativa al uso de insecticidas químicos para el manejo de esta plaga, es el uso de agentes de biocontrol, especialmente virus entomopatógenos de la familia Baculoviridae. A pesar de que los baculovirus han demostrado gran potencial insecticida como biocontroladores de lepidópteros plaga, el desarrollo de bioinsecticidas a base de estos ha sido limitado por múltiples factores, tales como su estrecho rango de hospederos (alta especificidad), costos de producción elevados, rápida inactivación por los rayos UV y altas temperaturas, así como el tiempo requerido para matar al insecto objetivo, en comparación con los insecticidas químicos. Debido a estos inconvenientes, resulta primordial realizar investigaciones encaminadas a evaluar su potencial insecticida e incrementar la eficacia de estos entomopatógenos bajo condiciones de laboratorio y campo. Dado que, se ha documentado que la efectividad de los baculovirus como bioinsecticidas es altamente dependiente del origen geográfico, siendo más efectivos contra insectos de la misma región. Aunado a lo anterior, se han documentado infecciones múltiples en un mismo insecto hospedero por Nucleopoliedrovirus y Granulovirus lo cual nos permite hipotetizar posibles efectos como el antagonismo, neutralismo, mutualismo, comensalismo, amensalismo, y sinergismo, derivados de la interacción entre diferentes baculovirus. A la fecha, los estudios referentes a la caracterización de baculovirus de *S. frugiperda* son limitados y los existentes se centran en Nucleopoliedrovirus, adicionalmente no se han documentado los efectos de evaluar Nucleopoliedrovirus y Granulovirus en combinación sobre larvas de *S. frugiperda* a diferentes intervalos de infección. Con base en lo anterior, se caracterizaron aislados nativos de Nucleopoliedrovirus y Granulovirus de *S. frugiperda* y se condujeron bioensayos con ambos géneros virales en combinación para evaluar su potencial insecticida sobre larvas de *S. frugiperda*.

1.2.1 Problemática

El maíz (*Zea mays* L.) es el grano más ampliamente cultivado e importante a nivel mundial, ya que es la base de la alimentación en gran parte del mundo (Yang *et al.*, 2017). *Spodoptera frugiperda*, es considerada como la principal plaga del maíz en México y otros países, al ocasionar pérdidas económicas considerables, reduciendo la producción hasta un 30%, al alimentarse de hojas, cogollos tiernos y mazorcas (Casmuz *et al.*, 2010; Ramirez-Cabral *et al.*, 2017). El control de este insecto requiere varias aplicaciones de insecticidas (piretroides, diamidas y benzoilureas), generando poblaciones resistentes, además, se ha documentado daños colaterales a insectos benéficos, especialmente, pérdidas poblacionales de polinizadores, depredadores y parasitoides al estar en contacto con los plaguicidas (Abdu-Allah y Pittendrigh, 2018; Ahmad *et al.*, 2018; Fernandes *et al.*, 2019; Hardke *et al.*, 2011). El uso de enemigos naturales representa una alternativa al control químico porque reducen la población de la plaga sin ocasionar efectos negativos en los seres humanos y el medio ambiente (Koffi *et al.*, 2020; Meagher Jr *et al.*, 2016; Rios-Velasco *et al.*, 2011a; Sisay *et al.*, 2018).

1.2.2 *Spodoptera frugiperda*

El gusano cogollero *S. frugiperda*, es un insecto plaga polífago y cosmopolita, el cual tiene una gran diversidad de hospederos (maíz, arroz, sorgo, caña de azúcar, algodón entre otros), siendo su hospedero principal el cultivo del maíz, por lo que es considerado como la principal plaga de este cultivo (Casmuz *et al*., 2010). Esta plaga se encuentra ampliamente distribuida en el continente americano y recientemente la plaga se ha convertido en una nueva especie invasora en África occidental y central, donde se registraron brotes por primera vez a principios del año 2016 (Georgen *et al*., 2016).

El gusano cogollero tiene un ciclo de vida que incluye cuatro etapas: huevo, larva, pupa y adulto. Los huevos son colocados generalmente en el envés de las hojas, una hembra llega a poner de 100 a 200 huevos. La larva tiene 6 etapas de crecimiento, solo de 1 a 2 larvas se encuentran por planta, debido a que conforme se desarrolla la larva se vuelven caníbales. La pupa es de color marrón brillante y el adulto es de color marrón oscuro. Su control se lleva a cabo de manera principal mediante productos químicos, aunque también se ha empleado el control biológico mediante el uso de bacterias (*Bacillus thuringenesis*), hongos (*Beauveria basssiana*) y baculovirus (Rijal, 2019).

1.2.3 Baculovirus

1.2.3.1 Clasificación. Dentro de los enemigos naturales reportados hacia *S. frugiperda*, destacan los virus entomopatógenos, las enfermedades por virus en insectos han sido encontradas ocasionadas al menos por 12 familias. Sin embargo, el conocimiento de la mayoría de estas es muy escaso (Blissard, 2000). Dentro de las familias de virus patógenos a insectos encontramos a la familia Entomopoxviridae, Reoviridae, Baculoviridae, entre otras, no obstante, de los virus entomopatógenos la familia Baculoviridae es la más numerosa y estudiada por su potencial uso para el control de plagas, dado que infectan únicamente a artrópodos y muchos son muy específicos (Caballero *et al*., 2001; Erlandson *et al.,* 2008).

La familia Baculoviridae es la más representativa entre los virus patógenos a artrópodos, el nombre deriva de la forma que poseen sus viriones (baculum=bastón), se caracteriza por poseer un genoma de ADN de doble cadena circular superenrollado, empaquetado dentro de una cápside baciliforme, cubierta por una envoltura lipoproteica (Caballero *et al*., 2001). Los baculovirus han sido usados contra plagas insectiles desde 1930 y a partir de 1983 se han empleado para expresar genes recombinantes (Hüser y Hofmann, 2003). Se han encontrado infectando más de 600 especies de insectos principalmente del orden Lepidoptera.

La familia Baculoviridae inicialmente con base en sus características morfológicas fue agrupada en dos géneros, nucleopolyhedrovirus y Granulovirus (Barreto *et al*., 2005). Más tarde, el análisis comparativo entre distintos genomas de baculovirus secuenciados y el estudio de su relación filogenética dio pie a la clasificación en cuatro grupos: Alphabaculovirus (NPVs de lepidópteros), Betabaculovirus (GVs de lepidópteros), Gammabaculovirus (NPVs himenópteros) y Deltabaculovirus (NPVs de dípteros) dado que se observó que los NPVs de dípteros e himenópteros debían estar en una agrupación distinta a la de los lepidópteros (Herniou *et al.*, 2003, Jehle *et al.*, 2006). Siendo los Alphabaculovirus (Nucleopoliedrovirus; NPVs) y Betabaculovirus (Granulovirus; GVs) los más estudiados (Barreto *et al.*, 2005; Herniou *et al.*, 2003; Jehle *et al.*, 2006).

Los baculovirus son una alternativa prometedora al uso de insecticidas químicos, al ser usados como bioinsecticidas seguros y efectivos para el control de plagas insectiles, además, la producción comercial de baculovirus para el control de plagas utilizando insectos hospedadores cultivados es factible, sin embargo, su uso se ha limitado dado su lento modo de acción y su estrecho rango de hospederos (Kamita *et al*., 2010; Grzywacz y Moore, 2017).

1.2.3.2 Nucleopoliedrovirus. Los Nucleopoliedrovirus (Alphabaculovirus), se han aislado de más de 600 especies de diferentes órdenes de insectos, principalmente del orden Lepidoptera, seguido de Hymenoptera, Díptera, Coleóptera y Trichoptera así como también, de ciertas especies del orden Decapoda clase Crustacea (Martínez *et al*., 2012). El tamaño de los cuerpos de oclusión (COs) varía entre 0.5 y 15 µm, con un tamaño promedio entre 0.8 y 2 µm, pueden presentar diversas formas según la especie del virus (cubo, tetraedro, icosaedro, dodecaedro o diversas formas irregulares).

Los Nucleopoliedrovirus (Alphabaculovirus) pueden ser simples o múltiples dependiendo del número de viriones contenidos en los cuerpos de oclusión (COs) que generalmente son poliédricos, es decir, son simples si contienen una nucleocápside por envoltura viral o múltiples cuando están conformados por dos o más nucleocápsides (Herniou *et al.*, 2003). La nucleocápside consiste en una cápside cilíndrica, cubierta en los extremos y en su interior contiene el núcleo donde se alberga

el material genético del virus (ADN) de forma enrollada y condensada, su estructura está conformada por una serie de anillos apilados, formados por subunidades proteicas, tiene un diámetro promedio entre 30 y 60 nm y una longitud entre 250 y 300 nm y su función es llevar la información genética del virus, hacia la célula huésped (Caballero *et al*., 2001).

Los viriones son los principales elementos infecciosos de los baculovirus tanto en la dispersión entre los diferentes individuos como a nivel celular en los diversos órganos y tejidos, el virión maduro se forma cuando la nucleocápsida adquiere la envoltura o membrana, que puede proceder de dos orígenes distintos dando lugar a dos fenotipos (ODV: viriones derivados de los cuerpos de inclusión y BV: viriones brotados), los cuales poseen el mismo genoma, pero difieren en el fenotipo y función dentro del ciclo de infección (Caballero *et al*., 2001; Haase *et al*., 2015).

Los viriones ODV se forman cuando las nucleocápsidas permanecen en la célula en que fueron sintetizadas adquiriendo una membrana de *novo,* y posteriormente quedan incluidos en una matriz de proteína que codifica el virus. pueden contener una sola nucleocápsida por virión (simple) o múltiples nucleocápsidas (múltiple) y son los responsables de la trasmisión horizontal. Por otra parte, los viriones BV se forman cuando la nucleocápsidas ya formadas, salen de la célula de huésped y adquieren la membrana a partir de la membrana del citoplasma de la célula huésped, estos viriones son los responsables de diseminar la infección a través del hemocele del insecto, de célula a célula (infección secundaria) (Wood *et al*., 1993; Kamita *et al*., 2010).

Al microscopio óptico se observan con facilidad dado su tamaño, y en contraste de fases producen un brillo característico al ser altamente refráctiles. Por otra parte, el microscopio electrónico contribuye a la determinación del tipo morfológico de los NPVs (simples o múltiples) (Torquato *et al*., 2006). Los nucleopolyhedrovirus son capaces de causar epizootias generalizadas que impactan grandemente el tamaño de varias poblaciones de insectos, sin embargo, también podrían presentar efectos negativos, por ejemplo, se ha visto que estos NPV, pueden infectar al gusano de la seda *Bombyx mori* (Lepidoptera: Bombycidae), afectando la producción de seda y, por lo tanto, es una gran preocupación para los productores de esta tela (Ikeda *et al*., 2015).

1.2.3.3 Granulovirus. Los Granulovirus (GVs) se han aislado de más de 100 especies pertenecientes al orden Lepidoptera. Su espectro de huéspedes es más restringido que el de los NPVs, aunque son pocos los estudios realizados al respecto. El tamaño y forma de los cuerpos de oclusión (COs) es de manera general homogéneo entre las diversas especies, con una forma granular u ovoide los GVs, contienen un solo cuerpo de oclusión compuestos por granulina (Moscardi, 1999).

Los Granulovirus producen COs en forma cilíndrica, llamados gránulos, que se encuentran típicamente en las células infectadas después de la ruptura de las membranas nucleares debido a la multiplicación extensa del virus, en comparación con los NPVs, los gránulos son pequeños, aproximadamente 0.3×0.5 μm, y típicamente contienen un solo virión, o raramente dos o más viriones (Ikeda *et al.*, 2015). En campo oscuro mediante observación al microscopio óptico, los COs de los GVs se pueden apreciar como puntos brillantes con movimiento browniano y en contraste de fases como pequeños puntos grises (Caballero *et al*., 2001).

La enfermedad ocasionada por los GVs denominada granulosis que se caracteriza por la presencia de inclusiones granulares diminutas en las células infectadas, fue detectada por primera vez por Paillot en 1926, en las larvas de *Pieris brassicae* (Lepidoptera: Pieridae) (Capinera, 2008). Los GVs tienen un espectro de huéspedes más limitado que los NPVs infectan a varias especies de insectos, pero a diferencia de los NPVs, estos solo han sido encontrado en insectos del orden Lepidoptera. Así pues, Los Granulovirus han sido poco estudiados en comparación con los Nucleopoliedrovirus (Caballero *et al.*, 2001).

1.2.3.4 Ciclo de infección. El mecanismo que utilizan los virus para llevar a cabo la infección en el núcleo de las células depende de su tamaño y estructura, así como de las señales celulares empleadas para desensamblar la cápside y el genoma (Cohen *et al*., 2011). El suelo es el principal reservorio de los baculovirus, estos, por acción del viento o parasitoides llegan hasta las plantas de los cultivos donde son ingeridos por el insecto plaga en forma de cuerpos de oclusión (COs) (Dias-Vasconcelos, 2011).

Los COs viajan a través del intestino del insecto hasta llegar al intestino medio donde las proteínas cristalizadas (poliedrina o granulina) son solubilizadas por acción del pH alcalino que prevalece en el mesénteron y a la acción de una proteasa alcalina propias del huésped. Una vez que se da el rompimiento de los COs, los viriones son liberados fusionándose a las células epiteliales mediadas por la acción de proteínas de anclaje de la membrana del virión. Dada la fusión son desprendidas las nucleocápsidas las cuales llegan al núcleo para iniciar la replicación. Tras la replicación en el núcleo de las células intestinales, los viriones brotan hacia la hemolinfa, causando la infección secundaria dentro del hemocele, las partículas virales entran por endocitosis, las nucleocápsides viajan hacia el núcleo y liberan el ADN, comenzando de nueva cuenta el ciclo de replicación, dando lugar a la formación de nuevos BV y ODV. Finalizando con el rompimiento del tegumento de la larva y la liberación de los cuerpos de oclusión al ambiente, los cuales quedan listos para iniciar de nueva cuenta el ciclo de infección (Caballero *et al*., 2001).

No obstante, se considera que el genoma de los NPVs y GVs podría entrar en el núcleo por mecanismos diferentes, las proteínas o receptores virales y celulares que intervienen en la fase inicial, el paso de unión de la cápside con el poro nuclear, y el desensamble de la nucleocápside, aunque se tienen algunos avances en su estudio aún no está bien esclarecido su mecanismo de acción (Cohen *et al*., 2011).

En los NPVs la replicación y morfogénesis de los cuerpos de oclusión ocurre en el núcleo de las células infectadas, produciendo una hipertrofia nuclear característica, donde la progenie viral aparece entre las 8 y 24 horas post-infección (hpi). En los GVs a diferencia de los NPVs la replicación inicia en el núcleo, pero generalmente inmediatamente se produce la ruptura de la membrana nuclear y por tanto el ciclo y morfogénesis viral continúa en la mezcla resultante de componentes citoplasmáticos y nucleares, observando la presencia de nucleocápsides a las 36 hpi (Sciocco-Cap, 2001).

1.2.3.5 Sintomatología. La infección por ambos géneros virales a menudo provoca la muerte del insecto hospedero (Kroemer *et al.*, 2015). Las larvas infectadas presentan síntomas característicos varios días después de la infección. Observándose el cese de la alimentación dejando de alimentarse de manera paulatina, cambio en la coloración del tegumento con la transformación fenotípica de

las larvas infectadas donde comienza con la hinchazón del cuerpo larvario y la posibilidad de obtener un color lechoso y blanquecino de la cutícula y fragilidad de la misma hasta su ruptura cuando la infección es avanzada, liberando los COs producidos durante la infección al medio ambiente por la descomposición de la cutícula (Gaganidze *et al*., 2014; Sauer *et al*., 2017; Vásquez *et al*., 2002). Los Nucleopoliedrovirus matan a su hospedero en ~5 días post-infección (dpi) (Barrera *et al.*, 2011; Ordóñez-García *et al.*, 2020). Mientras que los Granulovirus lo hacen a partir del día 7 hasta los 45 dpi (Hatem *et al.*, 2011; Ahmet B Inceoglu *et al.*, 2001; Pidre *et al.*, 2019).

No obstante, los Granulovirus pueden clasificarse en tres tipos, dependiendo del tropismo de infección que muestran hacia determinados tejidos del lepidóptero hospedero. Los del tipo I (de infección monoorganotrópica o muerte lenta) se restringen al tejido adiposo (cuerpo graso) del insecto y estos se siguen alimentando por periodos prolongados, por ende, su tamaño se incrementa y se convierte en un saco de partículas virales infectivas ya que el tegumento no se rompe, los mayores porcentajes de mortalidad se han documentado a partir de los 17 dpi, hasta los 28 dpi (Cuartas *et al.*, 2014; Pidre *et al.*, 2019). Las características patológicas de los GVs del tipo II (poliorganotrópicos) son similares a las de los Nucleopoliedrovirus, ambos infectan el cuerpo graso, matriz traqueal y epidermis, en consecuencia matan rápidamente (5-6 días) a sus hospederos, y el tipo III que son monoorganotrópicos están limitados al intestino medio del insecto, la muerte larval se produce a los 7 dpi, este tipo de virus ha sido reportado únicamente en *Harrisina brillians* (Barnes & McDunnough; Lepidoptera: Zygaenidae) (Federici y Stern, 1990; Sciocco-Cap, 2001).

1.2.3.6 Espectro de huéspedes. La diversidad de organismos causantes de enfermedades en otros animales, particularmente invertebrados, ha recibido mucha menos atención que en mamíferos, sin embargo, investigar las consecuencias de la diversidad de la infección para la gravedad de la enfermedad y la epidemiología probablemente sea crucial si queremos entender la ecología y la evolución de las interacciones huésped-patógeno, hay muchos ejemplos de epizootias de enfermedades en insectos, tal es el caso de los baculovirus, que generalmente tienen un espectro insecticida estrecho, por lo que conocer los mecanismos mediante los cuales controlan el rango de hospederos es un prerrequisito para mejorar sus aplicaciones, especialmente como pesticidas (Cory *et al*., 2005; Wu *et al*., 2016).

Según su relación filogenética, los baculovirus se clasifican en cuatro géneros, que también reflejan su rango de hospedadores y, en cierta medida, la morfología del cuerpo de los COs, los miembros de los géneros Alphabaculovirus y Betabaculovirus solo infectan a Lepidópteros, mientras que los virus de Gammabaculovirus y Deltabaculovirus son específicos para las especies de Himenópteros y Dípteros (Gueli *et al*., 2017).

Así mismo, para muchos virus de invertebrados, la infección *per os* es el primer paso de la invasión en un hospedero, y este es el paso clave para determinar el rango de hospederos que pudieran verse afectados, así como determinar la virulencia de un virus (Wang *et al*., 2017). Por otra parte, la dinámica de las interacciones patógeno-hospedero en los insectos está determinada por la densidad del hospedero y del patógeno, además de la transmisión y virulencia de este último (Myers y Cory, 2016). Los GVs tienen un espectro de huéspedes más limitado que los NPVs infectan a varias especies de insectos, pero a diferencia de los NPVs, estos solo han sido encontrado en insectos del orden Lepidoptera, no obstante, se ha constatado que algunos GVs como *Cydia pomonella*, *Heliothis armigera* y *Scotogramma trifolii* tienen un espectro de huéspedes más amplio abarcando a dos o más géneros.

Así pues, estos virus han sido poco estudiados en comparación de los NPVs, tanto biológica como molecularmente, en los casos en los cuales se ha verificado la infección cruzada de ambos géneros, no se ha confirmado la dosis requerida para causar la infección ni la identidad del virus causante (Caballero *et al*., 2001).

1.2.3.7 Genómica. Los virus presentan una variación genética generalizada debido a diversos procesos de adaptación. Se cree que las grandes poblaciones de virus de ADN albergan poca variación, aunque las poblaciones naturales pueden ser polimórficas, ya que la selección natural propicia que los genomas presenten mutaciones beneficiosas, de modo que estos genomas mejor adaptados permanezcan en una alta frecuencia en una población dada, ya que, después de la infección primaria ocasionada por pocas partículas infecciosas, las poblaciones de virus aumentan rápidamente, pero por lo general incurren en altos índices de mutación, que puede traer mutaciones

desfavorables para genomas individuales, pero también mutaciones beneficiosas permitiendo que el virus para evadir de la respuesta inmune del huésped (Chateigner *et al*., 2015).

El genoma de los baculovirus está formado por ADN circular con un tamaño entre 90 y 160 kb, siendo el tamaño del genoma similar entre los diversos virus baculovirus secuenciados, estos genomas son grandes en comparación a otros virus, pero pequeños respecto al de procariotas y eucariotas, los genes más conservados son el de la poliedrina (NPVs) o granulina (GVs) y la ubiquitina, la mayoría de los genes implicados en la replicación del ADN viral y la trascripción de los genes, están presentes en todos los baculovirus (Luque, 2000a).

El primer baculovirus secuenciado fue el de *Autographa califórnica* NPV (AcMNPV) (Ayres *et al*., 1994). Sin embargo, el número de genomas de baculovirus secuenciados ha ido en aumento, al año 2008 más de 40 genomas de baculovirus habían sido secuenciados, los cuales aportan información imprescindible para describir los genes de los diversos virus (Wolff *et al*., 2008). Más tarde, el comparativo del total de genes encontrados en 57 genomas secuenciados, revela que poseen 157 ORF (Miele *et al*., 2011).

