BIOLOGICAL CONTROL

Morphological, Biological, and Molecular Characterization of Type I Granuloviruses of *Spodoptera frugiperda*

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Abstract

Granuloviruses (GVs) Betabaculovirus associated with the fall armyworm (FAW), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), especially those of the type I, have scarcely been studied. These GVs might be an effective alternative for the biocontrol of this insect. In this study, the native GVs SfGV-CH13 and SfGV-CH28 were isolated from FAW larvae and characterized for morphology, molecular traits, and insecticidal activity. The elapsed time between symptomatic infection of larvae and stop feeding as well as the weight of larvae before death or prior to pupation were also evaluated. Both GVs had ovoid shape and a length of 0.4 μm. They had the same DNA restriction profiles and their genome sizes were about 126 kb. The symptomatic infection with the tested GVs mainly caused flaccidity of larva body and discoloration of integument. The integument lysis was only observed in 8% of infected larvae. Infected larvae gradually stopped feeding. Overall, these symptoms are characteristic of infections caused by type I GVs, which are known as monoorganotropic or slow-killing GVs. The median lethal dose (LD_{50}) values for SfGV-CH13 and SfGV-CH28 isolates were 5.4×10^2 and 1.1×10^3 OBs/larva, respectively. The median lethal time (LT_{50}) ranged from 17 to 24 days. LT_{50} values decreased as the viral dose was increased. The elapsed time from symptomatic infection until pupation and body weight of larvae (third instar) were higher with SfGV-CH28 than SfGV-CH13. Both granulovirus isolates were able to kill the FAW larvae from the 12th day.

Keywords Baculoviridae · Biocontrol · Granulovirus · Pathogenicity · Virulence · Fall armyworm

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Introduction

Entomopathogenic viruses of the Baculoviridae family are the most widely distributed and studied as biocontrol agents due to their high specificity and virulence against some pest insects (Inceoglu et al. 2006; Williams et al. 2023). This family groups four genera, but the Betabaculovirus (lepidopteran-specific granuloviruses, GVs) and Alphabaculovirus (lepidopteran-specific nucleopolyhedroviruses, NPVs) genera are the most commonly found in lepidopteran insects, including those of agricultural interest (Herniou et al. 2011; Jehle et al. 2006).

Betabaculoviruses are classified into three types according to their action mode and time required to cause symptomatic infection and death of the host insect (Federici 1997). Type II GVs are the most studied, due to their high virulence, being able to infect several tissues (e.g., fatty body, tracheal matrix, and epidermis) simultaneously, which consequently causes the death of their hosts in a short time (< 6 days)

even using low doses of occlusion bodies (OBs, ~ 2×10^2 OBs/larva) (Sciocco-Cap 2001). The *Cydia pomonella* GV (CpGV) and *Epinotia aporema* GV (EpapGV) are examples of type II GVs that have successfully allowed the biocontrol of the lepidopterans *Cydia pomonella* L. and *Epinotia aporema* (Wals.), respectively (Biedma et al. 2015; Jehle et al. 2017).

Type I GVs exert slow insecticidal activity, requiring 15–37 days, depending on dose and host instar, to cause symptomatic infection or death of host. This type of viruses is known as slow-killing viruses and can cause a horizontal transmission, which has higher efficacy for long-term control of insect pests since they can infect different larval instars. However, the susceptibility to baculoviruses decreases with increasing instar larval (Sporleder et al. 2007). Hatem et al. (2011) demonstrated that L3 larvae were 14.72 times more susceptible to type I GVs than L5 larvae. Type I GVs prolongs larval development, causing the death during late larval instars with the consequent greater production of infective OBs and causing deleterious effects on the reproductive capacity of surviving insects (Hatem et al. 2011; Hilton and Winstanley 2008; Takahashi et al. 2015). The time at which larvae infected by type I GVs stop feeding is variable; however, it has been observed that larvae infected with this type of viruses cause less damage to plants. This is contrary to the common belief that infected larvae continue to feed and damage host plants (Bhandari et al. 2010).