Los baculovirus comparten varios genes que son comunes entre los diversos baculovirus también llamados genes conservados, de un total de 13 genomas secuenciados 30 fueron homólogos, incluyendo genes que actúan durante las diferentes etapas, por ejemplo, la mayoría de los genes centrales codifican factores que son cruciales para el ciclo de vida y la singularidad de los baculovirus, e incluyen proteínas responsables de la replicación del ADN viral, transcripción del gen viral, arquitectura del virión, empaquetamiento del ADN viral y ensamblaje del virión e infección *per os* (Herniou *et al*., 2003; Ikeda *et al*., 2015). El conocimiento acerca del comportamiento de los genes (perdida, ausencia etc.) ha sido objeto de múltiples investigaciones en los últimos años, las cuales están encaminadas al descubrimiento y mejor entendimiento del comportamiento de estos virus a nivel genómico (Herniou *et al*., 2003; Miele *et al*., 2011).
Avances recientes en biotecnología ha hecho posible la expresión de genes en diferentes organismos, sin embargo, la expresión de genes pertenecientes a otro organismo aun es limitada y poco satisfactoria, por lo que la expresión de genes resulta un área de interés científico y tecnológico. Muchos genes implicados en la replicación y expresión del ADN están presentes en

todos los genomas de baculovirus, presumiblemente reflejando sus funciones críticas en la replicación del virus (Luque *et al*., 2001b). Además de los genes estructurales y los implicados en la replicación y trascripción del ADN, los baculovirus poseen otros genes que le proporcionan una ventaja selectiva.

En los GVs uno de los genes que ha recibido mayor atención es el gen enhancina (vef, viral enhancing factor) que contribuye a la propagación del virus en el organismo. Ya que VEF es una proteína que ocasiona la disrupción de la membrana peritrófica permitiendo el acceso de los viriones a las células (Luque *et al*., 2001a).

Por otra parte, con la llegada de la tecnología del ADN recombinante en el área de la ingeniería genética, se ha logrado potenciar el campo enfocado a la investigación de virus para el control de insectos, ya que un gran número de compañías privadas y varios grupos de académicos actualmente se encuentran contribuyendo en esta área, enfocándose principalmente en mejorar la velocidad de muerte sobre insectos plaga por parte de virus recombinantes, así como la expresión de diversas proteínas ajenas a los virus (Bonning y Hammock, 1996).

Diversos estudios esenciales se han realizado para la identificación final de los genes o componentes individuales de la estructura del virión lo que permite entender la arquitectura del virion y dar paso a posibles inserciones de genes ajenos que permitan mejorar las propiedades del virus (Rohrmann, 1992). El primer baculovirus que se secuenció totalmente fue el de *Autographa califórnica* NPV (AcMNPV) (Ayres *et al*., 1994).

Desde entonces, el número de secuencias completas ha crecido rápidamente, dentro de los GVs el genoma completo del GVs de *C. pomonella* (CpGV) posee un tamaño de 123500 pb, teniendo un alto nivel de conservación con otros virus al poseer 108 y 98 genes homólogos al GV *Xestia c-nigrum* (XcGV) y al GV *Plutella xylostella* (PxGV), respectivamente (Luque *et al.*, 2001b). Mientras que el GV de *Choristoneura occidentalis* (ChocGV) tiene un tamaño de 104710 pb presentando de igual modo regiones homologas a otros GVs (Escasa *et al*., 2006).

Al obtener las secuencias del genoma completo y compararlas entre virus, se relacionan entre sí los miembros de la familia de virus y se observan cómo han evolucionado desde su último ancestro común, para esto, se han explorado varios enfoques diferentes, la comparación de las secuencias genómicas de baculovirus completas ha proporcionado una amplia evidencia de la naturaleza de estos microorganismos, así, la historia evolutiva de los baculovirus se ha caracterizado por reordenamientos genómicos generalizados y frecuentes ganancias y pérdidas de genes, por lo tanto, a medida que el conocimiento de la función de los diversos genes de los baculovirus crece, los enfoques genómicos comparativos proporcionan una visión cada vez más profunda de su la biología, las interacciones que se presentan entre baculovirus-huésped y los factores que han dado forma y han impulsado la evolución de los baculovirus (Herniou *et al*., 2003).

1.2.3.8 Mejoramiento genético. A fin de incrementar la virulencia de los baculovirus se ha recurrido a la modificación genética mediante la tecnología del ADN recombinante y se han realizado diversos estudios en los cuales se ha probado la inserción o delación de ciertos genes, o la incorporación de toxinas activas a fin de hacerlos más eficientes, al reducir el tiempo requerido para que la infección cause la mortalidad o incapacite a las plagas de insectos y, como consecuencia, reduzca la cantidad de daños en los cultivos (Kamita *et al*., 2010; Kroemer *et al*., 2015).

De manera general cualquier gen que codifique una proteína con la capacidad de interrumpir el desarrollo normal de una larva contribuyendo a incrementar su virulencia, es candidato para su expresión como baculovirus recombinante (López-Ferber y Del Rincón-Castro, 2001). Los baculovirus recombinantes, han demostrado alta eficacia bajo condiciones de laboratorio, invernadero y campo, ocasionando una gran reducción del daño ocasionado por los insectos plaga (Inceoglu *et al*., 2001). Genes como la quitinasa y la catepsina del nucleopolyhedrovirus múltiple de *Choristoneura fumiferena*, fueron introducidos en el genoma del nucleopolyhedrovirus múltiple de *Anticarsia gemmatalis*, provocando la licuefacción en el hospedero e incrementando la actividad insecticida al observar una reducción del 60 % en la concentración letal media (CL_{50}) (Lima *et al*., 2013).

En China algunos baculovirus han sido modificados genéticamente para mejorar su infectividad y resistencia a la radiación ultravioleta (Sun, 2015). Para incrementar la eficacia de HeNPV se le inserto en su genoma una toxina de escorpión, observando un incremento considerable en la virulencia al disminuir el tiempo para causar la infección, en comparación con el baculovirus no recombinante (Sun *et al*., 2004). En un estudio realizado por Ali *et al.* (2015), se construyó un Nucleopoliedrovirus recombinante de *B. mori* (BmNPV) y un Nucleopoliedrovirus múltiple de *Autographa californica* (AcMNPV) que expresaba una cito-insecto-toxina (Cit_1a) específica del insecto a partir del veneno de la araña asiática *Lachesana tarabaevi*, donde, el BmNPV/Polh-Cit_1a mostró una reducción significativa del tiempo letal medio (LT_{50}) de larvas de gusanos de seda en comparación con las larvas tratadas con el control BmNPV que carecía del gen Cit_1a. Estos fenómenos se producen dado que, las larvas infectadas con estos virus recombinantes generalmente mueren antes que las larvas infectadas con virus de tipo salvaje no recombinantes, debido a la toxicidad de la proteína insecticida codificada en lugar de la patología de la infección en sí (Rhouma *et al*., 2005; Kroemer *et al*., 2015).

No obstante, el uso de baculovirus recombinantes puede presentar ciertos riesgos, dependientes del tipo de modificación genética que haya sufrido el virus, entre estos riesgos se pueden mencionar la presencia de proteínas que podrían ser toxicas para los consumidores o los depredadores de las larvas (López-Ferber y Del Rincón-Castro, 2001).

1.2.3.9 Usos de los baculovirus. Los baculovirus son ampliamente utilizado tanto en biotecnología como en control biológico, su valor aplicado proviene de millones de años de evolución influenciados por las interacciones con sus hospederos y el entorno (Thézé *et al*., 2018).

La agricultura juega un papel vital a nivel mundial. Los beneficios potenciales para la agricultura y los programas de salud pública a través del uso de bioplaguicidas son considerables, los bioplaguicidas más utilizados son los organismos vivos, que son patógenos para la plaga de interés, así mismo, la creciente demanda de cultivos sin residuos químicos es uno de los principales impulsores de este mercado (Dutta, 2015).

Tanto los Nucleopoliedrovirus como los Granulovirus son usados como bioinsecticidas (Kamita *et al.*, 2010). La primera introducción documentada de baculovirus en el medio ambiente que resultó en la supresión efectiva de una plaga ocurrió accidentalmente antes de la Segunda Guerra Mundial, para suprimir a *Diprion hercyniae* (Hymenoptera: Diprionidae) en Canadá, donde se introdujo una nucleopolihedrosis específica y desde entonces no se han requerido medidas de control contra esta especie de himenóptero (Sarwar, 2015). El caso más exitoso del uso de baculovirus en cultivos agrícolas, es el documentado en Brasil, donde a partir de 1990 se aplicó el Nucleopoliedrovirus múltiple de *Anticarsia gemmatalis* (AgMNPV) para el control de *Anticarsia gemmatalis* (Hübner; Lepidoptera: Noctuidae) en soya y para la década de los años 2000 se intensifico su uso en aproximadamente 2 millones de hectáreas (Del-Angel *et al.*, 2018). Otro caso de éxito se ha reportado para la palomilla de la manzana *Cydia Pomonella* (Lepidoptera: Tortricidae).

Cydia Pomonella es una de las principales plagas en huertos de manzana, pera y nogal en todo el mundo, en México se logró el aislamiento de un Granulovirus (CpGV) el cual resulto ser altamente virulento presentando una DL_{50} inferior a los 5 COs para larvas del primer instar, el espectro de huéspedes de este Granulovirus está limitado a siete especies de tortrícidos (Cherry & Williams, 2001). Posteriormente este GVs fue estudiado y evaluado en Europa y Norteamérica, así mismo varios productos comerciales son producidos en estos países y utilizados alrededor del mundo (Lacey *et al*., 2008).

CpGV es uno de los aislados más virulentos dentro de los Granulovirus, el cual se agrupa dentro del tipo II, infectando varios tejidos y provocando la ruptura del tegumento (Federici, 1997). El Granulovirus CpGV se emplea como componente activo de varios bioinsecticidas comerciales bajo el nombre de Carpovirus Plus, Madex, Carpovirusine y Madex Twen en países como Argentina, Uruguay y Chile (Kutinkova *et al*., 2008; Haase *et al*., 2015).

De igual modo en países de América Latina se han encontrado varios casos de éxito en los cuales se ha recurrido a la aplicación de bioinsecticidas a base de baculovirus, respecto a los GVs destacan *Erinnyis ello* y *Phthorimaea operculella* (Haase *et al*., 2015). En países como Perú, Bolivia, Venezuela y Argentina se emplea un bioinsecticida viral para el control de *P. operculella* (Zeller) (Lepidoptera: Gelechiidae), siendo este insecto considerado como la principal plaga en el cultivo

de la papa, así mismo en México se ha probado su alta efectividad bajo condiciones de laboratorio (Villamizar *et al*., 2005; Rodríguez *et al*., 2013).

En otros países como China, Guatemala, Argentina, Chile, Uruguay, Colombia y México se han documentado varios casos de éxito mediante el uso de baculovirus como bioinsecticidas, especialmente en el control de diversos lepidópteros plaga como *A. gemmatalis*, *Autographa californica* (Speyer)*, Cydia pomonella* (L.)*, Helicoverpa armigera* (Hübner)*, Heliothis virescens* (Fabricius)*, Mamestra brassicae* (L.), *Spodoptera litura* (Fabricius)*, Trichoplusia ni* (Hübner)*, Phthorimaea operculella* (Zeller)*, Spodoptera exigua* (Hübner)*, S. frugiperda*, entre otros (Haase *et al.*, 2015; Sun, 2015).

Destacando que para lograr incrementar el potencial de uso de los baculovirus en los diversos cultivos en América Latina es necesario el apoyo conjunto del gobierno, instituciones educativas, productores y la cooperación entre países a fin promover e implementar programas para el control biológico de diversas plagas de interés agrícola (Haase *et al*., 2015). Por otra parte, en China, el uso de baculovirus ha sido bajo en comparación con el de América Latina, sin embargo, ha ido ascendiendo en los últimos años, para el año 2014 se dio la autorización para la comercialización de 57 bioinsecticidas virales de 11 distintos virus (Sun, 2015).

1.2.3.10 Desarrollos biotecnológicos. Los baculovirus han evolucionado durante aproximadamente 450 millones de años para infectar, replicar y luego dispersarse en una amplia gama de insectos hospederos (Hitchman *et al*., 2011). Siendo la versatilidad la principal característica biotecnológica de los baculovirus (López *et al*., 2018). Dentro de las principales aplicaciones biotecnológicas de los baculovirus destacan el control biológico mediante la creación de bioinsecticidas, los baculovirus como vectores de expresión proteica y la expresión de genes de interés (Barrera *et al*., 2015).

Respecto a los Granulovirus a través de la reingeniería molecular, estos han evolucionado desde virus de insectos simples, útiles para el control biológico de plagas agrícolas, hasta herramientas biotecnológicas versátiles, capaces de infectar células de insectos y transducir células de mamíferos

para la expresión de diversas proteínas, actualmente, varios estudios se han dedicado al procesamiento y la formulación de los vectores a partir de estos virus, sin embargo, siguen siendo en su mayoría estudios preliminares y limitados (Aucoin *et al*., 2010).

Varios autores han realizado estudios exitosos sobre la evaluación de Granulovirus producidos experimental y comercialmente, en Europa, América del Norte, Argentina, Nueva Zelanda, Australia y Sudáfrica, donde se ha visto que el nivel de control de la población del insecto objetivo depende de la dosis, la frecuencia y el momento de aplicación del virus, dado lo anterior, se han buscado realizar mejoras en los Granulovirus, esto con distintas finalidades, desde ofrecer una mayor protección contra los diversos factores climáticos, principalmente la radiación UV mediante el uso de fotoprotectores, hasta mejoras genéticas.

En este sentido, las regiones promotoras del gen de granulina son de particular interés debido a los altos niveles de expresión de la proteína granulina, y gracias a esta información se amplió el potencial de la mejora genética de los Granulovirus, sin embargo, antes de que se libere el Granulovirus modificado genéticamente o cualquier otro baculovirus, se necesita una comprensión profunda de las posibles consecuencias del virus en el medio ambiente, por lo que actualmente se realizan un gran número evaluaciones para poder llevar a la comercialización dichos agentes de control biológico (Lacey *et al*., 2008).

En cuanto a la biotecnología agrícola, esta contribuye a una mayor productividad y por ende la reducción de costos, obteniendo productos finales de mayor calidad y sin repercusiones al medio ambiente. En México el uso de bioinsecticidas es muy bajo, y en su mayoría se centra en bacterias (*Bacillus thuringiensis*) y hongos (*Beauveria, Metarhizium y Paecilomyces*), en cuanto a los baculovirus su uso es casi nulo (Tamez-Guerra *et al*., 2001; Williams *et al*., 2013). Sin embargo, existen diversos estudios de éxito en los cuáles se ha demostrado el eficaz control de plagas agrícolas mediante el uso de estos agentes de control biológico, así como el mecanismo de acción y las diversas interacciones que se llevan a cabo.

Por otra parte, durante la infección hacia las células del insecto, en el sistema vector de expresión de baculovirus se expresan aproximadamente 500 genes recombinantes, siendo el promotor de la

poliedrina el más común, este vector de expresión es una herramienta utilizada en la producción de grandes cantidades de proteína recombinante de mamíferos para la investigación biomolecular, así como en el desarrollo de vacunas a nivel comercial para humanos y animales, por ejemplo, vacunas profilácticas contra el síndrome de inmunodeficiencia adquirida, la malaria, y la influenza, y como vacunas terapéuticas para el tratamiento del cáncer (Patterson *et al*., 1995; Koczka *et al*., 2018).

En este mismo sentido, investigadores han estado utilizando esta plataforma para expresar proteínas recombinantes, y miles se han purificado con éxito con diferente finalidad biotecnológica. La producción de proteínas recombinantes en larvas de insectos, ha ampliamente utilizada en países asiáticos, usando como vector de expresión el nucleopolyhedrovirus de *Bombyx mori*, infectando larvas del gusano de seda *B. mori,* obteniendo altos niveles de expresión de las proteínas en las larvas que son infectadas (Kost *et al*., 2005; Felberbaun, 2015).

En cuanto a los biopesticidas, estos se clasifican según su fuente (estructura) y el mecanismo por el cual mitigan o matan a las plagas, respecto a la utilización de baculovirus en estos productos, la aplicación de la biotecnología mediante el uso de la ingeniería genética se puede lograr la mejora de su actividad insecticida para su uso y comercialización, ya que se pueden desarrollar baculovirus recombinantes, eliminando el gen de la eclysteroid-glicosil-transferasa (egt) viral, el producto del gen egt viral previene la muda larvaria durante la infección, al inactivar la egt se aumenta la actividad de alimentación de las larvas infectadas, por lo que las larvas crecen y el inóculo viral se incrementa, observando que las se aumentan la eficiencia del control biológico del baculovirus en aproximadamente de un 20 a 30% (Sundari *et al*., 2016).

Con base en lo mencionado anteriormente, los baculovirus de *S. frugiperda* podrían ser una alternativa prometedora al uso de insecticidas químicos, debido a su inocuidad, especificidad y eficacia probada (Grzywacz y Moore, 2017; Kamita *et al.*, 2010). A la fecha se han caracterizado Nucleopoliedrovirus y Granulovirus de *S. frugiperda* con potencial insecticida (Barrera *et al.*, 2011; Ordóñez-García *et al.*, 2020; Rios-Velasco *et al.*, 2011b; Simón *et al.*, 2011). Sin embargo, los GVs han sido menos estudiados. Actualmente, solo se tiene el reporte del aislamiento y caracterización de tres aislados del tipo I o muerte lenta, procedentes de Colombia, Brasil y Argentina (Cuartas *et al.*, 2014; Pidre *et al.*, 2019). Además, se ha documentado que la efectividad

de los Nucleopoliedrovirus es altamente dependiente del origen geográfico, siendo más efectivos contra insectos de la misma región (Barrera *et al.*, 2011; Yasem de Romero *et al.*, 2009).

1.2.3.11 Interacción entre géneros de baculovirus. Aunado a lo anterior, se han encontrado larvas de *S. frugiperda* infectadas por Nucleopoliedrovirus y Granulovirus simultáneamente (Cuartas-Otálora *et al.*, 2019; Cuartas *et al.*, 2014; Ordóñez-García *et al.*, 2020; Valderrama *et al.*, 2010). Durante estas interacciones, las células del insecto infectado exhiben diversas respuestas contra la infección por los baculovirus, dependiendo si son infectadas por un aislado viral o por más de uno (infección múltiple) (Ikeda *et al.*, 2015). Las coinfecciones virales en larvas hospederas pueden ser interacciones intraespecíficas (entre géneros de baculovirus) e interespecíficas (entre Nucleopoliedrovirus simples y múltiples), las cuales pueden resultar en diversas categorías de relaciones biológicas (i) antagonismo, (ii) neutralismo, (iii) mutualismo, (iv) comensalismo, (vi) amensalismo y (vi) competencia (Cheng y Lynn, 2009; Salvador, 2010).

En diversos estudios se han probado combinaciones entre cepas de NPVs y GVs en diferentes proporciones y suministrados a distintos tiempos, buscando incrementar la virulencia de uno o de ambos aislados; sin embargo, los resultados han sido variables y contradictorios, pero en la mayoría de los casos han resultado favorables al disminuir los tiempos requeridos para causar la enfermedad en el insecto hospedero. Espinel-Correal *et al.* (2012) documentaron que al mezclar Granulovirus de *Phthorimaea operculella* (*Phop*GV) con el de *Tecia solanivora* (Povolny) (*Ts*GV) se incrementó la eficacia de ambos virus contra ambos lepidópteros. Biedma *et al.* (2015) al evaluar en interacción el Granulovirus de *Epinotia aporema* Wals. (*Epap*GV) y el Nucleopolyhedrovirus de *Anticarsia gemmatalis* (*Ag*MNPV*)* contra larvas de *A. gemmatalis*, reportaron un incremento en la mortalidad larval atribuido a daños sufridos en la membrana peritrófica de las larvas ocasionados por los Granulovirus. Goto *et al.* (2015), encontraron un incremento en la infectividad del Nucleopolyhedrovirus de *Mamestra brassic oae* (*Mb*NPV) adicionada con proteínas derivadas del Granulovirus *Xestia c-nigrum* (*Xc*GV) bajo condiciones de campo. Así mismo, Cuartas-Otálora y colaboradores (2019), observaron que la coinfección de larvas de *S. frugiperda* con dosis altas de Nucleopoliedrovirus de *S. frugiperda* (SfNPV) y dosis bajas de Granulovirus de *S. frugiperda* (SfGV) suministradas al mismo tiempo causó predominantemente síntomas de infección por

Nucleopoliedrovirus. Contrastantemente, Hackett *et al.* (2000), documentaron competencia por los recursos del hospedero al evaluar un Granulovirus de *Helicoverpa zea* (HzGV) con el Nucleopoliedrovirus de *H. zea* (HzNPV) en la co-infección de larvas de *H. zea*.

Con base en los resultados encontrados en investigaciones previas arriba descritas se observa que, mediante el uso de mezclas virales, es posible influenciar la actividad insecticida de uno o de ambos virus. No obstante, es necesario caracterizar previamente a los aislados virales de manera independiente para determinar su patogenicidad y virulencia y posteriormente conducir estudios que permitan dilucidar los efectos sobre la actividad insecticida de Nucleopoliedrovirus y Granulovirus de *S. frugiperda* al ser suministrados en mezcla y a diferentes tiempos de inoculación, para determinar si la efectividad insecticida de uno o de ambos aislados virales se ve incrementada o en su caso disminuida (antagonismo).

1.3 Hipótesis

La actividad insecticida del SfNPV sobre larvas de *Spodoptera frugiperda* se ve influenciada por el suministro del SfGV en co-infección y está estrechamente relacionada con la dosis y el tiempo en el que se lleva a cabo la infección.

1.4 Objetivo General

Caracterizar a los aislados de baculovirus *Sf*NPV y *Sf*GV de *Spodoptera frugiperda* y evaluar la actividad insecticida de ambos baculovirus en coinfección a diferentes dosis y tiempos de inoculación.

1.5 Objetivos Específicos

1. Caracterizar morfológicamente a los baculovirus *Sf*NPVs y *Sf*GVs, mediante microscopía electrónica de barrido y de transmisión.

2. Estimar la dosis letal media y el tiempo letal medio de *Sf*NPVs y *Sf*GVs contra larvas de *Spodoptera frugiperda*.

3. Caracterizar molecularmente a los Nucleopoliedrovirus y Granulovirus mediante enzimas de restricción.

4. Evaluar las DL_{50} y DL_{90} de los *Sf*GVs para determinar el tiempo en el que las larvas de *Spodoptera frugiperda* cesan su alimentación.

5. Evaluar el efecto en la actividad insecticida de *Sf*NPV y *Sf*GV en coinfección contra larvas de *Spodoptera frugiperda* mediante bioensayos de actividad biológica.

1.6 Sección Integradora del Trabajo

En el **Artículo I** (pp. 26-36) se describe la actividad biológica contra larvas del tercer estadio de *S. frugiperda* de ocho aislados de Nucleopoliedrovirus de *S. frugiperda* nativos de Chihuahua, México, a partir de los resultados generados se seleccionaron los dos aislados más virulentos para su caracterización morfológica mediante microscopía electrónica de barrido y de transmisión, biológica estimando la dosis letal media (DL_{50}) y el tiempo letal medio (TL_{50}) y molecular mediante un análisis con enzimas de restricción. De los ocho aislados evaluados, tres (SfCH15, SfCH18 y SfCH32) causaron una mortalidad >98% a las 168 h post-inoculación (hpi) usando una dosis de 9.2×10^4 COs/larva. Los aislados más virulentos fueron SfCH15 y SfCH32, los cuales presentaron una forma y tamaño irregular (1.02-2.24 μm), y una DL_{50} de 5.6×10^2 y 6.4×10^2 COs/larva, respectivamente. El TL_{50} más bajo (114.5 hpi) se observó con el aislado SfCH15 empleando la concentración más alta (2.5×10^6 COs/larva). Los perfiles de restricción de ADN para SfCH15 y SfCH32 fueron diferentes, con un tamaño de genoma de ~ 128 kb y 132 kb, respectivamente. Este estudio mostró que los aislados de NPVs nativos fueron altamente virulentos

contra larvas de *S. frugiperda*, siendo similar a otras cepas reportadas; sin embargo, se sugieren estudios de campo para confirmar su efecto insecticida.

En el **Artículo II** (pp. 37-67) se evaluaron dos aislados de Granulovirus SfGV-CH13 y SfGV-CH28, los cuales se caracterizaron morfológicamente mediante microscopía electrónica de barrido y transmisión, biológicamente empleando larvas del tercer estadio estimando la dosis y el tiempo letal medio y molecularmente mediante un análisis con enzimas de restricción, adicionalmente se evaluó el tiempo transcurrido entre la infección viral de las larvas y el cese de la alimentación, así como el peso de las larvas antes de la muerte o de la pupación, Ambos Granulovirus mostraron forma ovoide con una longitud de 0.4 µm, sus perfiles de restricción de ADN fueron similares y el tamaño de su genoma fue de aproximadamente 126 kb. La infección larval con los GVs causó flacidez del cuerpo y decoloración del tegumento. La lisis del tegumento solo se observó en el 8% de las larvas infectadas. En general, estos síntomas son característicos de las infecciones causadas por GVs de tipo I (monoorganotrópicos). Los valores de la DL_{50} para los aislados de SfGV-CH13 y SfGV-CH28 fueron de 5.4×10^2 y 1.1×10^3 COs/larva, respectivamente. El TL_{50} osciló entre 17 y 24 días, y disminuyó a medida que se incrementó la dosis. El DL_{90} extendió dos veces el tiempo de desarrollo larvario de *S. frugiperda*. Los resultados mostraron que ambos Granulovirus pueden ocasionar la muerte a más del 90% de las larvas de *S. frugiperda* a los 45 dpi empleando una dosis de 1.0×10^5 COs/larva.

En el **Artículo III** (pp. 68-88) se muestra la actividad insecticida de los aislados SfNPV-CH32 de Nucleopoliedrovirus y del Granulovirus (aislado SfGV-CH13) en coinfección contra larvas del tercer estadio de *S. frugiperda*. Para lo cual, diferentes dosis letales de ambos aislados virales se suministraron a las larvas a los tiempos cero (mismo tiempo), 12 y 24 h (después de haberse suministrado la dosis del primer aislado seleccionado). Los síntomas mostrados por las larvas co-infectadas correspondieron al virus suministrado inicialmente en mayor concentración. Los resultados obtenidos en las coinfecciones larvales con ambos aislados virales fueron variables y estuvieron en función de las dosis suministradas y el tiempo en que se suministraron. La actividad insecticida del NPV contra *S. frugiperda* al suministrarse junto con el GV al tiempo cero, no se vio influenciado. Sin embargo, al suministrar primero (12 o 24 h antes) el GV y luego el NPV, se observó un efecto antagónico en la actividad insecticida de este último. Por otra parte, se registró

un incremento en el tiempo de supervivencia en larvas inoculadas simultáneamente con la dosis más baja (DL_{10}) de NPV y alta (DL_{90}) de GV. Los resultados obtenidos sugieren que la dosis suministrada y el tiempo en el cual se lleve a cabo la infección influencian la actividad insecticida del NPV.

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2. MOLECULAR AND MORPHOLOGICAL CHARACTERIZATION OF MULTIPLE NUCLEOPOLYHEDROVIRUS FROM MEXICO AND THEIR INSECTICIDAL ACTIVITY AGAINST *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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ORIGINAL CONTRIBUTION

Molecular and Morphological characterization of multiple nucleopolyhedrovirus from Mexico and their insecticidal activity against *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract

The fall armyworm (FAW), *Spodoptera frugiperda* (Lepidoptera: Noctuidae), is the most important pest of maize in many countries. Entomopathogenic viruses mainly Baculoviruses family are excellent biological control agents and therefore a viable alternative for managing this pest. The aim of this study was to determine the biological activity of eight native nucleopolyhedrovirus (NPVs) against FAW larvae. Additionally, two of the most virulent isolates (SfCH32 and SfCH15) were characterized biologically by bioassays to estimate their median lethal dose (LD_{50}) and median lethal time (LT_{50}), morphologically by scanning electron microscopy (*SEM*) and transmission electron microscopy (TEM) and molecularly by restriction enzymes. Three (SfCH15, SfCH18 and SfCH32) of the eight tested native nucleopolyhedrovirus isolates caused mortalities >98% at 168-hr post-inoculation (hpi) with a dose of 9.2×10^4 OBs/larva. SfCH15 and SfCH32 isolates showed occlusion bodies (OBs) of irregular shape and size (1.02–2.24 μm). The SfCH15 and SfCH32 isolates showed similar median lethal dose (5.6×10^2–6.4×10^2 OBs/larva). The lowest median lethal time (114.5 hpi) was observed with the SfCH15 isolate at the highest concentration (2.5×10^6 OBs/larva). The DNA restriction profiles for SfCH15 and SfCH32 were different, with their genome size being ~128,000 bp and 132,000 bp, respectively. SfCH15 and SfCH32 isolates showed similar morphological characteristics and the highest virulence against fall armyworm. This study showed that native isolates were highly virulent against *S. frugiperda* larvae, being similar to other reported strains; however, field studies are required to confirm their insecticidal effect.

KEYWORDS

baculovirus, biological control, fall armyworm, genetic variability, morphology, virulence

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1 | INTRODUCTION

The maize (*Zea mays* L.) is the most important grain worldwide, either for human or for animal feeding (Yang, Balint-Kurti, & Xu, 2017). The fall armyworm (FAW), *Spodoptera frugiperda*, is the main insect pest of maize in many countries, including Mexico, causing crop losses greater than 30% (Casmuz et al., 2010). In America, several outbreaks have occurred that have caused losses in millions of dollars (Ramirez-Cabral, Kumar, & Shabani, 2017). Recent studies have demonstrated the continuous spreading of FAW (Goergen, Kumar, Sankung, Togola, & Tamò, 2016). The control of this insect requires several insecticide (pyrethroids, diacyl hydrazides, diamides and benzoylureas) applications, causing populations resistant, besides, there are collateral damage to beneficial insects, for example pollinator losses from contact with pesticides, and decreased bee activity and honey production (Abdu-Allah & Pittendrigh, 2018; Ahmad, Farid, & Saeed, 2018; Fernandes, Abreu, Christ, & Rosa, 2019; Hardke, Temple, Leonard, & Jackson, 2011).

The use of natural enemies of pests (i.e., fungi, bacteria, viruses, nematodes, protozoa, parasitoids and predator insects) as part of the integrated pest management (IPM) schemes represents an effective alternative to chemical control because they exert significant control of pests without negative effects on humans, animals and environment (Zimmermann, 2007).

The entomopathogenic viruses represent a viable alternative for control of FAW, especially the baculoviruses (Baculoviridae family). The baculoviruses are widely distributed and include four genera: Alphabaculovirus (lepidopteran-specific Nucleopolyhedrovirus (NPV), Betabaculovirus (lepidopteran-specific Granuloviruses), Deltabaculovirus (dipteran-specific NPV) and Gammabaculovirus (hymenopteran-specific NPV) (Jehle et al., 2006; Jiang, Yang, Ji, Zhang, & Wan, 2018; Wan, Jiang, & Li, 2016; Wan et al., 2019; Zamora-Avilés et al., 2017). Some studies have demonstrated that the nucleopolyhedrovirus (alphabaculovirus) caused mortalities under laboratory conditions of FAW larvae greater than 90% (Barrera, Simón, Villamizar, Williams, & Caballero, 2011; Escribano et al., 1999; Rios-Velasco et al., 2011). Under open field conditions, these viruses reduced up to 92% the damage caused by this pest (Gómez, Guevara, Cuartas, Espinel, & Villamizar, 2013). The nucleopolyhedrovirus (alphabaculovirus) is highly pathogenic and virulent, causing larval death in 4 days post-infection, depending on the viral isolate, the concentration of viral inoculum and larval instar (Barrera et al., 2011; Rios-Velasco et al., 2011; Yasem de Romero, Romero, Sosa Gómez, & Willink, 2009). They also show high specificity for the target insect and can be applied in the field using conventional equipment.

However, the effectiveness of the baculoviruses as bioinsecticides is highly dependent of their geographical origin as well as the origin of the target pest, being more effective against target insects from the same region (Barrera et al., 2011; Yasem de Romero et al., 2009). Barrera et al., (2011) evaluated two nucleopolyhedrovirus isolates, one Colombian (SfCOL) and another Nicaraguan (SfNIC), against Colombian FAW larvae, and they found that the native isolate (SfCOL) was the most virulent. Additionally, there are genetic variants among wild baculovirus isolates, which show differences in their pathogenicity and biological activity (Escribano et al., 1999; Martins, Montiel, Medeiros, Oliveira, & Simões, 2005). Yasem de Romero et al., (2009) evaluated nucleopolyhedrovirus isolates from several geographic regions in FAW larvae (three to five days old) and observed that the native isolate showed greater virulence than the isolates from another geographical regions.

In addition, during the replication step of the infection process, the baculoviruses exchange genetic material with other virus and other co-infecting microorganisms, resulting in a wide genetic diversity of isolates, which vary in pathogenicity and virulence (Ali, Abma-Henkens, van der Werf, Hemerik, & Vlak, 2018). This might explain the effect of the geographical origin on the insecticidal activity of baculoviruses. Thus, the baculoviruses from several regions have been isolated and characterized (Barrera et al., 2011; Escribano et al., 1999; Gómez-Valderrama, Guevara-Agudelo, Barrera-Cubillos, Cotes-Prado, & Villamizar-Rivero, 2010; Ordóñez-García et al., 2015; Rios-Velasco et al., 2011; Simón et al., 2011) for their activity as biological control agents (Figueiredo, Muñoz, Murillo, Mexia, & Caballero, 2009). Unfortunately, the research using native nucleopolyhedrovirus isolates of *S. frugiperda* in Mexico is limited to few studies, in which the characterization of FAW baculoviruses was incomplete (Rangel Núñez, Vázquez Ramírez, & Del Rincón Castro, 2014; Rios-Velasco et al., 2011). Thus, the aim of this study was to evaluate native nucleopolyhedrovirus from naturally infected FAW from Mexico and characterize their biological activity against FAW larvae under laboratory conditions, morphology and genetic differences.

2 | MATERIALS AND METHODS

2.1 | Insect rearing and propagation of viruses

The FAW larvae were obtained from a laboratory colony had been reared for 12 months (eight generations) in Cuauhtemoc, Chihuahua, Mexico. This colony was established using insects previously recollected from a infested maize plot (Latitude 28°12′44″N, Longitude 106°59′45″W, Altitude 2,125 m asl). They were maintained under controlled conditions (26 ± 2°C, >70% RH, 12:12 L: D photoperiod), fed with artificial diet (Southland Products Inc). Adult moths were maintained in 15-l cylindrical jars and fed with sugar solution (150 mg/ml).

Eight nucleopolyhedrovirus isolates obtained in a previous study (Ordóñez-García et al., 2015) from Chihuahua, Mexico, were propagated in fourth instar FAW larvae by the droplet feeding method (Hughes & Wood, 1981). For the inoculation, 0.5 µl of viral suspensions (~10^8 occlusion bodies OBs) was supplied to larvae, which had been starved for 12 hr. The OBs were mixed with Fluorella blue (0.01 mg/ml) and sucrose (100 mg/ml) before use. The infected larvae were individually placed into the cups with artificial diet for feeding and were maintained under controlled ambient conditions,

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as indicated above for 12 days. Then, the dead larvae were collected at 24 hr intervals and stored at −20°C.

The larvae were macerated in sterile mortars using sterile distilled water containing 1 mg/ml of sodium dodecyl sulphate (SDS). The excess of larval cuticle was removed by filtering the extract through muslin. The filtrates were placed into 10 ml polypropylene tubes and centrifuged (8,500 × g, 4°C, 10 min). The pellet with the OBs was re-suspended in 10 ml of sterile distilled water and stored at −80°C. Then, the OBs were purified by continuous sucrose (purity ≥99.5%) gradients (40 and 66%, w/w) using a gradient former (CBS Scientific, GM 200) according to Muñoz, Martínez, Murillo, Ruíz De Escudero, and Vilaplana (2001) with modifications. Twenty millilitres of these gradients were placed into 30-ml polyallomer tubes and then 5 ml of viral suspension were deposited on the surface of the gradients and ultracentrifuged (70,000 × g, 4°C, 90 min) using a 70 Ti rotor (Beckman Coulter, Optima XPN-100).

The bands containing OBs were recovered using a Pasteur pipette and placed into 10 ml polypropylene tubes to be washed twice with sterile distilled water using centrifugation (8,500 × g, 4°C, 10 min). Finally, the obtained OBs were re-suspended in 1 ml aliquots of sterile distilled water and stored at −80°C, until use. A fully characterized Nicaraguan strain (genotype SfMNPV-B) (Barrera et al., 2011; Escribano et al., 1999; Simón et al., 2011) abbreviated in this study as SfNIC and a partially characterized strain (SfAN1) (Rios-Velasco et al., 2011) from Coahuila, Mexico, were used as reference.

2.2 | Insecticidal activity

The insecticidal activity of NPVs (eight native isolates and the strains SfNIC and SfAN1) was tested in third instar FAW larvae. The bioassays were performed by the droplet feeding method, according to Hughes and Wood (1981). Each larva was supplied with a 0.5 µl drop containing 100 mg/ml of sucrose, 0.01 mg/ml of Fluorella blue and the viral inoculum to obtain a dose of 9.2 × 10⁴ OBs/larva. Previously, OBs were released by sonication (Branson 1510) for 30 s and were counted in triplicate in a Neubauer chamber (Marienfeld), in a phase-contrast microscope (Carl Zeiss, Axio Scope A1) at 400× magnifications (Muñoz et al., 2001). Preliminary tests demonstrated that this dose was able to cause ≤90% of dead larvae (data not shown). A total 825 third instar FAW larvae were used in the experiment. Twenty-five larvae per replicate for each isolate and twenty-five larvae were used as control group (without viral inoculum) per replicate. The bioassays were performed by triplicate. Only larvae that consumed the whole inoculum and showed intestinal tract with blue colour as confirmed by observation under a stereomicroscope (Leica G26) were considered in the experiment. These larvae were individually maintained inside 29.5-ml plastic cups containing artificial diet under controlled ambient conditions described above.

Larval mortality was recorded every 12 hr, monitoring the typical symptoms of viral infection (Barrera et al., 2011) and examining the tissues in an optical microscope to confirm the presence of OBs. Based on the shortest time (168 hpi) required by the isolates

to cause 100% of larval death with a dose of 9.2 × 10⁴ OBs/larva, the NPVs of SfCH15 and SfCH32 isolates were selected to be subsequently characterized for their median lethal dose (LD₅₀), median lethal time (LT₅₀), morphology and genetic differences.

2.3 | Determination of the median lethal dose and median lethal time

The LD₅₀ and LT₅₀ were determined only for the most virulent isolates (SfCH15 and SfCH32) and for the reference strains (SfAN1 and SfNIC). The LD₅₀ values of NPVs were determined in third-instar FAW larvae using five viral doses (2.1 × 10¹, 8.6 × 10¹, 2.15 × 10², 2.15 × 10³ and 8.6 × 10³ OBs/larva) lower than those that caused 100% larval mortality in the experiment of the insecticide activity, above described. The LT₅₀ values were determined in larvae of the same instar using four viral doses (8.6 × 10³, 8.6 × 10⁴, 6.5 × 10⁵ and 2.5 × 10⁶ OBs/larva), which caused ≥90% mortality between the 5th and 9th d post-inoculation (dpi). The bioassays were performed in triplicate using 25 larvae per replicate for each viral dose. Twenty-five larvae were used as control group per replicate. For both determinations, larval mortality was recorded every 8 hr until death, checking typical symptoms (as mentioned above) or until they reached the pupal stage (14 dpi) to determinate the LD₅₀ (Barrera et al., 2011).

2.4 | Morphological characterization of baculoviruses isolates

The isolates SfCH15 and SfCH32 (putatively more virulent) were identified according to their morphological characters using an optical microscope (Carl Zeiss, Axio Scope A1) at 1,000× magnifications and characterized by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM analysis, one drop of the viral suspension was placed on an aluminum pin and allowed to dry previous to be covered with gold in an (Auto Sputter Coater 108). The analysis was carried out using a SEM (FEI, Helios Nanolab 600 DualBeam). Fifty OBs were evaluated for size. For TEM analysis, the OBs were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hr, post-fixed in 1% osmium tetroxide for 1 hr. The samples were dehydrated using ethanol and then embedded in Epon–Araldite resin. Ultrathin sections were cut and mounted on grids. The samples were stained with 2% aqueous uranyl acetate and lead citrate, and observed with a transmission electron microscope (JEOL, JEM-200CX) at 80 kV. The number of nucleocapsids per envelope and their size were measured.

2.5 | Extraction of viral DNA

One millilitre of the dispersion of purified OBs (~10⁹ OBs/ml) was mixed with 1 ml of 0.1 M sodium carbonate (Na₂CO₃), 1 ml of 0.1 M

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sodium chloride (NaCl) at pH 10.8 and 1 ml of buffer ethylenedi-aminetetraacetic acid-tromethamine hydrochloride (TE) (0.01 M tromethamine (Tris)-hydrochloric acid (HCl), 0.001 M ethylenedi-aminetetraacetic acid (EDTA)) at pH 7.6. The mixture was incubated at 28°C, for 2 hr under agitation (140 rpm) and an equal volume of buffer (TE) (1 ml) was added. The released virions were purified by continuous sucrose (purity ≥99.5%) gradients (20 and 66%, w/w). Twenty millilitres of the formed gradient were placed into 30 ml polyallomer tubes, and then, 5 ml of the virion suspensions were deposited on the surface, previous to be ultracentrifuged (81,000 × g, at 4°C, 1.5 hr) using an ultracentrifuge (Beckman Coulter, Optima XPN-100). The bands of virions were collected with a Pasteur pipette and washed once with sterile distilled water by ultracentrifugation (81,000 × g, at 4°C, 40 min). The pellets with virions were re-suspended in 500 µl of sterile distilled water and stored at −20°C, until use.