Type I GVs firstly infect the midgut epithelium and, then, the infection migrates to the fatty tissue of the insect, with this tissue being the most important infection site (Federici 1997; Sciocco-Cap 2001). Other insect tissues are rarely damaged by type I GVs, allowing the development of the larvae and favoring the replication of the new infective viruses in the insect host. The infections caused by type I GVs do not generally cause integument lysis but induce flaccidity of insect body (Sciocco-Cap 2001). Type I GVs are recognized as a factor of natural mortality for lepidopteran pests despite of their slow insecticidal activity.

It has been demonstrated that some type I GVs can increase the insecticidal effectiveness of other GVs and NPVs (Espinel-Correal et al. 2012; Shapiro 2000), by enhancing the metalloprotease activity in OBs, which favors the digestion of the peritrophic membrane and facilitates the access of the virions into intestinal cells (Bivian-Hernández et al. 2017; Ishimwe et al. 2015). Therefore, this mixture significantly alters the development of the symptomatic infection and might be used alone as new viral bioinsecticides or as a cocktail with other viruses (Caballero et al. 2001; Cuartas et al. 2014; Haase et al. 2015).

The co-infection of *S. frugiperda* larvae with type I GVs and NPVs has been documented (Gómez et al. 2010). *Spodoptera frugiperda* GVs have scarcely been studied (Cuartas et al. 2014; Ferrelli et al. 2018; Pidre et al. 2019), as

compared to *S. frugiperda* NPVs (García-Banderas et al. 2020; Williams et al. 2023). To date, only three *S. frugiperda* type I GVs from different geographic origins (Colombia, Brazil, and Argentina) have been studied (Cuartas et al. 2014; Pidre et al. 2019).

Spodoptera frugiperda is the main pest of corn (maize; *Zea mays* L.) in Mexico and other countries, causing crop losses exceeding 30% (Blanco et al. 2014; Casmuz et al. 2010). The negative effects of *S. frugiperda* in agriculture have been mitigated by physical and mechanical strategies (e.g., destruction of eggs and neonate larvae and use of pheromone for mass trapping), cultural management of the crop (e.g., early crop planting, weed removal, and use of mineral fertilizers), and the application of botanical extracts and broad-spectrum insecticides, with the latter being this most extensively strategy used with satisfactory results in suppressing this insect (Zhang et al. 2021).

However, the excessive use of pesticides causes environment contamination and insecticide-resistant pest populations (Gutiérrez-Moreno et al. 2019; Hafez et al. 2021; Hussain et al. 2021; Ju et al. 2021). The demand for environmental-friendly strategies, like biological control, to suppression this insect is increasing.

Several insects (parasitoids and predators) and parasitic nematodes have extensively been documented as natural enemies of *S. frugiperda* (Kenis et al. 2022; Ordóñez-García et al. 2015). Microbial biocontrol strategies for *S. frugiperda* have been mainly focused on entomopathogenic fungi and bacteria (Kenis et al. 2022; Sagar et al. 2020).

Another promising biocontrol strategy is the use of baculoviruses (NPVs and GVs). However, baculoviruses in the same way that entomopathogenic fungi and bacteria can be susceptible to environmental factors, both UV radiation and heat exposure, with these being the most important factors responsible for their ineffectiveness and low persistence on field conditions (Bustillos-Rodríguez et al. 2023). Nevertheless, GVs are underestimated, especially the type 1 GVs due to their low speed to kill their hosts (Hussain et al. 2021). However, these viruses can be considered as an alternative in integrated pest management programs for the suppression of *S. frugiperda* larval. Thus, the objective of this work was to characterize two type I granulovirus isolates and determine their insecticidal activity against *S. frugiperda* larvae.

Material and Methods

Insect Rearing and Propagation of Granuloviruses

The FAW larvae were obtained from a laboratory colony maintained under controlled conditions (26 ± 2°C, > 70% RH, 12:12 L:D) and fed with artificial diet (Southland Products Inc; Lake Village, AR, USA).