For the extraction of viral DNA, the virion dispersions were mixed with 400 µl of buffer for proteinase K (0.01 (Tris), 0.005 M (EDTA), 5 mg/ml (SDS)) and incubated at 65°C for 15 min. Then, 100 µl of proteinase K (2 mg/ml) (Invitrogen Life Technologies Corp) was added and the reaction mixture was incubated at 37°C for 2 hr. An aliquot (500 µl) of a mixture of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the reaction, previous to be centrifuged

(17,000 × g, at 4°C, 5 min). The aqueous phase was collected in a new microtube, and an equal volume of isopropyl alcohol and 100 µl of 3 M sodium acetate were added to the samples previous to be incubated at −20°C for 2 hr. The mixture was centrifuged (17,000 × g, 4°C, for 10 min). The pellet was washed with 1 ml of 70% ethyl alcohol using centrifugation (17,000 × g, 4°C, 3 min), and the pellet was re-suspended in 30 µl of sterile ultrapure water and stored at −20°C until use. Finally, the DNA quality was examined by electrophoresis on a 1% agarose gel.

2.6 | Restriction endonuclease analysis

The SfCH32 and SfCH5 isolates were digested with *Bam*HI and *Hind*III enzymes (Invitrogen Life Technologies Corp). One microgram of DNA was digested with 10 U of the enzymes, at 37°C for 2 hr. The reaction was stopped by adding 2 µl of loading buffer 10X (Thermo-Fisher Scientific). The obtained restriction fragments were examined by electrophoresis on a 0.6% agarose gel at 13 V for 16–18 hr, using TAE buffer (40 mM Tris-acetate, 1 mM EDTA at pH 8.0). A molecular weight marker of 1 kb DNA Ladder, (Thermo-Fisher Scientific) and ethidium bromide solution (10 mg/ml) were used to visualize the DNA on agarose gels using the image system (Bio-Rad, ChemiDoc™
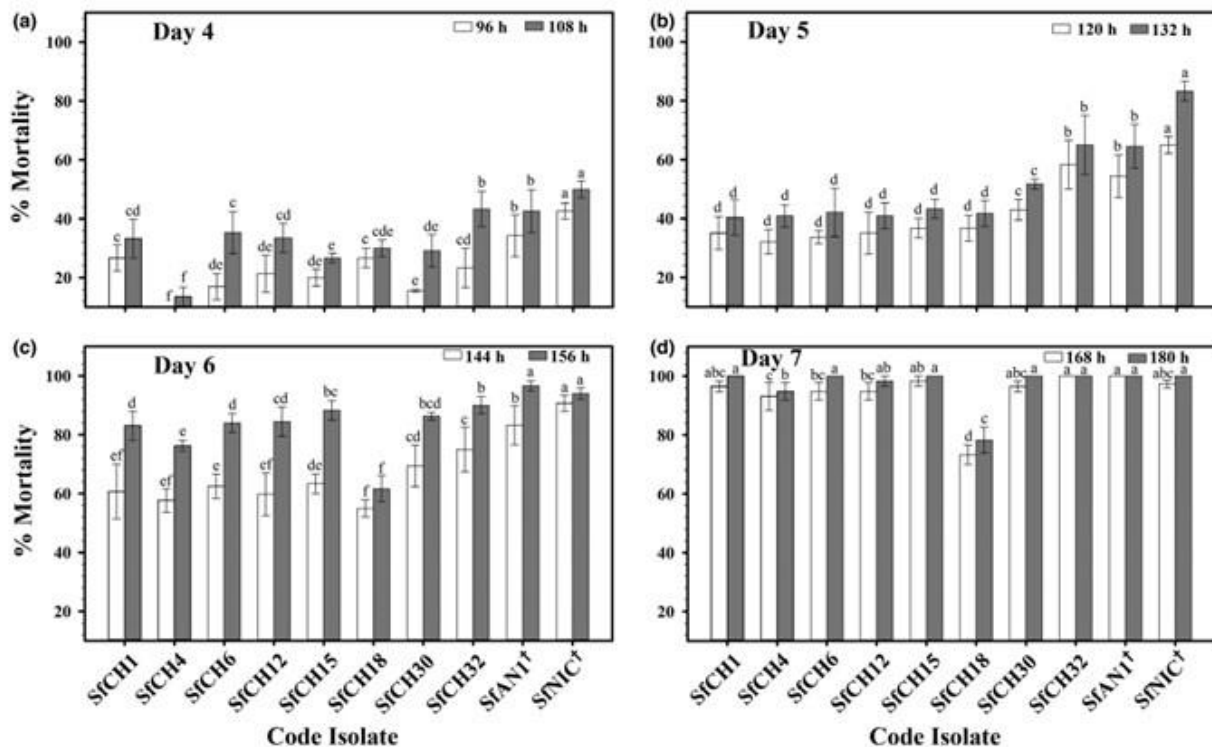
FIGURE 1 Mortality rate of *Spodoptera frugiperda* larvae infected with native NPV isolates and reference strains. All values are arithmetic means ± standard error of third instar fall armyworm larvae, treated with Mexican baculovirus isolates evaluated from 4th to 8th day post-inoculation. Values with the same literal between treatments to same evaluation time are statistically equal according to Tukey's test (p < .05). Mortality percentages recorded in the first three days are not shown because these values were low (<15%). [†]Strains (SfAN1 and SfNIC). In control larvae not mortality was recorded

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XRS+). The fragments size of SfCH15 and SfCH32 isolates were estimated by comparing the bands with those obtained with the molecular weight marker.

2.7 | Statistical analysis

The experiment was conducted under a completely randomized design. The obtained data in the experiment of insecticidal activity were analysed by an analysis of variance (ANOVA), and the means were separated by Tukey's test ($p < .05$). The LD_{50}, LT_{50} and fiducial limit values were analysed using log-probit regressions. All dates were analysed using SAS software (SAS, 2002). Mortality was corrected by the Abbott (1925) formula.

3 | RESULTS

3.1 | Biological characterization

During the infection process, the infected larvae showed a dark brown colour and lysis of the integument was observed. However, this symptom was not observed immediately in larvae treated with the Nicaraguan strain, which showed a darker colour.

The larval mortality values caused in the experiment of insecticidal activity by tested nucleopolyhedrovirus are shown in Figure 1. Significant differences ($p < .001$) between treatments at 96 hpi were observed where larval mortality ranged from 6.7% to 42.6% (Figure 1a). The SfCH32 isolate and SfAN1 strain caused 100% mortality at 168 hpi. The SfCH1, SfCH6, SfCH15 and SfCH30 isolates and the SfNIC strain also caused a mortality of 100% but at 180 hpi ($p < .001$) (Figure 1d). The mortality was ≥98% at 192 hpi (data not shown) for the other tested NPVs, increasing to 100% at 204 hpi. The typical symptoms reported for FAW larvae infected with NPVs were similar to those caused by tested native isolates and reference strains. Larvae showed low mobility, and some of them stopped feeding after 64 hpi and showed changes in colouration (from whitish to brown and finally to black), high fragility and rupture of the integument, vomiting and diarrhoea, among other symptoms. The integument of the larvae infected with the Nicaraguan strain showed a lower fragility to rupture.

The LD_{50} for the four tested baculoviruses ranged from 5.6×10^2 to 8.0×10^2 OBs/larva (Table 1), being SfCH32 isolate showing the lowest LD_{50}. The LT_{50} decreased as the dose was increased (Table 2). In larvae treated with 2.5×10^6 OBs/larva of SfCH15 or SfCH32, the LT_{50} was 114.5 and 118.4 hpi, respectively, while those treated with the lowest dose (8.6×10^4 OBs/larva) showed a LT_{50} of up to 66.8 hr. The lowest LT_{50} was obtained with SfNIC strain.

3.2 | Morphological characterization

By observing virus isolates using the optical microscope, eight native baculoviruses were identified (SfCH1, SfCH4, SfCH6, SfCH12,

SfCH15, SfCH18, SfCH30 and SfCH32) as NPVs; these showed OBs of polyhedral shape. The NPVs of isolates (SfCH15 and SfCH32) purified were characterized by SEM and TEM. The SEM measurements revealed that the OBs of SfCH15 and SfCH32 showed irregular shape (cubic and pyramidal) and that their size ranged from 1.02 to 2.24 µm and from 1.58 to 2.11 µm, respectively (Figure 2a, c).

The TEM measurements allowed for determining that both isolates were NPVs of multiple types due to the presence of several nucleocapsids in each OB. The number of virions contained in the OBs of SfCH15 and SfCH32 was 10–14 and 5–10, respectively. The number nucleocapsids for SfCH15 ranged from 2 to 6 per envelope, with each nucleocapsid showing an average length of 0.28 µm. The number of nucleocapsids for SfCH32 varied from 1 to 2, with an average length of 0.3 µm (Figure 2b, d).

3.3 | Restriction endonuclease analysis

The isolates SfCH15 and SfCH32 showed slight differences in the restriction patterns. Digestion of SfCH15 generated 12 and 8 fragments, depending of the restriction enzyme (Figure 3a, b). On the other hand, 13 and 7 fragments were observed in digestion reactions of SfCH32 (Figure 3a, b). The digestion of this isolate with the HindIII enzyme generated a fragment of ~2,500 bp, which was similar to that observed for the Nicaraguan strain (SfNIC) (Figure 3a). This fragment was not observed in the digestion reaction of SfCH15. However, the digestion of this isolate with BamHI generated a fragment of ~30,000 bp (Figure 3b), which was absent in SfCH32. The DNA size for SfCH15 and SfCH32 was ~128,000 bp and ~132,000 bp, respectively.

4 | DISCUSSION

The symptoms observed for infected larvae were similar to those reported by others for FAW larvae infected with NPVs (Gómez-Valderrama et al., 2010; Rios-Velasco et al., 2011; Rowley, Popham, & Harrison, 2011). In the present study, it was found that the lysis of the integument was observed immediately after death of larvae treated with both native NPVs (SfCH15 and SfCH32). This symptom was less evident in larvae infected with SfNIC, because the integument lysis was not observed immediately after death. Contrary, the larvae treated with this strain showed recurrent vomiting and diarrhoea before death, as compared with those treated with native isolates and SfAN1. Some studies have demonstrated that the integument lysis in larvae is induced by cathepsin and chitinase encoded by baculoviruses (Caballero, Williams, & López-Ferber, 2001; Kang, Tristem, Maeda, Crook, & O'Reilly, 1998). Thus, tested baculoviruses showed insecticidal activity in vivo against FAW, and therefore, they represent a potential alternative to control FAW in cornfields, as suggested by others (Rohrmann, 2013).

The percentages of larval mortality obtained in this study with the dose of 9.2×10^4 OBs/larva were higher than that (72%) reported

TABLE 1 Median lethal dose (LD_{50}) of Mexican nucleopolyhedrovirus isolates against third instar FAW larvae, evaluated at 14-day post-inoculation

| Isolate code | LD_{50} | Fiducial limits (95%) | | χ^2 | df | Slope ± (SE) | Intercept ± (SE) |
		Lower	Upper				
SfCH15	6.4×10^2	4.8×10^2	8.7×10^2	4.4	3	1.1 ± 0.08	−3.0 ± 0.22
SfCH32	5.6×10^2	4.3×10^2	7.4×10^2	4.6	3	1.2 ± 0.08	−3.3 ± 0.23
SfAN1	6.7×10^2	5.1×10^2	8.9×10^2	3.2	3	1.1 ± 0.08	−3.2 ± 0.23
SfNIC	8.0×10^2	6.0×10^2	1.1×10^3	4.7	3	1.1 ± 0.08	−3.0 ± 0.23

Note: Probit regressions were fitted using SAS program. The LD_{50} was estimated from a minimum of five doses.
Abbreviations: df, degrees of freedom; SE, standard error; χ^2, goodness of fit test.

TABLE 2 Median lethal time (LT_{50}) of nucleopolyhedrovirus isolates against third instar FAW larvae treated with four viral doses

| Doses (OBs/larva) | Isolate code | LT_{50} (hr) | Fiducial limits (95%) | |
			Lower	Upper
8.6×10^3	SfCH15	181.3	175.5	187.5
	SfCH32	181.8	176.6	187.1
	SfAN1	176.5	169.1	184.3
	SfNIC	105.2	102.0	108.4
8.6×10^4	SfCH15	134.3	129.5	139.1
	SfCH32	150.2	146.0	154.2
	SfAN1	141.1	133.3	148.8
	SfNIC	99.1	97.0	101.2
6.5×10^5	SfCH15	125.4	120.6	130.1
	SfCH32	143.7	130.3	157.3
	SfAN1	129.3	127.0	131.6
	SfNIC	93.7	91.7	95.6
2.5×10^6	SfCH15	114.5	109.7	119.2
	SfCH32	118.4	113.2	123.4
	SfAN1	119.0	115.8	122.2
	SfNIC	88.1	86.4	89.9

Note: The data of each isolate corresponds to the average of three replicates. LT_{50} was expressed in hours.

by Rios-Velasco et al., (2011) for FAW larvae of third instar at 25 dpi using a concentration of 4.0×10^6 OBs/mm^2 of SfAN1. This difference in mortalities could be attributed to differences in the used bioassay technique because surface diet contamination technique has some limitations, including the heterogeneous dispersion of OBs on diet surface, causing variability of the number of OBs consumed by the insect (Ibarra & Del Rincón-Castro, 2001). A larval mortality of 100% after 8.5 days with a dose of 9.2×10^4 OBs/larva was observed in the present study. Barreto, Guimaraes, Teixeira, Paiva, and Valicente (2005) evaluated the mortality of 3rd- and 4th-instar FAW larvae infected with 22 baculoviruses from Brazil and observed that doses of 10^3 and 10^4 OBs/ml caused mortalities from 9% to 90.7% and from 11.5% to 94%, respectively, and doses of 10^6 OBs/ml or higher caused mortalities >72%. The mortality values were higher

than those (39.6% at 168 hpi) reported by Figueroa, Coronado, Pineda, Chavarrieta, and Martinez-Castillo (2015), who evaluated a SfNPV from Nicaragua at 10^6 OBs/ml.

Regarding to the virulence of the isolates, the LD_{50} values for tested native isolates (SfCH15 and SfCH32) were similar to those of the reference strains (SfNIC and SfAN1), demonstrating a high virulence of native isolates against third instar FAW larvae. The median lethal dose was similar between tested isolates and strains, except for the native isolate SfCH32, which showed the lowest LD_{50} and was 1.14, 1.20 and 1.43 times more virulent than SfCH15, SfAN1 and SfNIC, respectively. The LD_{50} values were also lower than those reported by Vásquez, Zeddam, and Tresierra (2002) using a dose of 4.97×10^4 OBs/larva in third instar FAW larvae, being the isolates SfCH32 and SfCH15, 89 and 78 times more virulent, respectively. On the other hand, Rangel Núñez et al., (2014) tested three exotic nucleopolyhedrovirus strains from Argentina, USA and Honduras against a Mexican population of FAW, where the Argentine strain was the most virulent with a LC_{50} of 4.9×10^4 OBs/ml on first instar larvae.

The LT_{50} values for isolated nucleopolyhedrovirus were similar to those (5.5–7.1 d) obtained by Kamiya et al., (2004) in third instar Spodoptera litura larvae treated with NPV from S. litura using a concentration of 1×10^8 OBs/ml. The relationship between LT_{50} and dose of tested baculoviruses was similar to that observed by Takatsuka, Okuno, Nakai, and Kunimi (2003) for S. litura (Fabricius) (Lepidoptera: Noctuidae) infected with several viral concentrations. This trend was more linear with SfNIC in the present study. Barrera et al., (2011) obtained a median time to death of 168 hpi for second instar FAW larvae treated with 1.5×10^6 OBs/ml of a Colombian NPV. This value is similar to those found for isolates SfCH15, SfCH32 and SfAN1; nevertheless, the Nicaraguan strain showed a lower LT_{50}. The LT_{50} values (88.1–105.2 hpi) observed for SfNIC are into the range (78–135 hpi) reported by Simón, Williams, López-Ferber, Taulemesse, and Caballero (2008) at concentrations that resulted in mortalities from 70% to 80% in larvae infected with genotypes of the Nicaraguan isolate. These authors also mentioned that an individual insect could be infected by multiple genotypes. Thus, a Nicaraguan isolate obtained from S. frugiperda multiple nucleopolyhedrovirus (SfMNPV-NIC) was found to be composed by at least nine different genotypes, which showed more virulence when they were tested

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FIGURE 2 Occlusion bodies of SfCH15 isolate (a-b), and SfCH32 (c-d) isolate seen by *SEM* and TEM at scale 20,000× and 50,000× magnifications, respectively. Nucleocapsid (NC) and virions (V)
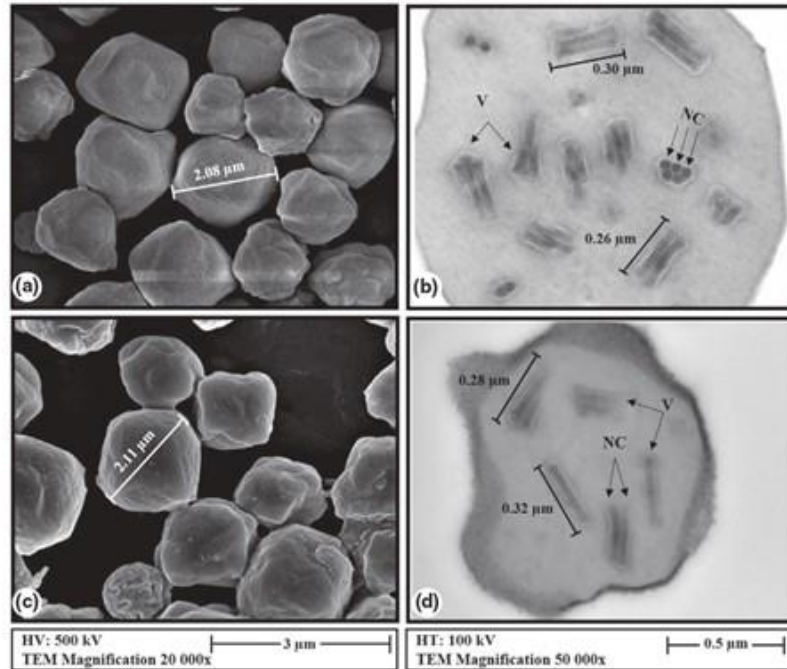

together as compared with their individual effects (Escribano et al., 1999; Simón et al., 2008). However, in these mixtures, the genotype SfMNPV-B was the most predominant and highly virulent (Barrera et al., 2011; Simón et al., 2008). The occurrence of genotype variants in the same viral isolate from different geographical origins has already been demonstrated (Takatsuka et al., 2003).

The NPVs with multiple nucleocapsids could cause a faster infection than those having single nucleocapsid (Blissard & Theilmann,

2018). The SfCH15 isolate was more virulent than SfCH32 at doses higher than 8.6×10^3 OBs/larva, since its LT_{50} was always the lowest (Table 2), probably due to the greater number of nucleocapsids contained in this isolate.

Morphological measurements revealed irregular shapes for SfCH15 and SfCH32. The shape and number of virions in OBs depend of baculoviruses genus and species (Caballero et al., 2001). The size values for OBs are particularly similar to those (1.93–2.14 μm)

FIGURE 3 Restriction endonuclease analysis profiles of DNA from SfCH32 and SfCH15 isolates. M molecular marker size 1 kb Plus DNA Ladder (Thermo Scientific), a) second, third and fourth lines showed digestions of SfNIC, SfCH15 and SfCH32, respectively, generated by *Hind*III enzyme on a 0.6% agarose gel, 18 hr/13V; b) second and third lines indicated the digestions of SfCH32 and SfCH15, generated by Bam*HI* enzyme on a 0.6% agarose gel, 16 hr/13 V. $indicated different band among isolates
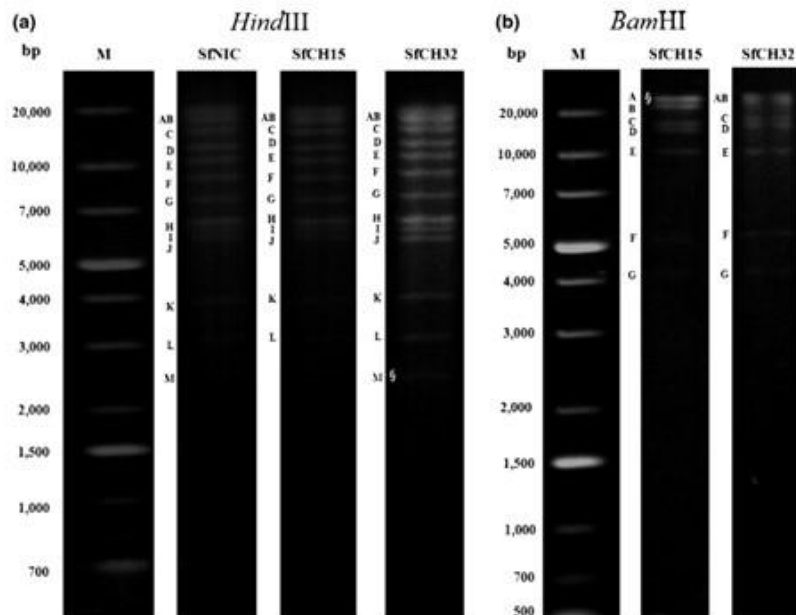
reported by Gómez-Valderrama et al., (2010). The number of virions and nucleocapsids in each OB varied among tested baculoviruses. Up to 6 nucleocapsids per viral envelope were observed in SfCH15, while single or two nucleocapsids were observed in SfCH32 (Figure 2b, 4d). Kumar, Rao, Sireesha, and Kumar (2011) found OBs containing 2–7 nucleocapsids packed within a virion in three lepidopteran pests (*Helicoverpa armigera*, *S. litura* and *Amsacta albistriga*).