Two granulovirus isolates (SfGV-CH13 and SfGV-CH28) were obtained from naturally infected FAW larvae in two corn plots in Chihuahua, Mexico (latitude 28°12′44″N, longitude 106°59′45″W, and altitude 2125 m asl; latitude 28°40′59″N, longitude 106°48′50″W, and altitude 2079 m asl for SfGV-CH13 and SfGV-CH28, respectively). These GVs were found in mixture with NPVs, co-infecting FAW larvae. For this reason, the GVs were separated from NPVs by filtration using filter papers (pore sizes of 1.5 and 0.45 µm, respectively) and, then, by sucrose gradients (40 and 66%, w/w) using a gradient former (CBS Scientific, GM 200) according to Muñoz et al. (2001) and Ordóñez-García et al. (2020). Twenty milliliters of these sucrose solutions was placed into 30-ml polypropylene tubes and then 5 ml of the viral suspension was deposited on the surface of the gradients and centrifuged ($40,310 \times g$, 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 sterile distilled water (SDW) by centrifugation ($40,310 \times g$, 4°C, 40 min). The purification was confirmed by optical microscopy (Carl Zeiss AxioScope A1; Carl Zeiss, Gottingen, Germany) at $1000 \times$ magnifications. The OBs obtained from both GV isolates were replicated in fourth instar FAW larvae by the droplet feeding method (Hughes and Wood 1981). Larvae killed by GV infection were collected and macerated in sterile mortars using SDW containing SDS (1%). The excess of larval cuticle was removed by filtration using muslin and centrifugation ($8500 \times g$, 4°C, 10 min). The pellet of OBs was re-suspended in 10 ml of SDW and stored at -80°C.

Bioinsecticidal Activity

The insecticidal activity of both GV isolates was determined by estimating the median lethal dose (LD_{50}) and the median lethal time (LT_{50}) in third instar FAW larvae using the droplet feeding method (Hughes and Wood 1981). Six viral doses (1×10^1, 5×10^1, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 OBs/larva) were tested to estimate the LD_{50}, and three higher doses (1×10^5, 1×10^7, and 5×10^7 OBs/larva) were used to determine the LT_{50}. These doses were selected because they caused a larval mortality greater than 90% in preliminary studies (data not shown). Each FAW larva was starved for 12 h and, then, supplied with 0.5 µl of purified viral suspensions (Ordoñez-García et al. 2020). The OBs were mixed with Fluorella blue (0.001%, w/v) and sucrose (10%, w/v) before use. Previously, OBs were disaggregated by sonication (Branson 1510, CT, USA) for 30 s and were counted in triplicate in a Neubauer chamber (Marienfeld, Germany), using a phase-contrast microscope (Carl Zeiss, AxioScope A1; Gottingen, Germany) at $1000 \times$ magnifications (Muñoz et al. 2001). 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. Both bioassays were performed in triplicate for each viral dose using 25 FAW larvae per replicate and another 25 larvae were used as the control group (fed with Fluorella without viral inoculum) per replicate. Only larvae consuming the whole viral inoculum and showing intestinal tract with blue color, as confirmed by observation under a stereomicroscope (Leica G26), were considered in the experiment. Due to long time to cause mortality by this viral genus, the number of larvae killed by the action of tested GVs was recorded every 24 h, or until they reached the pupal stage (Barrera et al. 2011).

The days required for FAW larvae to reach the pupal stage were also determined, using the LD_{50} for the granuloviruses SfGV-CH13 and SfGV-CH28 isolates (5.4×10^2 and 1.1×10^3 OBs/larva, respectively), while the maximum weight reached by FAW larvae was determined using the LD_{90} for tested GVs (4.3×10^5 and 9.5×10^5 OBs/larva, respectively). Both LD_{50} and LD_{90} for both GV isolate were previously determined. From the sixth day post-infection (dpi), the weight of the larvae was recorded every 24 h. The infected larvae were placed into new cups with artificial diet to check if they were still feeding. These bioassays were performed in triplicate, using a total of 25 larvae per replicate and another 25 were used as control larvae (fed with Fluorella without viral inoculum) per replicate following the methodologies and conditions described above.

Morphological Characterization

The granulovirus isolates were identified according to their morphological characters, which were determined using optical microscopy (Carl Zeiss AxioScope A1 at $1000 \times$ magnifications), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) microscopy. For SEM analysis, one drop of the viral suspension was placed on the sample holder, dried, covered with a gold layer (Auto Sputter Coater 108; Cressington Scientific Instruments, Watford, UK), and immediately visualized using FEI Helios Nanolab 600 DualBeam microscope (FEI Company; Hillsboro, OR, USA). At least 50 OBs were considered for the measurement of size. For TEM analysis, the OBs were fixed using a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde, and then, the samples were placed on 1% osmium tetra-oxide. The samples were dehydrated with ethanol and embedded with a resin. Ultrathin sections were cut and observed using a JEOL JEM-200CX (JEOL Ltd; Tokyo, Japan) microscope at 80 kV.

Extraction of Virions and Viral DNA

The release of virions from OBs was performed by mixing the viral suspensions with 1 ml of 0.1 M sodium carbonate (Na_2CO_3), 1 ml of 0.1 M sodium chloride (NaCl) at pH 10.8,

and 1 ml of buffer TE (0.01 M tromethamine (Tris) hydrochloric acid (HCl), 0.001 M ethylenediaminetetraacetic acid (EDTA)) at pH 7.6. The mixture was incubated at 28°C for 2 h under agitation (140 rpm) and then an equal volume of buffer TE (1 ml) was added (Ordóñez-García et al. 2020). The released virions were purified by continuous sucrose gradients (20 and 66%, w/w) according to Muñoz et al. (2001), with modifications. Briefly, 20 ml of the formed gradient was placed into 30-ml polypropylene tubes, and immediately, 5 ml of the virion suspension was deposited on the surface and was centrifuged ($40,310 \times g$, at 4°C, 1.5 h). The bands of virions were collected with a Pasteur pipette and washed twice with SDW using centrifugation ($40,310 \times g$, at 4°C, 40 min). The pellets with virions were re-suspended in 500 μl of SDW and stored at -20°C until use.

For the extraction of viral DNA, the virion samples were mixed with 400 μl of buffer of proteinase K (0.01 M Tris, 0.005 M EDTA, 0.5% SDS) and incubated at 65°C for 15 min. Then, 100 μl of proteinase K (2 mg/ml) (Invitrogen Life Technologies Corp; Carlsbad, CA, USA) was added and the reaction mixture was incubated at 37°C for 2 h. An aliquot of 500 μl of a mixture phenol:chloroform:isoamyl alcohol (25:24:1) was added to the reaction and then it was centrifuged ($17,000 \times g$, at 4°C, 5 min). The aqueous phase was collected in a new microtube, and 500 μl of isopropyl alcohol and 100 μl of 3M sodium acetate were added to the samples previous to be incubated at -20°C for 2 h. The mixture was centrifuged ($17,000 \times g$, 4°C, 10 min) and the pellet was washed with 70% ethyl alcohol using centrifugation ($17,000 \times g$, 4°C, 5 min). The pellet was re-suspended in 30 μl of sterile double distilled water (ddH$_2$O). The quality of viral DNA was examined by electrophoresis on 1% agarose gels. The DNA concentration was determined by a A260 NanoDrop One spectrophotometer (Thermo Fisher Scientific; MA, USA).