The restriction profile of DNA showed differences among tested baculoviruses. This analysis is widely used to differentiate baculoviruses, including baculoviruses isolated from a single insect specimen (Herniou, Olszewski, Cory, & O'Reilly, 2003; Simón et al., 2011). The SfCH15 and SfCH32 native isolates showed two different bands after digestion with *Hind*III and *Bam*HI enzymes, demonstrating the existence of small genetic differences among them. Small differences in the baculoviruses genome could cause significant difference in their pathogenicity and virulence (Behle & Popham, 2012). Nevertheless, sequencing studies, of these isolates could be useful to establish a better relationship between genetic differences and their virulence. The isolates SfCH32 and SfNIC did not show different restriction profiles after digestion with *Hind*III although they were obtained from different geographical regions. This demonstrated that these isolates are genetically related each other, as demonstrated in other studies (Figueiredo et al., 2009). Other restriction enzymes might lead to different restriction patterns for these isolates. However, these restriction enzymes (*Hind*III and *Bam*HI) were chosen because they are found inside the main enzymes that have been reported in previously characterized SfMNPV isolates (Escribano et al., 1999). Other authors have also demonstrated that the genetic variability of baculoviruses allowed for explaining their differences in virulence as a function of the geographical origin (Barrera et al., 2011; Escribano et al., 1999; Simón et al., 2011). Takatsuka et al., (2003) found different restriction profiles in *S. litura* NPVs isolates from Japan, Vietnam and Malaysia, due to the presence of mixtures of genotypes of baculoviruses. The genome size for the isolates SfCH15 and SfCH32 was similar to those (132,254–133,899 bp) reported for other *S. frugiperda* NPVs from different geographical origins (Barrera et al., 2011; Simón et al., 2011). Further studies are required to determinate the exact size of their genomes.

The eight native NPV isolates were pathogenic and virulent against FAW larvae, but the isolates SfCH15 and SfCH32 showed the highest insecticidal activity and corresponded to multiple NPVs. These isolates were genetically different, according to their restriction profiles. Their LD_{50} were similar to those obtained with the Nicaraguan strain, which is worldwide recognized as highly virulent. These results suggest that the native isolates of viruses could be used as biological control agents against *S. frugiperda*; however, studies under field conditions are required to confirm the insecticidal effect of these isolates, since it has been reported that UV radiation and temperature reduce might insecticidal activity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

MOG designed and conducted the experiment and wrote the manuscript, CRV conceived and designed the experiment, JJOP and JCBR wrote the draft manuscript, CHAM, DIBR, MASM and OJCC analysed data and conducted statistical analyses and GGM contributed with biological material. All authors read and approved the manuscript. And all authors have agreed to authorship and the order of authorship.

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DATA AVAILABILITY STATEMENT

Raw data are provided as a supplementary file.

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52

3. MORPHOLOGICAL, BIOLOGICAL, AND MOLECULAR CHARACTERIZATION OF TYPE I GRANULOVIRUSES OF *Spodoptera frugiperda*

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1   **Morphological, biological, and molecular characterization of Type I granuloviruses of**

2   *Spodoptera frugiperda*

3

4   Running title: Characterization of *Spodoptera frugiperda* granulovirus

5

6   **Abstract**

7   The fall armyworm (FAW), *Spodoptera frugiperda,* is the main pest of corn (maize) in Latin

8   America. The Granulovirus genus is an important member of the Baculoviridae family. The

9   granuloviruses associated with FAW, especially those of Type I, have scarcely been studied

10   but they might represent an alternative for the biocontrol of this insect. In this study, the

11   native granuloviruses SfGV-CH13 and SfGV-CH28 were isolated from FAW larvae and

12   characterized for morphology, molecular traits, and insecticidal activity. The elapsed time

13   between infection of larvae and stop feeding as well as the weight of larvae before death or

14   prior to pupation were also evaluated. Both granuloviruses showed ovoid shape with a length

15   of 0.4 μm. They showed the same DNA restriction profiles and their genome sizes were about

16   126 kb. The infection with tested GVs mainly caused flaccidity of larva body and

17   discoloration of integument. The integument lysis was only observed in 8 % of infected

18   larvae. Infected larvae gradually stopped feeding. Overall, these symptoms are characteristic

19   of infections caused by Type I granuloviruses, which are known as monoorganotropic or

20   slow-death. The LD_{50} values for SfGV-CH13 and SfGV-CH28 isolates were 5.4×10^2 and

21   1.1×10^3 OBs/larva, respectively. The LT_{50} ranged from 17 to 24 d, with LT_{50} decreasing as

22   the dose was increased. The elapsed time since infection until pupation (LD_{50}) and body

54

23 weight of larvae (third instar) were higher with SfGV-CH28 than SfGV-CH13. Both

24 granulovirus isolates were able to kill the FAW larvae from the 12th day.

25 **Keywords:** Baculoviridae, Biocontrol, Granulovirus, Pathogenicity, Virulence

1. Introduction

27   Entomopathogenic viruses belonging to the Baculoviridae family are the most widely

28 distributed and studied worldwide as biocontrol agents due to their high specificity and

29 virulence against some insects (Inceoglu et al., 2006). This family groups four genera, with

30 Betabaculovirus, (lepidopteran-specific granulovirus, GV), and Alphabaculovirus,

31 (lepidopteran-specific nucleopolyhedrovirus, NPV) being the most common genera of the

32 family found in lepidopteran insects, including those of agricultural interest (Jehle et al.,

33 2006).

34   Granuloviruses are classified into three types according to their action mode and time

35 required to cause infection and death of the host insect (Sciocco-Cap et al., 2001). The most

36 studied are Type II, which are highly virulent, because they can infect several tissues at the

37 same time (e.g. fatty body, tracheal matrix, and epidermis), causing the death of the host in

38 a short time (four to six days) even at low doses of occlusion bodies (OBs, $\sim 2 \times 10^2$

39 OBs/larva) (Federici, 1997; Sciocco-Cap et al., 2001). The CpGV and EpapGV are examples

40 of Type II GVs that have successfully allowed the biocontrol of the lepidopterans *Cydia*

41 *pomonella* L., and *Epinotia aporema* (Wals.), respectively (Jehle et al., 2017; Sciocco-Cap

42 et al., 2001). Types I and III GVs have scarcely been studied probably because are rare in

43 nature. Type I GVs present a slow insecticidal activity due to require a long time (15-37 d,

44 depending on dose and larval instar) to cause infection or death of host. This type of viruses

45 are known as slow-killing and can generate a horizontal transmission, which has higher

46   efficacy for long-term control of insect pests since they can infect larvae at different instars,

47   retard their development, and cause the dead at the final instar producing a greater number

48   of OBs (Hatem et al., 2011; Hilton & Winstanley, 2008; Takahashi et al., 2015). Type I GVs

49   firstly infect the midgut epithelium and, then, the infection migrates to the fatty tissue of the

50   insect, with this tissue being the most important infection site (Federici, 1997; Sciocco-Cap,

51   2001). Other tissues are rarely damaged by Type I GVs, allowing the development of the

52   larvae and favoring the replication of the viruses in the insect and the protection of plants for

53   a longer time. The infections caused by Type I GVs do not generally cause integument lysis

54   but induce flaccidity of insect body (Sciocco-Cap, 2001). Type I GVs are recognized as factor

55   of natural mortality for lepidopteran pests despite of their slow insecticidal activity. Type I

56   GVs and NPVs can co-infect the same host larva (Gómez et al., 2010). This mixture

57   significantly alters the development of the infection and might be used as new viral

58   bioinsecticides or cocktails with other viruses (Caballero et al., 2001; Cuartas et al., 2014;

59   Haase et al., 2015).

60   Some studies have demonstrated that some types of GVs can to increase the insecticidal

61   effectiveness of other GVs and NPVs (Espinel-Correal et al., 2012; Shapiro, 2000). By

62   enhancin (metalloprotease) protein present in OBs, which digest the peritrophic membrane

63   of the insect and facilitate the access of the virions into intestinal cells (Bivian-Hernández et

64   al., 2017; Ishimwe et al., 2015). The granuloviruses of *Spodoptera frugiperda* have scarcely

65   been studied, as compared to *Spodoptera frugiperda* NPVs. To date, only three Type I

66   granuloviruses (isolates from Colombia, Brazil, and Argentina) have been associated with

67   the fall armyworm (FAW), *Spodoptera frugiperda* J. E. Smith (Lepidoptera: Noctuidae)

68   (Cuartas et al., 2014; Pidre et al., 2019). This insect is the main pest of corn (maize; *Zea mays*

69   L.) in Mexico and other countries, causing crop losses exceeding 30% (Casmuz et al., 2010).

70 Currently, the control of FAW is mainly based on the use of broad-spectrum chemical

71 insecticides, obtaining satisfactory results in pest control but causing negative consequences

72 on the environment and the health of agricultural workers (Hardke et al., 2011). Thus, the

73 objective of this work was to characterize two Type I granulovirus isolates and determine

74 their insecticidal activity against *Spodoptera frugiperda*.

75 **2. Materials and methods**

76 *2.1 Insect rearing and propagation of granuloviruses*

77 The FAW larvae were obtained from a laboratory colony maintained under controlled

78 conditions (26 ± 2 °C, >70% RH, 12:12 L: D) and fed with artificial diet (Southland Products

79 Inc; Lake Village, AR, USA).

80 Two granulovirus isolates (SfCH13 and SfCH28) were obtained from infected FAW

81 larvae in corn plots from Chihuahua, Mexico (latitude 28°12'44"N, longitude 106°59'45"W,

82 and altitude 2,125 m asl; latitude 28°40'59"N, longitude 106°48'50"W and altitude 2,079 m

83 asl for SfCH13 and SfCH28). These GVs were found in mixture with NPVs, co-infecting the

84 same FAW larva. For this reason, the GVs were separated from NPVs by filtration using

85 filter papers (pore sizes of 1.5 and 0.45 μm, respectively) and, then, by sucrose gradients (40

86 and 66%, w/w) using a gradient former (CBS Scientific, GM 200) according to Muñoz et al.

87 (2001) and Ordóñez-García et al. (2020). Twenty milliliters of these sucrose solutions were

88 placed into 30 ml polypropylene tubes and then 5 ml of the viral suspension were deposited

89 on the surface of the gradients and centrifuged ($40,310 \times g$, 4°C, 1.5 h). The bands containing

90 OBs were recovered using a Pasteur pipette and placed into 30 ml polypropylene tubes to be

91 washed twice with sterile distilled water (SDW) by centrifugation ($40,310 \times g$, 4°C, 40 min).

92 The purification was confirmed by an optical microscope (Carl Zeiss AxioScope A1; Carl

93   Zeiss, Gottingen, Germany) at $1,000 \times$ magnifications. The OBs obtained from both GV

94   isolates were propagated in fourth instar FAW larvae by the drop feeding method (Hughes

95   & Wood, 1981). Larvae killed by GVs infection were collected and macerated in sterile

96   mortars using SDW containing SDS (1%). The excess of larval cuticle was removed by

97   filtration using muslin and centrifugation ($8,500 \times g$, 4 °C, 10 min). The pellet of OBs was

98   re-suspended in 10 ml of SDW and stored at -80 °C.

99   *2.2 Bioinsecticidal activity*

100   The insecticidal activity of GV isolates was determined by estimating the median lethal

101   dose (LD_{50}) and the median lethal time (LT_{50}) in third instar FAW larvae using the drop feed

102   method (Hughes & Wood, 1981). Six viral doses (1×10^1, 5×10^1, 1×10^2, 1×10^3, 1×10^4,

103   and 1×10^5 OBs/larva) were tested to estimate the LD_{50}, and three higher doses (1×10^6, $1 \times$

104   10^7, and 5×10^7 OBs/larva) were used to determine the LT_{50}. These doses were selected

105   because they caused a larval mortality greater than 90% (data not shown). Each FAW larva

106   was starved for 12 h and, then, supplied with 0.5 µl of purified viral suspensions (Ordoñez-

107   García et al., 2020). The OBs were mixed with Fluorella blue (0.001%, w/v) and sucrose

108   (10%, w/v) before use. Previously, OBs were disaggregated by sonication (Branson 1510,

109   Connecticut, USA) for 30 s and were counted in triplicate in a Neubauer chamber

110   (Marienfeld, Germany), using a phase-contrast microscope (Carl Zeiss, AxioScope A1;

111   Gottingen, Germany) at $1000 \times$ magnifications (Muñoz et al., 2001). The infected larvae

112   were individually placed into 29.5 ml plastic cups and fed with artificial diet and maintained

113   under the controlled ambient conditions described above. Both bioassays were performed in

114   triplicate for each viral dose using 25 FAW larvae per replicate. Only larvae consuming the

115   whole inoculum and showing intestinal tract with blue color, as confirmed by observation

116 under a stereomicroscope (Leica G26), were considered in the experiment. The number of

117 larvae killed by the action of tested GVs was recorded every 24 h, or until they reached the

118 pupal stage (Barrera et al., 2011). An additional study was conducted to determine the elapsed

119 time between the infection of larvae with the LD_{50} (5.4×10^2 and 1.1×10^3 OBs/larva for

120 SfCH13 and SfCH28, respectively) and LD_{90} (4.3×10^5 and 9.5×10^5 OBs/larva,

121 respectively) and stopped feeding. The weight of larvae before death was also evaluated. This

122 bioassay was performed in triplicate, using a total of 25 larvae per replicate following the

123 methodologies and conditions described above. The infected larvae were weighed every 24

124 h, from the sixth day post-infection (dpi). The larvae were placed into new cups with artificial

125 diet to check if they were still feeding.

126 *2.3 Morphological characterization*

127 The granulovirus isolates were identified according to their morphological characters

128 using an optical microscope Carl Zeiss AxioScope A1 at 1000 × magnifications. The

129 morphological characteristics of tested granuloviruses were determined by scanning electron

130 microscopy (SEM) and transmission electron microscopy (TEM). For SEM analysis, one

131 drop of the viral suspension was placed on the sample holder, dried, covered with a gold layer

132 (Auto Sputter Coater 108; Cressington Scientific Instruments, Watford, UK), and

133 immediately visualized by a scanning electron microscope FEI Helios Nanolab 600

134 DualBeam (FEI Company; Hillsboro, OR, USA). At least 50 OBs were considered for the

135 measurement of size. For TEM analysis, the OBs were fixed using a mixture of 2.5%

136 glutaraldehyde and 2% paraformaldehyde, and then, the samples were placed on 1% osmium

137 tetra-oxide. The samples were dehydrated with ethanol and embedded with a resin. Ultrathin

138   sections were cut and observed by a transmission electron microscope JEOL JEM-200CX

139   (JEOL Ltd; Tokyo, Japan) at 80 kV.

140   *2.4 Extraction of virions and viral DNA*

141   The releasing of virions from OBs was performed by mixing the viral suspensions with 1

142   ml of 0.1 M sodium carbonate (Na_2CO_3), 1 ml of 0.1 M sodium chloride (NaCl) at pH 10.8,

143   and 1 ml of buffer (TE) (0.01 M tromethamine (Tris) hydrochloric acid (HCl), 0.001 M

144   ethylenediaminetetraacetic acid (EDTA) at pH 7.6. The mixture was incubated at 28 °C for

145   2 h under agitation (140 rpm) and then an equal volume of buffer (TE) (1 ml) was added

146   (Ordóñez-García et al., 2020). The released virions were purified by continuous sucrose

147   gradients (20 and 66%, w / w) according to Muñoz et al. (2001), with modifications. Briefly,

148   20 ml of the formed gradient were placed into 30 ml polypropylene tubes, and immediately,

149   5 ml of the virion suspension were deposited on the surface and were centrifuged (40,310 ×

150   g, at 4 ° C, 1.5 h). The bands of virions were collected with a Pasteur pipette and washed

151   twice with SDW using centrifugation (40,310 × g, at 4 °C, 40 min). The pellets with virions

152   were re-suspended in 500 μl of SDW and stored at -20 °C until use.

153   For the extraction of viral DNA, the virion samples were mixed with 400 μl of buffer of

154   proteinase K (0.01 M Tris, 0.005 M EDTA, 0.5% SDS) and incubated at 65 °C for 15 min.

155   Then, 100 μl of proteinase K (2 mg/ml) (Invitrogen Life Technologies Corp; Carlsbad, CA,

156   USA) was added and the reaction mixture was incubated at 37 °C for 2 h. An aliquot of 500

157   μl of a mixture phenol: chloroform: isoamyl alcohol (25: 24: 1) was added to the reaction

158   and then it was centrifuged (17,000 × g, at 4 °C, 5 min). The aqueous phase was collected in

159   a new microtube, and a volume of isopropyl alcohol (500 μl) and 100 μl of 3M sodium acetate

160   were added to the samples previous to be incubated at –20 ° C for 2 h. The mixture was

161   centrifuged (17,000 × *g*, 4 ℃, 10 min) and the pellet was washed with 70% ethyl alcohol

162   using centrifugation (17,000 × *g*, 4°C, 5 min). The pellet was re-suspended in 30 μl of sterile

163   ultrapure water. The quality of viral DNA was examined by electrophoresis on 1% agarose

164   gels. The DNA concentration was determined by a A260 NanoDrop One spectrophotometer

165   (Thermo Fisher Scientific; Waltham, MA, USA).

166   *2.5 Restriction endonuclease analysis*

167   Both GV isolates were digested with *Hind*III, *Bam*HI, and *Pst*I enzymes (Invitrogen Life

168   Technologies Corp; Carlsbad, CA, USA). One microgram of viral DNA was digested with

169   10 U of the enzymes, at 37 °C for 2 h. The reaction was stopped by adding 2 μl of loading

170   buffer 10X (Thermo-Fisher Scientific; Waltham, MA, USA). The obtained restriction

171   fragments were analyzed by electrophoresis on 1% agarose gels at 25 V for 7 h, using TAE

172   buffer (40 mM Tris-acetate, 1 mM EDTA at pH 8.0). A molecular weight marker of

173   GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) and 10 μl 10,000X SYBR Safe

174   DNA gel stain (Invitrogen) were used to visualize the DNA on agarose gels using the image

175   system (Bio-Rad ChemiDoc™ XRS⁺; Hercules, CA, USA). The fragment sizes and number

176   of both GVs isolates were estimated by comparing the bands with those of the molecular

177   weight marker using the Image Lab software version 5.2.1 (Bio-Rad ChemiDoc™ XRS⁺;

178   Hercules, CA, USA).

179   *2.6 Statistical analysis*

180   The experiment was conducted under a completely randomized design. The data on

181   insecticidal activity were analyzed by an analysis of variance (ANOVA), and the means were

182   separated by a Tukey's test ($p < 0.05$). The LD_{50}, LD_{90}, LT_{50} and fiducial limit values were

183 analyzed using log-Probit regressions (Finney, 1971). All data were analyzed using SAS

184 software (SAS, 2002). Mortality was corrected by the Abbott (1925) formula.

3. Results

185

186 *3.1 Insecticidal activity*

187 The isolates caused different larvae mortality at four of the six tested doses (Fig. 1). Larval

188 mortality caused by the highest dose (1×10^5 OBs/larva) of both GVs started on 12 dpi and

189 on 15 dpi with the lowest dose (1×10^1 OBs/larva). The death of larvae ended on 45 dpi (data

190 not shown) and averaged ~8% at that time point. Both isolates caused the same infection

191 symptoms (Fig. 2). GV-infected larvae gradually stopped feeding and their bodies become

192 flabby. The integument lysis was only observed in 8% of infected larvae, which showed fatty

193 body shedding after 20 dpi and remained alive in this condition for at least two days (Figs.

194 2b and 2d). These larvae also showed swelling and an atypical milky whitish color on their

195 cuticle, with this color being quite different to that of control larvae (Fig. 2).

196 The estimated LD_{50} for the SfGV-CH13 and SfGV-CH13 isolates were 5.4×10^2 and 1.1

197 $\times 10^3$ OBs/larva, respectively (Table 1). The LT_{50} decreased as the viral dose was increased.