Restriction Endonuclease Analysis

Both GV isolates were digested with *Hind*III, *Bam*HI, and *Pst*I enzymes (Invitrogen Life Technologies Corp; Carlsbad, CA, USA). One microgram of viral DNA was digested with 10 U of the enzymes, at 37°C for 2 h. The reaction was stopped by adding 2 μl of loading buffer 10X (Thermo-Fisher Scientific; Waltham, MA, USA). The obtained restriction fragments were analyzed by electrophoresis on 1% agarose gels at 25 V for 7 h, using TAE buffer (40 mM Tris–acetate, 1 mM EDTA at pH 8.0). A molecular weight marker of GeneRuler 1 kb DNA Ladder (Thermo Fisher Scientific) and 10 μl of SYBR Safe DNA gel stain (Invitrogen) were used to visualize the DNA on agarose gels using the image system (Bio-Rad ChemiDoc™ XRS⁺; Hercules, CA, USA). The fragment sizes and number for both GV isolates were estimated by comparing their bands with those

of the molecular weight marker GeneRuler 1kb DNA Ladder (Thermo Fisher Scientific) using the Image Lab software version 5.2.1 (Bio-Rad ChemiDoc™ XRS⁺; Hercules, CA, USA).

Statistical Analysis

The bioassays were conducted under a completely randomized design. The data on insecticidal activity were analyzed by an analysis of variance (ANOVA), and the means were separated by a Tukey test ($p < 0.05$). The LD$_{50}$, LD$_{90}$, LT$_{50}$, and fiducial limit values were analyzed using log-Probit regressions (Finney 1971). All data were analyzed using SAS software (SAS 2002). Mortality was corrected by the Abbott (1925) formula.

Results

Insecticidal Activity

Both granulovirus isolates caused significant larval mortality at four of the six tested doses (Fig. 1). No mortality was observed for control larvae. Larval mortality caused by the highest dose (1×10^5 OBs/larva) of both GVs started on 12 dpi and on 15 dpi with the lowest dose (1×10^1 OBs/larva). The death of larvae ended on 45 dpi (data not shown). Both isolates caused the same infection symptoms (Fig. 2). GV-infected larvae gradually stopped feeding and their bodies become flabby. The integument lysis was only observed in 8% of infected larvae, which showed fatty body shedding after 20 dpi and remained alive in this condition for at least 2 days (Fig. 2b and 2d). These larvae also showed swelling and an atypical milky whitish color on their cuticle, with this color being quite different to that of control larvae (Fig. 2).

The estimated LD$_{50}$ for the SfGV-CH13 and SfGV-CH28 isolates were 5.4×10^2 and 1.1×10^3 OBs/larva, respectively (Table 1). Based on overlapping fiducial limits, there were no significant differences in LD$_{50}$ between SfGV-CH13 and SfGV-CH28 isolates. However, the LD$_{50}$ for the SfGV-CH13 isolate was 1.85 times lower than that for the SfGV-CH28 isolate (Table 1). The LT$_{50}$ decreased as the viral dose was increased. LT$_{50}$ of 414.7 h (= 17.3 days) and 476.2 (= 19.8 days) were obtained with the highest dose (5×10^7 OBs/larva) of SfGV-CH13 and SfGV-CH28, respectively. The lowest dose (1×10^5 OBs/larva) of these isolates led to LT$_{50}$ of 570.3 h (= 23.8 days) and 591.8 h (= 24.8 days) (Table 2). Larval mortality times ranged from 414.7 h (17.3 days) to 570.3 h (23.8 days) for the SfGV-CH13 isolate and from 476.2 h (19.8 days) to 591.8 h (24.7 days) for the SfGV-CH28 isolate. The LD$_{50}$ and LD$_{90}$ of both GVs caused a delay in larval development. The maximum body weight prior to pupation and the time required by the larvae to reach

Fig. 1 Larval mortality caused by different doses of the granulovirus isolates SfGV-CH13 and SfGV-CH28 at 45 days post-infection. Same small letters on standard error ($\pm$) bars indicate that there is no statistical difference between isolates at the same dose, according to Tukey's test ($p < 0.05$). The numbers 1 to 6 in the x-axis indicate the following isolate doses: 1×10^1, 5×10^1, 1×10^2, 1×10^3, 1×10^4, and 1×10^5 OBs/larva, respectively. In the larvae used as controls, no mortality was recorded
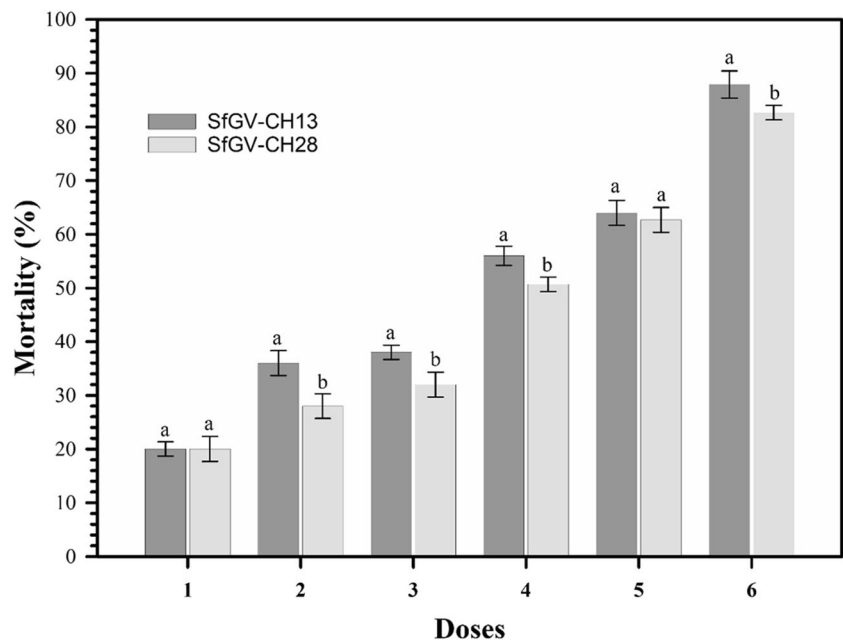

Fig. 2 Appearance of *Spodoptera frugiperda* larvae infected with the tested isolates at a dose of (1×10^4 OBs/larva). Larvae infected with the SfGV-CH13 isolate at 30 days post-infection (dpi) (**a**); larvae infected with the SfGV-CH13 showing exposition of fat body at 32 dpi (**b**); larvae infected with the SfGV-CH28 isolate at 30 dpi (**c**); and larvae infected with the SfGV-CH28 isolate showing damage in the abdominal segments at 22 dpi (**d**). Red arrows indicate injuries in the posterior abdominal segments and external exposure of the fat body

pupation after infection with LD_{50} depended on isolate, with the SfGV-CH28 isolate causing the highest changes in these response variables (Table 3). No significant differences were observed in the time required to reach the pupal stage by control larvae (15.17 days) and larvae treated with LD_{50} of the SfGV-CH13 isolate (15.26 days). However, the time required by the SfGV-CH28 isolate to cause larval death (17.82 days) was significantly different to those for the other treatments. These same significant differences occurred in the weight that the larvae reached before reaching the pupal stage. The body weight of these larvae averaged 0.51 and 0.47 g/larva, respectively for the control group and

Table 1 Median lethal dose (LD_{50}) and lethal dose ninety (LD_{90}) of tested type I granulovirus isolates against third-instar *Spodoptera frugiperda* larvae

Dose tested	Isolate	Dose obtained	Fiducial limits (95%)		χ^2	df	Slope $\pm$ (SE)	Intercept $\pm$ (SE)
			Lower	Upper				
LD_{50}	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	1.0×10^3	6.0×10^2	2.0×10^3	1.1	4	0.4 ± 0.08	-1.3 ± 0.12
LD_{90}	SfGV-CH13	4.3×10^5	1.3×10^5	2.2×10^6	4.5	4	0.4 ± 0.04	-1.2 ± 0.13
	SfGV-CH28	9.5×10^5	2.7×10^5	5.8×10^6	1.1	4	0.4 ± 0.08	-1.3 ± 0.12

LD_{50}, median lethal dose expressed as OBs/larva. These values were obtained from a minimum of six doses (treatments) and estimated at 45 days post-infection (dpi)