198 LT_{50} of 17.3 and 19.8 d were obtained with the highest dose (5×10^7 OBs/larva) of SfGV-

199 CH13 and SfGV-CH28, respectively. The lowest dose (1×10^5 OBs/larva) of these isolates

200 led to LT_{50} of 24.4 and 24.8 d (Table 2). The LD_{50} and LD_{90} of both GVs caused a delay in

201 larval development. The maximum body weight prior to pupation and the time required by

202 the larvae to reach pupation after infection with LD_{50} depended on isolate, with SfGV-CH28

203 isolate causing the highest changes in these response variables (Table 3). The times required

204 to reach the pupal stage by control larvae and larvae treated with LD_{50} of the SfGV-CH13

205 isolate were 15.17 and 15.26 days, respectively. The body weight of these larvae averaged

206   0.51 and 0.47 g/larva, respectively. The larvae treated with the SfGV-CH28 isolate required

207   2.5 days more to reach pupation and their body weight was up to 0.1 g higher than those of

208   the other experimental groups.

209   The infection with tested isolates at LD_{90} reduced the body weight of FAW larvae,

210   according to the measurements carried out at 6, 10 and 15 dpi (Fig. 3). However, the body

211   weight of larva at 25 dpi was similar for all experimental groups (Fig. 3). Nevertheless, 20%

212   of larvae infected with the SfGV-CH13 isolate showed a body weight ≥ 0.60 g between 19

213   and 27 dpi and 28% of the larvae infected with both isolates stopped feeding for a period of

214   3 to 6 days, after 14 dpi (data no shown).

215   *3.2 Morphological characterization*

216   OBs of both isolates showed a homogeneous ovoid shape. The length of OBs ranged from

217   0.36 to 0.49 μm for the SfGV-CH13 isolate and from 0.37 to 0.45 μm for the SfGV-CH28

218   isolate, with an average length of 0.4 μm for both GVs isolates (Figs. 4a and 4c). Both isolates

219   showed a single virion (~0.3 μm) per OB (Figs. 4 b and 4d).

220   *3.3 Molecular characterization*

221   Both isolates showed the same DNA restriction profiles. Thirteen, 14, and 16 restriction

222   fragments were observed with the enzymes *Hind*III, *Bam*HI, and *Pst*I, respectively (Fig. 5).

223   The DNA size for tested isolates was about 126 kb.

224   **4. Discussion**

225   Tested isolates killed FAW larvae since 12th dpi. Similar times for starting of death for

226   insects infected by Type I GVs (7-14 dpi) have already been reported (Inceoglu et al., 2001).

227   In our study, the larvae died from 12 to 45 dpi, with this time range being higher than that

228   reported for nucleopolyhedroviruses (3-8 dpi) infecting the FAW larvae (Barrera et al., 2011;

229 Ordóñez-García et al., 2020). The data for insecticidal activity demonstrated that tested

230 isolates were Type I, causing a monorganotropic infection and an slow death of the insect

231 (Alletti et al., 2017). The infection caused by tested isolates was similar to those documented

232 for this type of GVs, with such symptoms differing significantly from those observed in

233 insects infected with NPVs (Barrera et al., 2011; Ordóñez-García et al., 2020; Pidre et al.,

234 2019). The symptoms commonly observed in larvae infected with GVs include the cessation

235 of feeding, larvae swelling, little or no liquefaction of insect body, and darkening of insect

236 body (Sauer et al., 2017; Wang et al., 2008).

237 Tested GVs did not cause liquefaction of larvae body, probably due to tested the isolates

238 did not infect the epidermis, with this preventing the deposition of chitinase on the peritrophic

239 membrane (Sciocco-Cap, 2001). Rohrmann (2019) observed that some NPVs contained

240 gp37, a chitinase favoring the fusion of virions with cells of the middle intestine. The creamy-

241 yellow appearance observed on larvae infected with tested GV isolates can be attributed to

242 the accumulation of high quantities of OBs on the fatty body tissues of larvae (Sciocco-Cap,

243 2001). This fatty tissue completely detached from the rest of the larva body in 8% of the

244 larvae infected with tested GVs. Pidre et al. (2019) also observed severe lesions in the last

245 abdominal segments (fatty tissue) of FAW larvae infected with a granulovirus isolate from

246 Argentina. Tested GVs showed different virulence, with SfGV-CH13 isolate being was two

247 times more virulent than the SfGV-CH28 isolate. The LD_{50} and LT_{50} values obtained with

248 tested isolates were lower than those reported by Cuartas et al. (2014) for Brazilian and

249 Colombian GVs isolates from *S. frugiperda*. They (Cuartas et al., 2014) reported LC_{50} of 4.5

250 $\times 10^5$ and 1.6×10^5 OBs/ml with mean times to death (MTD) of 29 and 33 d for the VG008

251 and VG014 isolates, respectively. Hackett et al. (2000) observed shorter survival times (15.3

252 - 18.3 d) for *Helicoverpa armigera* larvae infected with a *H. armigera* Type I granulovirus

253 (HaGV) that those observed in our study. The low rate of larval death could be related to the

254 tropism of Type I granuloviruses, which only infect the midgut and fatty body of the host

255 insect, as compared to Type II granuloviruses and the nucleopolyhedrovirus that infect many

256 tissues and cause a rapid death of the host (Sciocco-Cap, 2001). Some insects can also delay

257 the infection of Type 1 GVs as a first defense mechanism, blocking viral replication in cells

258 during the early stages of infection (Hinsberger et al., 2019; Moore, 2002; Pauli et al., 2018;

259 Wang et al., 2008). The larvae infected with the SfGV-CH28 isolate required more time to

260 reach the pupal stage and showed a slower weight gain rate than those of the other

261 experimental groups. Wang et al. (2008) observed that infected larvae can live more and be

262 bigger than uninfected larvae. However, larvae infected at LD_{90} of both isolates showed a

263 lower weight at 15 dpi than control larvae, with this indicating that a lower viral dose (LD_{50})

264 can increase the survival time and body weight prior to death as compared to the highest viral

265 dose (LD_{90}). This infection-mediated lengthening of the developmental process of larvae,

266 involving more time to reach pupal or adulthood stages, is favorable for the management of

267 insect pests, because the larvae are exposed to their natural enemies more time (Machado et

268 al., 2020).

269 The ovoid shape, size, and the number of virions observed for tested OBs were similar to

270 those observed for other OBs of lepidopteran granuloviruses (Barrera et al., 2014; Ikeda et

271 al., 2015; Luque et al., 2001; Moscardi, 1999). The size of tested GVs was into the range (0.3

272 × 0.5 μm) observed by Ikeda et al. (2015). It was also similar to that (0.43 μm) of *Spodoptera*

273 *frugiperda* granulovirus (SfGV) (Cuartas et al., 2014). However, other sizes have been

274 reported. Wang et al. (2008) and Barrera et al. (2014) observed sizes of 0.24-0.34 and 0.30

275 μm for *Spodoptera litura* granulovirus (SpltGV) and *Erinnyis ello* granulovirus (EeGV),

276 respectively.

277   Restriction enzyme analysis allowed the molecular identification of tested isolates, which

278   showed the same number and positions of the fragments after digestion with the enzymes

279   *Hind*III, *Bam*HI, and *Pst*I (Fig. 5). This similarity might be related to the geographical origins

280   of the isolates, which was similar for them. Barrera et al. (2014) did not observe differences

281   in the restriction profiles of three granulovirus isolates due to they were genotypic variants

282   of the same viral strain. On the other hand, Cuartas et al. (2014) found differences in the

283   restriction fragments of *S. frugiperda* VG008 and VG014 granuloviruses from Colombia and

284   Brazil. Ordóñez-García et al. (2020) also observed small differences in the restriction patterns

285   (*Hind*III and *Bam*HI enzymes) of SfCH15 and SfCH32 NPV isolates, both obtained from

286   nearby area in Mexico. However, tested isolates showed different virulence despite of their

287   molecular similarity, suggesting that they were possibly different genotypes. Ali et al. (2018)

288   stated that the genotypes of viruses differ in dose response and time required to kill the host

289   (biological activity), as observed in our study. Tested isolates showed significant differences

290   in mortality percentages at some doses, as well as at the LD_{50} and LT_{50}, with SfGV-CH13

291   isolate being more virulent than the SfGV-CH28 isolate. The genome sizes for tested isolates

292   were about ~ 126 kb. This size was smaller to those estimated by Cuartas et al. (2014) for

293   VG008 (135 kb) and VGO14 (132.6 kb). Pidre et al. (2019) estimated a size of at least 135

294   kb for an Argentinian isolate of *Spodoptera frugiperda* granulovirus. However, complete

295   genome sequencing of GV isolates is necessary to obtain more accurate values.

296   **Conclusion**

297   Tested isolates showed characteristics of Type I GVs because they infected only one tissue

298   and caused a slow death of the insect. They killed more than 90% of *S. frugiperda* caterpillars

299   at 45 dpi at a dose of 1.0×10^5 OBs/larva. Both isolates were genetically identical, according

300 to their DNA restriction profiles. The LD$_{90}$ extended two times the larval development time

301 of *S. frugiperda*. The LD$_{50}$ values obtained with both GVs were similar to those reported for

302 NPVs, however, their LT$_{50}$ were lower than those previously reported for other *Spodoptera*

303 *frugiperda* granulovirus. Our results suggest that tested isolates might be used as biocontrol

304 agents against *S. frugiperda* despite of their high LT$_{50}$

Declaration of Competing Interest

311 The authors declare no conflict of interest

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464

465 **FIGURE LEGENDS**

466 **FIGURE 1.** Larval mortality caused by different doses of the granulovirus isolates SfGV-

467 CH13 and SfGV-CH28 at 45 days post-infection. Equal literals on standard error bars

468 indicate that there is no statistical difference between isolates at the same dose, according to

469 Tukey's test ($p < 0.05$). The numbers 1 to 6 in the x-axis indicate the following isolate doses:

470 1×10^1, 5×10^1, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 OBs/larva, respectively.

471

472 **FIGURE 2.** Appearance of FAW larvae infected with tested isolates with at a dose of ($1 \times$

473 10^4 OBs/larva). Larvae infected with the SfGV-CH13 isolate at 30 days post-infection (dpi)

474 (a); Larvae infected with the SfGV-CH13 showing exposition of fat body at 32 dpi (b);

475 Larvae infected with the SfGV-CH28 isolate at 30 dpi (c) and; Larvae infected with the

476 SfGV-CH28 isolate showing damage in the abdominal segments at 22 dpi (d).

477

478 **FIGURE 3.** Effect of the lethal dose (LD_{90}) on the larval development of *S. frugiperda*

479 larvae infected with native granuloviruses evaluated until 25 days post-infection.

480

481 **FIGURE 4.** Morphology of the GVs. SEM (a) and MET (b) micrographies (50,000 and

482 20,000 × magnifications, respectively) of the SfGV-CH13 isolate. SEM (c) and MET (d)

483 micrographies (50,000 and 20,000 × magnifications, respectively) of the SfGV-CH28

484 isolate. NC, nucleocapsid. V, virion.

485

486 **FIGURE 5.** DNA restriction profiles of SfGV-CH13 and SfGV-CH28 isolates on a 1%

487 agarose gel (7h/25V). First line shows the molecular marker size. Second to fourth lines

488 show the restriction profiles of the SfGV-CH13 isolate generated by *Hind*III, *Bam*HI, and

489 *Pst*I enzymes; respectively. Fifth to seventh lines show the restriction profiles of the SfGV-

490 CH28 isolate generated by *Hind*III, *Bam*HI, and *Pst*I enzymes; respectively.
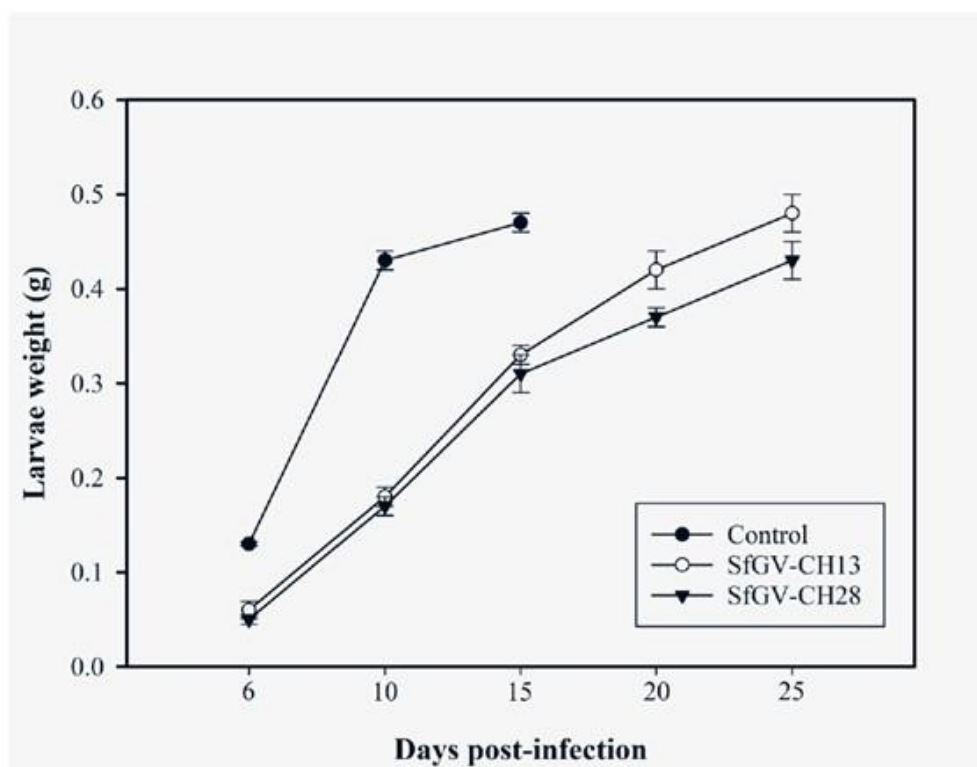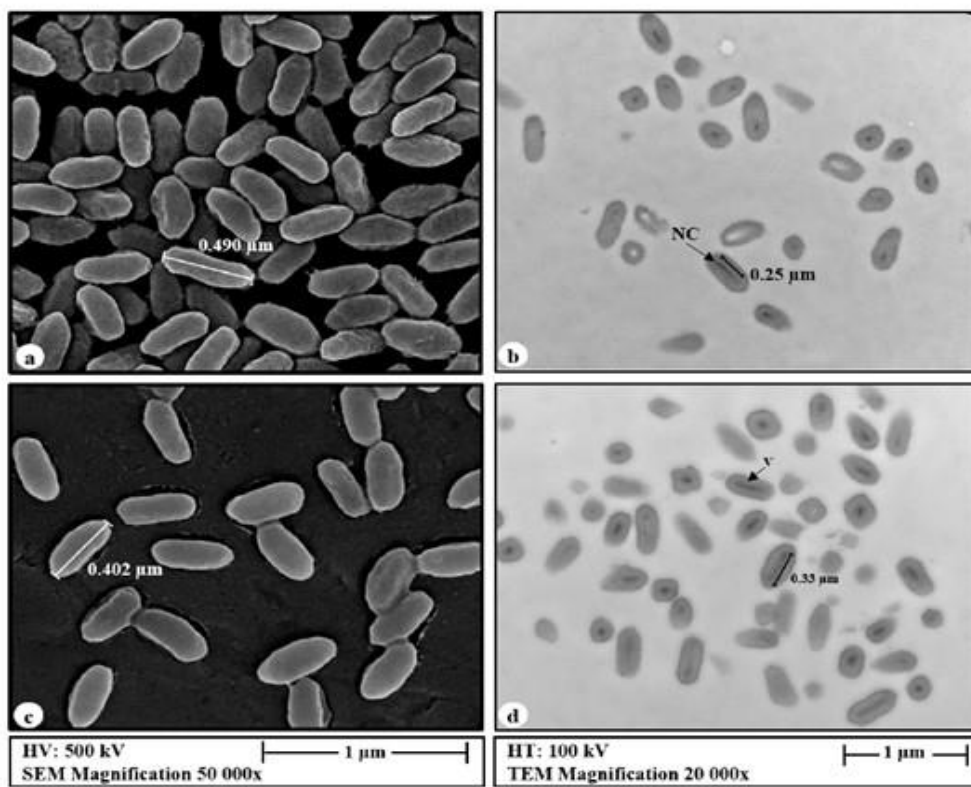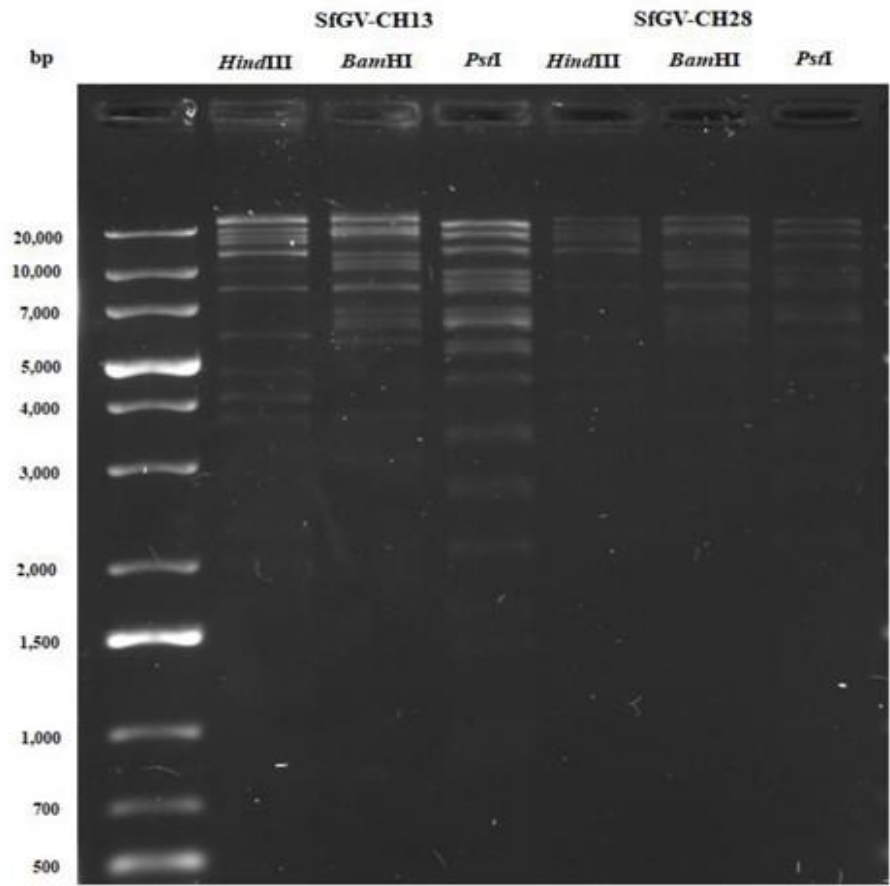
491   Fig 1.

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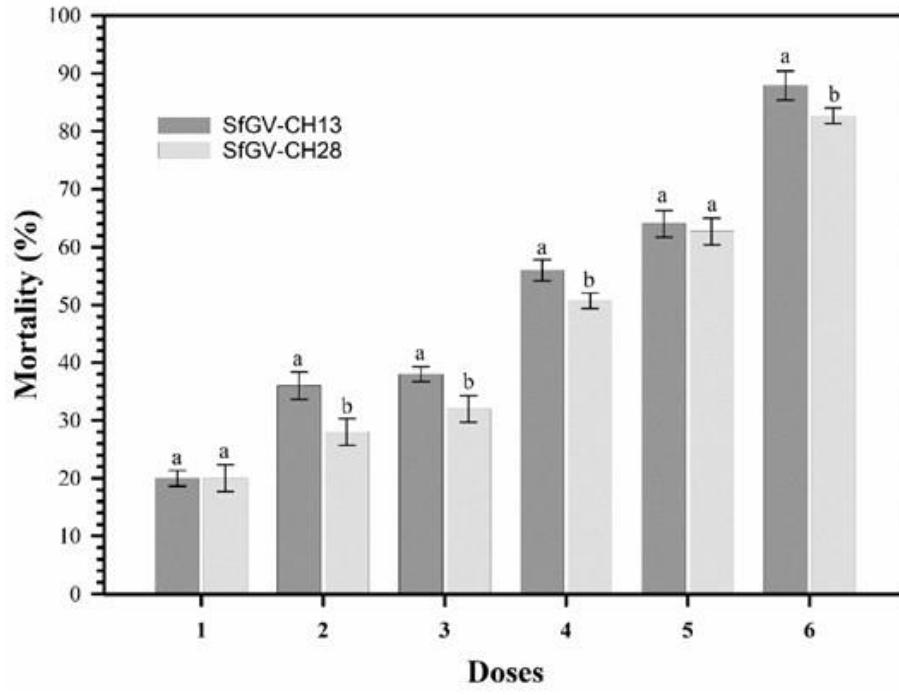
499   Fig 5.

501 **Table 1**

502 Median lethal dose (LD_{50}) of tested granulovirus isolates against third instar *S. frugiperda*
503 larvae

Isolate	LD_{50}	Fiducial limits (95%)		χ^2	df	Slope ± (SE)	Intercept ± (SE)
		Lower	Upper				
SfGV-CH13	5.4×10^2	3.1×10^2	9.5×10^2	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
SfGV-CH28	1×10^3	6×10^2	2×10^3	1.1	4	0.4 ± 0.08	-1.3 ± 0.12

504   LD_{50}: Median lethal dose expressed as OBs/larva. These values were obtained from a minimum of 6 doses
505 (treatments) and estimated at 45 day post-infection.
506 Probit regressions were fitted using SAS program. The LD_{50} was estimated from a minimum of five
507 doses.
508 χ^2 = Goodness of fit test.
509 df = degrees of freedom
510 SE = Standard error

511 **Table 2**

512 Median lethal time (LT_{50}) of the doses of tested granulovirus isolates tested against third
513 instar *Spodoptera frugiperda* larvae.

Isolate	Doses (OBs/larva)	LT_{50} (h)[a]	LT_{50} (d)[b]	Fiducial limits (95%)	
				Lower	Upper
SfGV-CH13	1×10^5	585.1	24.4	489.9	514.5
	1×10^7	502.2	20.9	489.9	514.5
	5×10^7	414.7	17.3	403.6	425.9
SfGV-CH28	1×10^5	596.0	24.8	578.3	613.7
	1×10^7	540.3	22.5	522.0	558.4
	5×10^7	476.2	19.8	455.9	495.7

514 Data correspond to the average of three replicates. [a]LT_{50} Median Lethal Time expressed in hours; [b]LT_{50} Median
515 Lethal Time expressed in days.

516 **Table 3**

517 Development of *S. frugiperda* larvae with the median lethal dose of two
518 native granuloviruses.

Isolate	Days to reach the pupal stage ± (SE)	Maximum weight before pupal stage ± (SE)
SfGV-CH13	$15.17 \pm 0.09b$	$0.51 \pm 0.02b$
SfGV-CH28	$17.82 \pm 0.40a$	$0.62 \pm 0.01a$
Control	$15.26 \pm 0.05b$	$0.47 \pm 0.01b$

519 All values are arithmetic means ± standard error. Values with the same literal between
520 treatments to same evaluation time are statistically equal according to the Tukey's test (p
521 < 0.05).
522 SE = Standard error

4. INDIVIDUAL AND COMBINED ENTOMOPATHOGENIC ACTIVITY OF A *Spodoptera frugiperda* MULTIPLE NUCLEOPOLYHEDROVIRUS AND A TYPE I *Spodoptera frugiperda* GRANULOVIRUS ON *Spodoptera frugiperda* LARVAE

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Individual and combined entomopathogenic activity of a *Spodoptera frugiperda* multiple Nucleopolyhedrovirus and a Type I *Spodoptera frugiperda* Granulovirus on *Spodoptera frugiperda* larvae

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Abstract

The fall armyworm (*Spodoptera frugiperda*) is the main pest of maize. Nucleopolyhedrovirus (NPV) and Granulovirus (GV) are entomopathogenic viruses belonging to Baculoviridae family and are recognized as biocontrol agents for this insect. In this study, the insecticidal activity of several doses of a SfMNPV (LD_{10}, LD_{50} and LD_{90}) and a Type I SfGV (LD_{50} and LD_{90}), alone and in co-infection, was evaluated on *S. frugiperda* larvae. In the case of the co-infection assays, one virus was applied at 0h and then, the second virus was supplied at different times (0, 12 and 24 h), in order to test the effect of the co-infection time on the insecticidal activity of the viruses. The symptoms observed in the co-infected larvae depended on the viral dose supplied at 0 h. Larvae treated with the highest dose (LD_{90}) of NPV and co-infected with GV at LD_{50} only showed symptoms of nucleopolyhedrovirus infection at 14 dpi. However, larvae initially infected with the highest dose of GV (LD_{90}) and subsequently co-infected with NPV (LD_{50} and LD_{10}) at different times showed symptoms of infection with both viruses. The insecticidal activity of GV and NPV alone or in combination depended on the viral doses and the time elapsed between the first and second inoculation. The insecticidal activity was not influenced when both viruses were supplied

at high doses (LD_{50} and LD_{90}) at the same time at the beginning of the experiment. The infection with GV at high doses (LD_{50} and LD_{90}) before infection with NPV mostly caused antagonist effects, independently of the time of NPV infection. Few treatments caused a synergistic effect, especially when larvae were firstly infected with NPV at medium and high doses (LD_{50} and LD_{90}) followed by the co-infection with medium and high doses (LD_{50} and LD_{90}) of GV after 24h. This synergistic effect was lost when the initial infection was carried out with the lowest dose (LD_{10}) of NPV and the highest dose of GV (LD_{90}), except when the GV was inoculated first and the NPV 24 h later.

Keywords: Baculoviruses, Biological control; Fall armyworm, Interaction, Pest;

1. Introduction

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is the main pest of maize (corn; *Zea mays* L.) and other crops in Mexico and other countries, causing crop losses exceeding 30 % (Goergen *et al.*, 2016; Harrison *et al.*, 2019; Nboyine *et al.*, 2020; Ordóñez-García *et al.*, 2020; Rijal, 2019). The control of this insect has been based on chemical insecticides, which cause negative effects on human health and the environment, with the latter including the elimination of beneficial insects (Sree and Varma, 2015). This has forced to search for environment-friendly methods for the control of FAW. The use of natural enemies (fungi, bacteria, viruses, nematodes, protozoa, parasitoids and predator insects) of FAW as a strategy of control represents has advantages over chemical insecticides (Zimmermann, 2007). The Baculoviridae members are known among entomopathogenic viruses as excellent biocontrol agents of this pest and have been considered as primary components in lepidopteran pest management programs (Natra *et al.*, 2015). The Baculoviridae family groups four genera, with the Alphabaculovirus (Nucleopolyhedrovirus; NPVs) and Betabaculovirus (Granulovirus; GVs) genera being lepidopteran-specific, and the most widely distributed and studied worldwide (Inceoglu *et al.*, 2006; Sood *et al.*, 2019). The biological activity of both viruses genera has been widely documented on many lepidopteran pests (Barrera *et al.*, 2014; Bernal *et al.*, 2013; Harrison *et al.*, 2012; Jukes *et al.*, 2014; Zamora-Avilés *et al.*, 2017). NPV isolates are the most widely used and studied as biocontrol agents of FAW (Barrera *et al.*, 2011; Behle and Popham, 2012; Martínez *et al.*, 2012; Méndez *et al.*, 2002; Rangel-Núñez *et al.*, 2014; Ordóñez-García *et al.*, 2020; Vieira

et al., 2012). The widespread use of *S. frugiperda* NPVs is a consequence of their high virulence, infecting several tissues at the same time (e.g. fatty body, tracheal matrix, and epidermis) and causing the death of the insect in a short time (four to six days) even at low doses of occlusion bodies (OBs) (Rios-Velasco *et al.*, 2011b; Simón *et al.*, 2008). The use of type I *S. frugiperda* GVs as agent for pest control has only been documented by Barrera *et al.* (2014) and Pidre *et al.* (2019). The type I GVs infect mainly the fatty tissue, causing a slow death of the insects (Federici, 1997). Studies with other baculoviruses have demonstrated that the co-infection of insects with different types of these viruses can cause effects of potentiation, synergism, and antagonism (Biedma *et al.*, 2015; Espinel-Correal *et al.*, 2012; Hackett *et al.*, 2000). Cuartas-Otálora *et al.* (2019) observed that mixtures of two baculoviruses genera positively influenced the insecticidal capacity of one or both of them. Some types of GVs can easily digest the peritrophic membrane of the insect by the an enhancin (metalloprotease) protein present in their OBs, facilitating the access of virions of NPVs and others GVs into intestinal cells and, consequently, increasing the insecticidal activity of these viruses (Cheng and Lynn, 2009; Ishimwe *et al.*, 2015). In general, the current knowledge on the insecticidal activity of viral genera of *S. frugiperda* in co-infection is scarce, disperse, and conflicting. The aim of the present study was to evaluate the insecticidal activity of a SfMNPV and a Type I SfGV isolates alone and in combination against FAW larvae at different doses and infection times.

2. Materials and methods

2.1 Insect rearing

The FAW larvae were obtained from a laboratory colony, which had been reared for 12 months (eight generations) in Cuauhtemoc, Chihuahua, Mexico. The colony was established using larvae collected from a maize plot (latitude 28°12′44″N, longitude 106°59′45″W, altitude 2,125 m above sea level). Larvae were maintained under controlled conditions (26 ± 2 °C, >70% RH, 12:12 L: D photoperiod) and fed with artificial diet (Southland Products Inc.). Adult moths were fed with a sugar solution and maintained in 15 L cylindrical plastic containers.

2.2 Virus propagation

A GV (SfCH13 isolate) and a NPV (SfCH32 isolate) were used in the study. These isolates were described previously (Ordóñez-García *et al.,* 2015). The isolates were propagated separately in fourth instar FAW larvae by the droplet feeding method (Hughes and Wood, 1981). For the inoculation, 0.5 µL of viral suspension (~10^8 and ~10^{10} OBs of NPVs and GVs, respectively) were supplied to larvae that had been starved for 12 h. The OBs were mixed with Fluorella blue (0.001%, w/v) and sucrose (10%, w/v) before use. The infected larvae were individually placed into 29.5 mL plastic cups containing artificial diet and maintained under the controlled ambient conditions described above. The dead larvae were collected and stored at –20 °C. Then, these larvae were macerated in sterile mortars using sterile distilled water (SDW) containing 1 mg/mL of sodium dodecyl sulphate (SDS). The larval cuticle was removed by filtration through muslin. The filtrates were placed into 10 mL polypropylene tubes and centrifuged ($8,500 \times g$, 4 °C, 10 min). The pellet with the OBs was re-suspended in 10 mL of SDW and stored at –80 °C. Then, the OBs were purified by continuous sucrose gradients (40 and 66%, w/w) using a gradient former (CBS Scientific, GM 200). Five milliliters of the viral suspension were deposited on the surface of 20 mL of the gradients previously collected into 30-mL polyallomer tubes and ultracentrifuged ($40,310 \times g$, 4°C, 1.5 h). The bands containing OBs were recovered using a Pasteur pipette and placed into 30 mL polypropylene tubes to be washed twice with SDW by ultracentrifugation ($40,310 \times g$, 4°C, 40 min). Finally, the pure OBs were re-suspended in 1 mL aliquots of SDW and stored at –80 °C until use.

2.3 Bioassays

The insecticidal activity of the Granulovirus (SfGV-CH13) isolate was evaluated using the median lethal dose (LD_{50}) and LD_{90} at 45 days post-infection (dpi) and the LD_{10}, LD_{50}, and LD_{90} for Nucleopolyhedrovirus (SfMNPV-CH32) isolate at 14 dpi. The doses tested of NPV and GV were determined in our previous study and by preliminary experiments, respectively (Ordóñez-García *et al.*, 2020) (Table 1). The co-infection of third instar FAW larvae with SfGV and SfMNPV baculoviruses was also evaluated. These bioassays were performed by suppling the lethal doses of both isolates at 0, 12, and 24 h after suppling the first virus (GV or NPV). All bioassays were performed using the droplet feeding method (Hughes and Wood, 1981). FAW larvae used in the bioassays were starved for 12 h and, then, supplied with 0.5 µl or 1 µl of purified viral suspensions,

previously counted in triplicate in a Neubauer chamber (Marienfeld, Germany), using a phase-contrast microscope (Carl Zeiss, AxioScope A1; Gottingen, Germany) at 400 and 1000 × magnifications for NPV and GV, respectively (Muñoz *et al.*, 2001; Ordóñez-García *et al.*, 2020). The OBs were disaggregated by sonication (Branson 1510, Connecticut, USA) for 30 s after being counted and then, mixed with Fluorella blue (0.001%, w/v) and sucrose (10%, w/v) before use in bioassays.

In the case of the co-infection, the inoculation with both isolates (0.5 µl of each viral suspension) at 0h was performed at the same time. At 12 or 24 h, the viral suspensions were supplied as described in Table 2. At 12 and 24 h, the larvae infected with the first viral isolate were individually placed into 29.5 ml plastic cups and fed with artificial diet for 4 h and 12 h and then, starved for 8 h and 12, respectively, before supplying 0.5 µl of the second viral suspension. At all-time points, the larvae were maintained under the controlled ambient conditions described above. Only larvae consuming the whole inoculum and showing intestinal tract with blue color, as confirmed by observation under a stereomicroscope (Leica G26) were considered in the experiment. The number of dead larvae was recorded every 8 h until 14 dpi in all treatments. After this day, the larval mortality in treatments with GVs alone (T1 and T2) and in mixture (T7-T20; Table 2) was monitored every 24 h until larvae death (~ 58 d).

Three hundred seventy five larvae were used for monitoring the individual biological activity of the lethal doses indicated above for NPV and GV isolates, i.e., 75 larvae per treatment (T1-T5; Table 2), and 1,125 larvae to monitor the effects of virus mixtures (T6-T20) in larvae at different times (0, 12 and 24 h; Table 2). Larvae (75) treated with 0.5 µl of Fluorella blue and sucrose solution were used as absolute control group (T21; Table 2). The infected larvae were individually placed into 29.5 ml plastic cups and fed with artificial diet and maintained under the controlled ambient conditions described above.

2.4 Experimental design and statistical analysis

The experiment was conducted under a completely randomized design. In total, 21 treatments were evaluated in triplicate using 25 larvae per replicate. The obtained data were analyzed by an analysis of variance (ANOVA) and the means were separated by a Tukey´s test ($p < 0.05$). The lethal doses and fiducial limit values were analyzed using log-Probit regressions (Finney, 1971). All data were analyzed using SAS using SAS software (SAS, 2002).

3. Results

3.1 Individual insecticidal activity of the baculovirus

Untreated larvae did not die and reached the pupal stage14 days after starting the bioassays. The infection with GVs at LD_{50} and LD_{90} caused a larval mortality of 9.3 and 20% at 14 dpi, and 46.7 and 93.2% at 45 dpi, respectively. The larval mortality with SfNPV at LD_{10}, LD_{50} and LD_{90} was 9.3, 52 and 86.7% at 14 dpi, respectively. The larval mortality percentages observed at tested doses of both viral isolates were expected. The larvae infected with SfGV-CH13 at LD_{50} and LD_{90} survived by long time (28 and 43 dpi, respectively) and showed typical symptoms of GV infection, including cessation of feeding, slow movement, and a soft and yellowish-white (pale) cuticle (Fig. 1a). On the other hand, the larvae infected with the SfNPV-CH32 isolate showed slow movement, vomiting, diarrhea, lysis of their brown and black cuticle (Fig. 1b), and some larvae stopped feeding after ~3 dpi.

3.2 Insecticidal activity of the baculovirus mixtures

Larvae infected with a high dose of nucleopolyhedrovirus (LD_{90}) and a medium dose of granulovirus (LD_{50}) (Table 2) showed typical symptoms of nucleopolyhedrovirus infection at 14 dpi and after this time, they showed only symptoms of granulovirus infection. On the other hand, the larvae co-infected with the medium dose of NPV (LD_{50}) and the highest dose of the GV (LD_{90}) (T11 to T15) as well as the larvae co-infected with the lowest dose of NPV (LD_{10}) and the highest dose of the GV (LD_{90}) (T16 to T20) showed symptoms of infection with both viruses at 14 dpi. The highest larval mortality at 14 dpi was observed in larvae initially inoculated with the highest dose (LD_{90}) of NPV and 24 h after with the lowest dose (LD_{50}) of GV (T10) (Fig. 2b). This mortality was similar to that caused by NPV alone at the highest dose (T5) (Fig. 2a), and by larvae inoculated at the same time with the lowest dose (LD_{50}) of GV and the highest dose (LD_{90}) of NPV (T6). However, in these three treatments (T5, T6 and T10), the maximum mortality was reached between 8 and 9 dpi (Fig. 3a). Retarding 12 and 24 h the co-infection with NPV at the highest dose in larvae previously infected with the lowest dose of GV (T7 and T8) caused a gradual reduction of the larval mortality, which was of 60 and 54.7% in these treatments, respectively. This trend revealed a clear effect of antagonism. This effect was observed since 6 dpi when the larval mortality ranged from 10.7 to 21.8% for T7 and T8. These mortalities were significantly lower than that caused by the

infection with NPV alone (82.7%; T5) (Fig. 3a). However, when the highest dose of NPV was initially (0 h) applied in larvae and the co-infection with the lowest dose of GV was retarded 12 and 24h (T9 and T10), the mortally gradually increased until 69.3 and 93.4%, respectively (Fig. 2b).

On the other hand, when the viruses were applied at the same time but their doses were inverted (T11), i.e., GV at LD_{90} and of NPV at LD_{50}, the mortality was lower than that observed for the co-infection assay involving GV at LD_{50} and NPV at LD_{90} (T5), demonstrating the high relevance of NPV on infection development. In fact, all co-infection treatments involving the medium and highest doses (LD_{50} and LD_{90}) of NPV (T6-T15) caused a higher mortality than the infection with GV alone at all of tested doses, independently of the infection time with NPV. Retarding 12 and 24 h the coinfection with the medium dose of NPV (LD_{50}) in larvae previously infected with the highest dose of GV (LD_{90}) (T12 and T13) reduced slightly the larval mortality, from 46.7 to 37.3 and 32%, as compared when both viruses were supplied at the same time (T11). However, it was not significant, probably due to the advanced development of GV infection. The mortality in treatments involving these concentrations of viruses (GV at LD_{90} and NPV at LD_{50}) was only increased by firstly infecting the larvae with NPV and retarding in 24 h the coinfection with GV (T15) (Fig. 2c).

The lowest mortality values in co-infection assays were observed with the lowest concentration of NPV (LD_{10}), independently of the suppling time, reaffirming the importance of this virus on infection development (Fig. 2d). The larvae co-infection with the highest dose of GV (LD_{90}) and the lowest dose of NPV (LD_{10}) at different inoculation times extended up to ~13 days (55 to 58 dpi) the larval survival time, as compared with the co-infection (~45 dpi) with the medium LD_{50} and highest LD_{90} doses of NPV and GV. The mortality values were generally similar in treatments involving this NPV (LD_{10}) dose (T16-T20), except when the co-infection with NPV (T18) was retarded 24 h in larvae firstly infected with the highest dose of GV (LD_{90}). In this treatment an increase in larval mortality was observed, highlighting an increase in mortality of 22.7%, as compared to the expected value (10%) with infection with NPV alone at LD_{10} of (Fig. 2d, Fig. 3c).

4. Discussion

No mortality was recorded in for the control larvae of *S. frugiperda* (T21), which reached the pupal stage approximately 14 days after inoculation. Similar values were reported by Du Plessis *et al.*

(2020). On the other hand, the infection symptoms varied with the treatment. Larvae treated only with GV or NPV developed infection symptoms characteristic of each infecting virus, as reported by others (Barrera *et al.*, 2011; Pidre *et al.*, 2019; Wang *et al.*, 2008). Larvae treated with the highest dose (LD_{90}) of NPV and co-infected with GV at LD_{50} (i.e., T5-T10) only showed symptoms of nucleopolyhedrovirus infection at 14 dpi, probably as a consequence of the high dose of nucleopolyhedrovirus supplied to the larvae. Similarly, Cuartas-Otálora *et al.* (2019) observed that the coinfection of larvae with high doses of NPV and low doses of GV of *S. frugiperda* supplied at the same time predominantly caused symptoms of nucleopolyhedrovirus infection. Nevertheless, the larvae initially infected with the highest dose of GV (LD_{90}) and subsequently co-infected with NPV (LD_{50} and LD_{10}) at different times showed symptoms of infection characteristic of both viruses. In our study, the inoculation order defined the success of each one of the viral isolates, as reported by others (Dias-Vasconcelos, 2001).

Different interaction effects were observed in the experiment, depending on the dose and inoculation time of each virus. In this sense, the high insecticidal activity of NPV was not largely influenced by GV when both viruses were supplied at the same time at high concentrations (LD_{50} and LD_{90}). An antagonistic effect was observed in our study with the high doses (LD_{50} and LD_{90}) of both viral isolates when the NPV was supplied 12 or 24 h after the infection with GV. This trend was observed since 6 dpi. This effect of antagonism was clearly observed in T7, T8, T12 and T13, causing a decrease in larval mortality, probably because in these treatments were the replication of the GV started 12 and 24 h, as compared to that of NPV causing a competition among the viruses for the same replication site (Dias-Vasconcelos, 2001).

This antagonistic effect has been scarcely documented in studies on interaction of baculoviruses (Hackett *et al.*, 2000; Whitlock, 1977). This effect has been widely documented in interactions between baculovirus and parasitoid insects, finding that both compete for host resources, especially for replication sites, with the first of them infecting or parasitizing the host causing the predominant effect (Dias-Vasconcelos, 2001). Hackett *et al.* (2000) documented an antagonistic effect in *H. zea* larvae co-infected with a *Helicoverpa armigera* granulovirus (HaGV-9.6 × 10^6 OBs per cup) and a *Helicoverpa zea* nucleopolyhedrovirus (HzSNPV-10^3 OBs per cup) and attributed the antagonism to a competition between both viruses for resources of the host insect and a HaGV protein inhibiting the HzSNPV replication. They observed that the antagonistic effect was still present even when the HaGV was supplied 36 h after HzSNPV, time in which the nucleopolyhedrovirus should fully be

established in the host and penetrated the peritrophic membrane. In our study, the antagonism effect was not observed when the GV was supplied 24 h after delivery of NPV (i.e., T10) probably due to an advanced replication level of NPV because nucleopolyhedroviruses replicate their DNA between 6 hpi and 18 dpi (Sciocco-Cap, 2001). Whitlock (1977) observe an antagonistic effect in *Heliothis armigera* larvae co-infected with OBs from a NPV and a GV, noting an important decrease of larval mortality (~50%) as a consequence of the antagonism.

A synergistic effect was observed in T10, T15 and T18, where the second viral isolate (GV or NPV) was supplied 24 h later. An increase in the insecticidal activity of NPV was observed in these treatments due to the co-infection. In treatments 10 and 15, where larvae were first supplied with the GV (LD_{50} and LD_{90}) and 24 h later they were coinfected with the NPV (LD_{50} and LD_{90}), the synergism could be given due to the advantage of the NPV in the infection time, although GV probably favored the NPV infection due to some granuloviruses can rapidly degrade the peritrophic membrane. The high concentration of the GV (LD_{90}) and the elapsed time before inoculation of the NPV probably favored the entry of NPV in T18. Guo *et al.* (2007) observed increases in the insecticidal capacity (virulence) of *Spodoptera litura* nucleopolyhedrovirus (SlNPV) on *S. litura* larvae when the larvae were co-infected with a granulovirus of *Xestia c-nigrum* (XcGV), noting the rupture of peritrophic matrix of larvae treated with XcGV alone or with both viruses in coinfection. Espinel-Correal *et al.* (2012) documented an increase in the insecticidal activity when the Peruvian *Phthorimaea operculella* granulovirus (PhopGV) and Colombian *Phthorimaea operculella* granulovirus (PhopGV) co-infected *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolny) larvae, as compared with the individual infection with the viruses. Similar results were reported by Biedma *et al.* (2015), who co-infected *A. gemmatalis* larvae with a *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) used at a low dose (50 OBs/larva) and a *Epinotia aporema* granulovirus (EpapGV) at 6000 OBs/larva, finding an increase in the mortality rates (from 42- to 81%) and a decrease in the median survival time (from 7.7 to 5.5 days) as a consequence of the co-infection. Cuartas-Otálora *et al.* (2019) also documented an enhancement in the insecticidal activity (~27% larval mortality) after co-infection of second instar FAW larvae with the isolate SfCOL of *S. frugiperda* multiple nucleopolyhedrovirus (SpfrMNPV) and low doses of the isolate VG008 of *S. frugiperda* granulovirus (SpfrGV) and they attributed this enhancement to proteins from OBs of *S. frugiperda* granulovirus VG008, which caused holes in the peritrophic membrane facilitating the access of NPV virions. On the other hand, an increase in the larval

survival time was observed in some treatments (T17-T20), where the larvae were supplied either 12 or 24 h later with the lowest dose of NPV (LD_{10}) or 12, or 24 h later with the highest dose of GV (LD_{90}). These viral proportions might favor a higher number of OBs since the larvae survived for longer periods and probably reached a larger size, as reported previously (Lasa *et al.*, 2007). Similar results were reported by Hackett *et al.* (2000), who found that the survival time of *H. zea* larvae increased as the dose of GV was increased. The increase in larvae survival time could be associated with a sublethal effect of the Type I granulovirus, causing changes in the physiological processes associated with the development stages, development times, and death larvae. However, the mechanisms driving these effects of sublethal doses are poorly understood, and in most cases, the effects have been related to hormonal and enzymatic changes associated with viral infections (Hatem *et al.*, 2011).

5. Conclusions

The results demonstrated that the insecticidal activity of SfMNPV was influenced by the inoculation time and dose. The supply of both viruses at the same time did not influence the insecticidal activity of NPV. An antagonism effect is caused by the retardation of 12-24h in the supply of high doses (LD_{50} and LD_{90}) of the SfMNPV isolate to larvae previously infected with high doses (LD_{50} and LD_{90}) of GV or when the supply of the SfGV isolate (LD_{50} and LD_{90}) is retarded 12 h in larvae infected with high doses of SfMNPV (LD_{50} and LD_{90}). A synergistic effect was caused by high doses (LD_{50} and LD_{90}) of SfGV supplied at 24h to larvae previously treated with high doses (LD_{50} and LD_{90}) of SfMNPV. The use of high doses (LD_{90}) of GV in combination with low doses (LD_{10}) of NPV increases ~3-4 times the larval development time.

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FIGURE CAPTIONS

FIGURE 1. Appearance of FAW larvae infected with tested isolates alone and in mixtures. Larva infected with the SfGV-CH13 isolate alone at a dose of 4.3×10^5 OBs/larva at 11 days post-infection (dpi) (a); Larva infected with the SfNPV-CH32 isolate alone at a dose of 6.6×10^3 OBs/larva at 6 dpi (b); Dead larvae at 6 dpi, when larvae were infected with GV and NPV at the same time at doses of $LD_{50}:LD_{90}$ (c), $LD_{90}:LD_{50}$ (d), and $LD_{90}:LD_{10}$ (e), respectively.

FIGURE 2. Larval mortality caused by the isolates SfGV-CH13 (GV) and SfNPV-CH32 (NPV) at different concentrations (LD_{10}, LD_{50} and LD_{90}) alone (a) and confecting (b, c and d) the larvae after 0, 12, and 24 h of delivery of the first virus. Data were taken at 14 days post-infection. $^\blacklozenge$, the viruses were delivered at the same time (0h), *, delivered 12 h after of the infection with the first isolate. ‡, delivered 24 h after of the infection with the first isolate. Equal literals on standard error bars indicate that there is no statistical difference between isolates at the same dose, according to Tukey's test ($p < 0.05$).

FIGURE 3. Larval mortality caused by the isolates SfGV-CH13 and SfNPV-CH32 alone and in co-infection from 3 to 14 days post-infection. See Table 2 for treatment assignment.
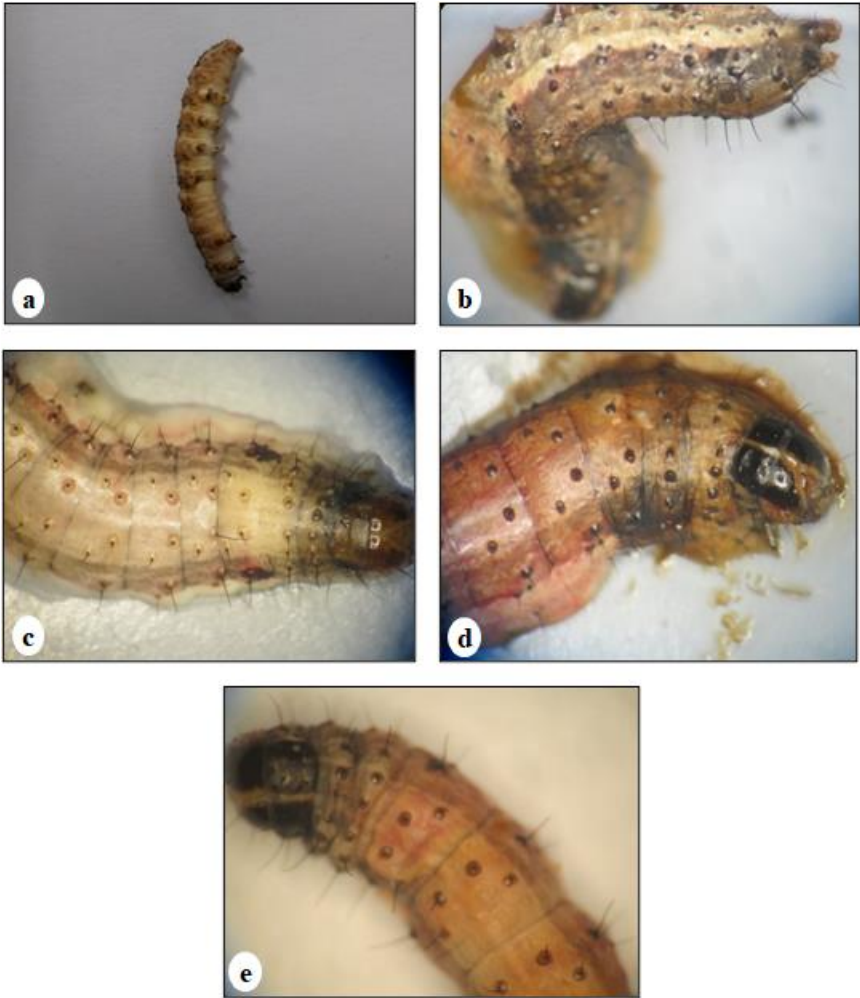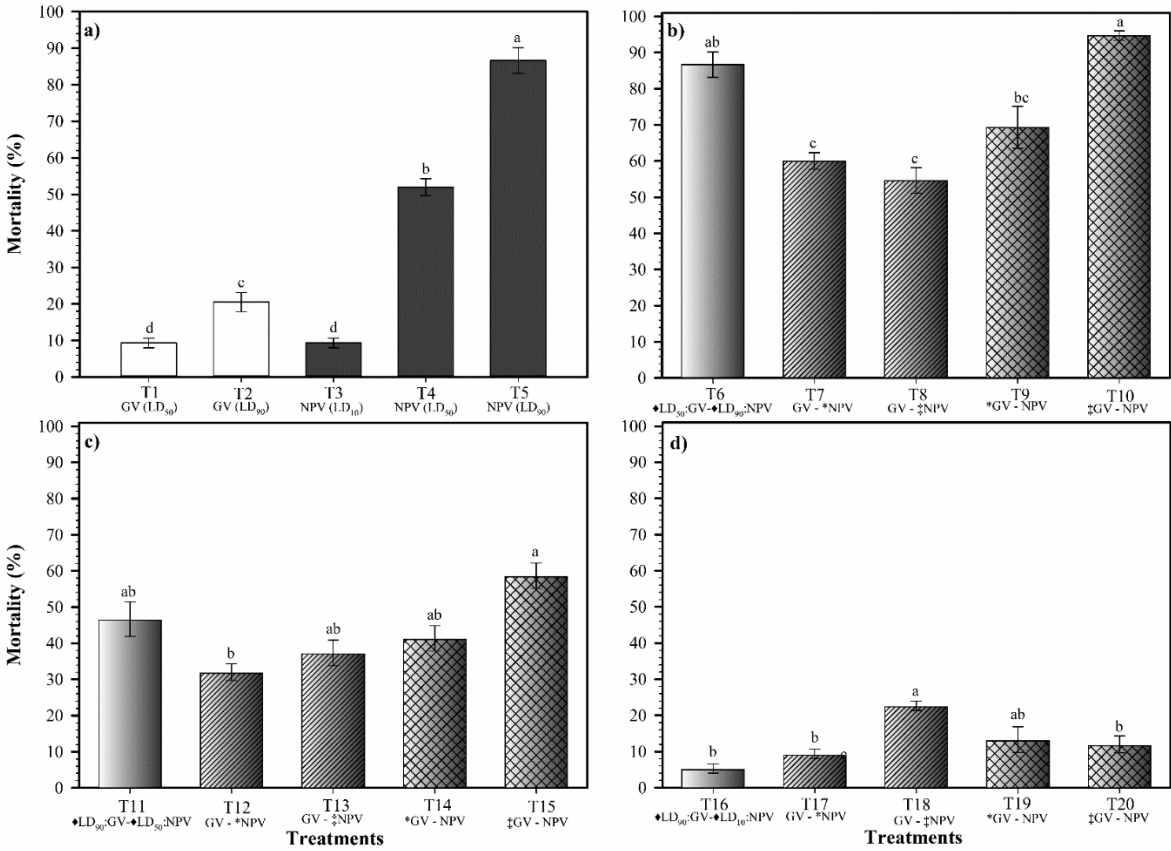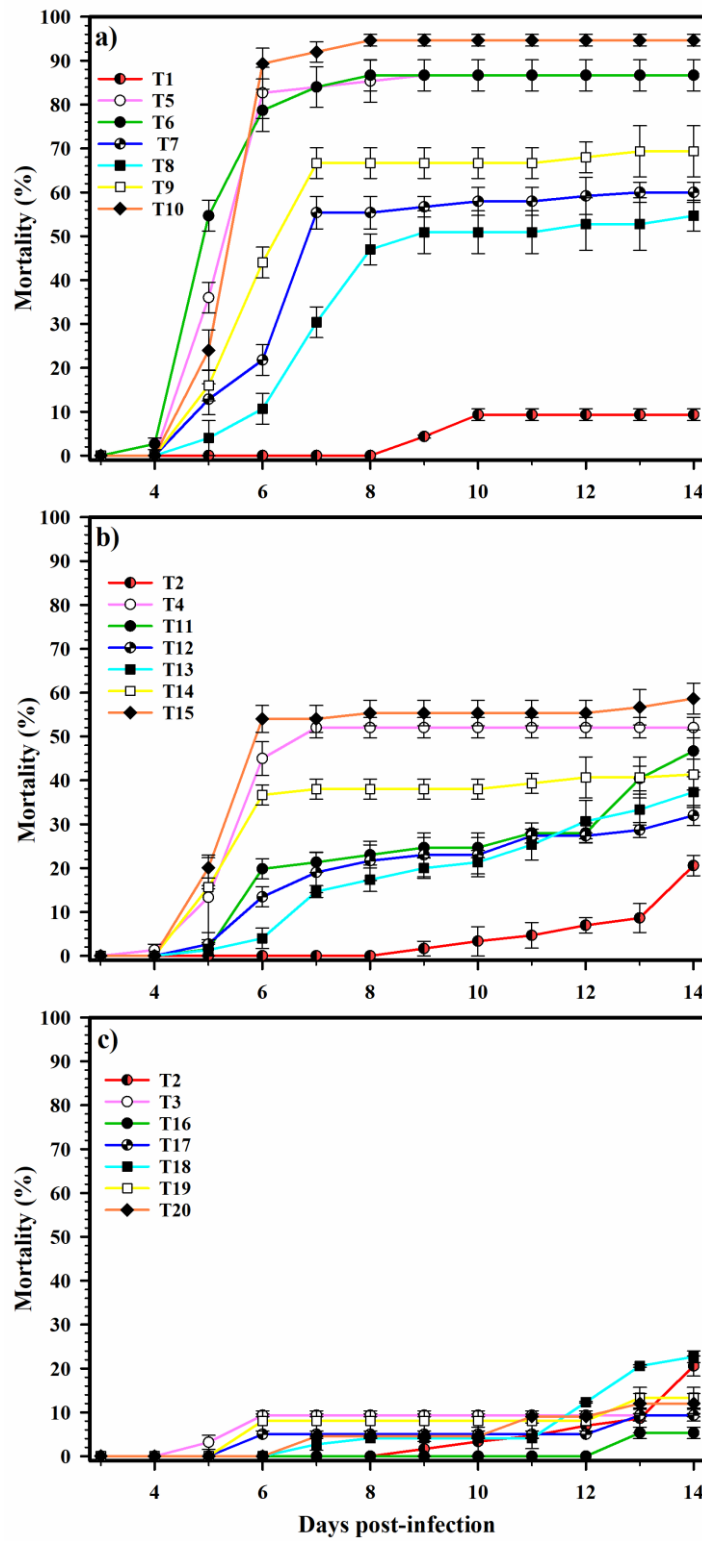
Fig 1.

Fig 2.

Fig 3.

Table 1. Lethal doses of tested granulovirus and nucleopolyhedrovirus isolates against third instar *S. frugiperda* larvae

Isolate	Lethal Doses	Doses (OBs/larva)	Fiducial limits (95%)		χ^2	df	Slope ± (SE)	Intercept ± (SE)
			Lower	Upper				
SfGV-CH13	LD$_{50}$	5.4×10^2	3.1×10^2	9.5×10^2	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
	LD$_{90}$	4.3×10^5	1.3×10^5	2.3×10^6	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
	LD$_{10}$	4.7×10^1	2.6×10^1	6.7×10^1	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
SfNPV-CH32	LD$_{50}$	5.6×10^2	4.3×10^2	7.4×10^2	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
	LD$_{90}$	6.6×10^3	4.4×10^3	1.1×10^4	4.5	4	0.4 ± 0.04	-1.2 ± 0.13

Granulovirus LD$_{50}$ and LD$_{90}$ lethal doses expressed as OBs/larva. These values were obtained from a minimum of 6 doses (treatments) and estimated at 45 day post-infection.

Nucleopolyhedrovirus LD$_{10}$, LD$_{50}$ and LD$_{90}$ lethal doses expressed as OBs/larva. These values were obtained from a minimum of 5 doses (treatments) and estimated at 14 day post-infection.

Probit regressions were fitted using SAS program.

χ^2 = Goodness of fit test.

df = degrees of freedom

SE = Standard error

Table 2. Treatments evaluated in the bioassays against third instar *S. frugiperda* larvae

Treatment	Dose OBs/larva		Inoculation of viral isolates	Interaction type
	SfGV-CH13	SfNPV-CH32		
T1	5.4×10^2 (LD$_{50}$)	--	Alone	--
T2	4.3×10^5 (LD$_{90}$)	--	Alone	--
T3	--	4.7×10^1 (LD$_{10}$)	Alone	--
T4	--	5.6×10^2 (LD$_{50}$)	Alone	--
T5	--	6.6×10^3 (LD$_{90}$)	Alone	--
T6	⬧ LD$_{50}$	⬧ LD$_{90}$	Same time	Neutralism
T7	LD$_{50}$	* LD$_{90}$	GV at 0h + NPV at 12 h	Antagonism
T8	LD$_{50}$	‡ LD$_{90}$	GV at 0h + NPV at 24h	Antagonism
T9	*LD$_{50}$	LD$_{90}$	NPV at 0h + GV at 12 h	Antagonism
T10	‡ LD$_{50}$	LD$_{90}$	NPV at 0h + GV at 24 h	Synergism
T11	⬧ LD$_{90}$	⬧ LD$_{50}$	Same time	Neutralism
T12	LD$_{90}$	* LD$_{50}$	GV at 0h + NPV at 12 h	Antagonism
T13	LD$_{90}$	‡ LD$_{50}$	GV at 0h + NPV at 24h	Antagonism
T14	* LD$_{90}$	LD$_{50}$	NPV at 0h + GV at 12 h	Antagonism
T15	‡ LD$_{90}$	LD$_{50}$	NPV at 0h + GV at 24 h	Synergism
T16	⬧ LD$_{90}$	⬧ LD$_{10}$	Same time	Neutralism
T17	LD$_{90}$	* LD$_{10}$	GV at 0h + NPV at 12 h	Neutralism
T18	LD$_{90}$	‡ LD$_{10}$	GV at 0h + NPV at 24h	Synergism
T19	* LD$_{90}$	LD$_{10}$	NPV at 0h + GV at 12 h	Neutralism
T20	‡ LD$_{90}$	LD$_{10}$	NPV at 0h + GV at 24 h	Neutralism
T21	Uninfected larvae		Control	

⬧, supplied at the same time
*, supplied 12 h after of the infection with the first isolate.
‡, supplied 24 h after of the infection with the first isolate.
-- There is no interaction because only one isolate was evaluated

5. CONCLUSIONES GENERALES

- El tamaño de los viriones y las nucleocápsides fue consistente con lo reportado en la literatura para NPVs y GVs de *Spodoptera frugiperda*.

- Los NPVs aislados son del tipo múltiple lo que podría estar relacionado con su mayor eficacia hacia *S. frugiperda*. Mientras que los GVs corresponden al tipo I (monoorganotrópicos).

- Los NPVs fueron más virulentos hacia larvas de *S. frugiperda* que los GVs, pero en menor grado que el aislado Nicaragüense.

- Los síntomas manifestados por las larvas infectadas por NPVs y GVs fueron consistentes con aquellos reportados para estos virus de *S. frugiperda*, excepto con los síntomas causados por el aislado de Nicaragua, destacando la necrosis, vómito y diarrea frecuentes.

- Los NPVs provocaron licuefacción de la cutícula larval de *S. frugiperda*, mientras que los GVs, únicamente causaron flacidez en la larva.

- Las dosis bajas de GVs (10^2 COs/larva) permiten que más del 50 % de la población de larvas lleguen al estado de pupa.

- El tiempo requerido para causar mortalidad en larvas del tercer estadio de *S. frugiperda* fue tres veces menor para los NPVs (~5 d) que para los GVs (~17 d).

- Las coinfecciones evidenciaron la replicación de ambos géneros virales en las larvas, donde a los 14 dpi prevalecieron los síntomas de la dosis viral más alta suministrada inicialmente.

- La actividad insecticida del NPV se vio alterada por el GV, cuando este se suministró a las larvas de *S. frugiperda*, 12 o 24 h antes de suministrar el NPV, evidenciando un antagonismo.

- Las altas dosis de Granulovirus (DL_{90}) en combinación con bajas dosis de Nucleopoliedrovirus (DL_{10}), a diferentes tiempos de inoculación (12 y 24 h) incrementan ~ de tres a cuatro veces el tiempo de supervivencia de las larvas.

6. RECOMENDACIONES

- Evaluar la expresión diferencial de genes durante la coinfección entre NPVs y GVs, para determinar los genes reprimidos o sobreexpresados en ambos aislados.

- Cuantificar la expresión de genes de virulencia durante la coinfección entre NPVs y GVs mediante PCR en tiempo real.

- Determinar la cantidad de cuerpos de oclusión replicados de cada género viral durante el proceso de coinfección y muerte del insecto.