Probit regressions were fitted using SAS program

χ^2, goodness of fit test

df, degrees of freedom

SE, standard error

Table 2 Median lethal time (LT_{50}) of the doses of type I granulovirus isolates tested against third instar *Spodoptera frugiperda* larvae

Isolate	Doses (OBs/larva)	LT_{50}:h (days)	Fiducial limits (95%)	
			Lower	Upper
SfGV-CH13	1×10^5	570.3 h (23.8 d)	555.9	585.1
	1×10^7	502.2 h (20.9 d)	489.9	514.5
	5×10^7	414.7 h (17.3 d)	403.6	425.9
SfGV-CH28	1×10^5	591.8 h (24.7 d)	573.2	611.0
	1×10^7	540.3 h (22.5 d)	522.0	558.4
	5×10^7	476.2 h (19.8 d)	455.9	495.7

Data correspond to the average of three replicates. LT_{50}, median lethal time expressed in hours and days

Table 3 Development period from 3rd instar of *Spodoptera frugiperda* larva to pupal stage treated with the median lethal dose of two native type I granuloviruses

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

All values are arithmetic means $\pm$ SE. Values in the same column with the same letter are not statistically different according to Tukey's test ($p < 0.05$). *SE*, standard error

Fig. 3 Weight of control larvae and larvae upon infection with LD_{90} of the SfGV-13 and SfGV-28 isolates

carried out at 6, 10, and 15 dpi (Fig. 3). However, the body weight of larvae at 25 dpi was similar for both isolates (SfGV-CH13 and SfGV-CH28) (Fig. 3). However, it was observed that 20% of the larvae infected with the SfGV-CH13 isolate at the LD_{90} had a weight ≥ 0.60 g in between 19 and 27 dpi and 28% of the larvae infected with both isolates stopped feeding for a period of 3 to 6 days, after 14 dpi (data no shown).

Morphological Characterization

OBs of both isolates showed a homogeneous ovoid shape. The length of OBs ranged from 0.36 to 0.49 µm ± 0.01 (standard error, SE) for the SfGV-CH13 isolate and from 0.37 to 0.45 µm ± 0.01 (SE) for the SfGV-CH28 isolate, with an average length of 0.4 µm for both GV isolates (Fig. 4a and c). Both isolates showed a single virion (~ 0.3 µm) per OB (Fig. 4b and d).

SfGV-CH13 isolate. The larvae treated with the SfGV-CH28 isolate required 2.5 days more to reach pupation and their body weight was up to 0.1 g higher than those of the other experimental groups (Table 3).

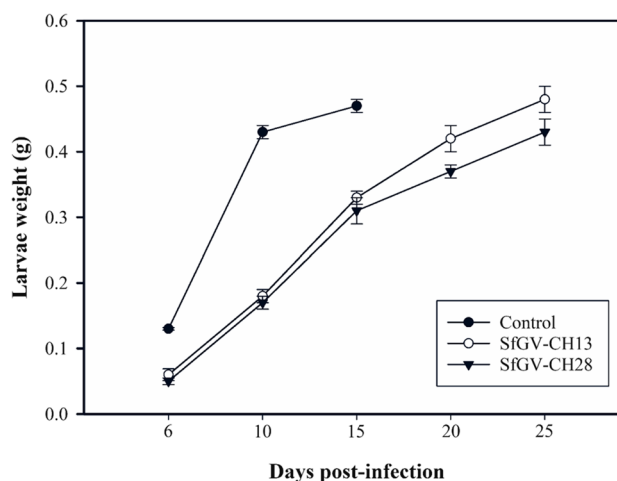The infection with the tested isolates at LD_{90} reduced the body weight of FAW larvae, according to the measurements

Fig. 4 Morphology of the granuloviruses. SEM (**a**) and MET (**b**) micrographs (50,000 and 20,000× magnifications, respectively) of the SfGV-CH13 isolate. SEM (**c**) and MET (**d**) micrographs (50,000 and 20,000× magnifications, respectively) of the SfGV-CH28 isolate. NC, nucleocapsid; V, virion
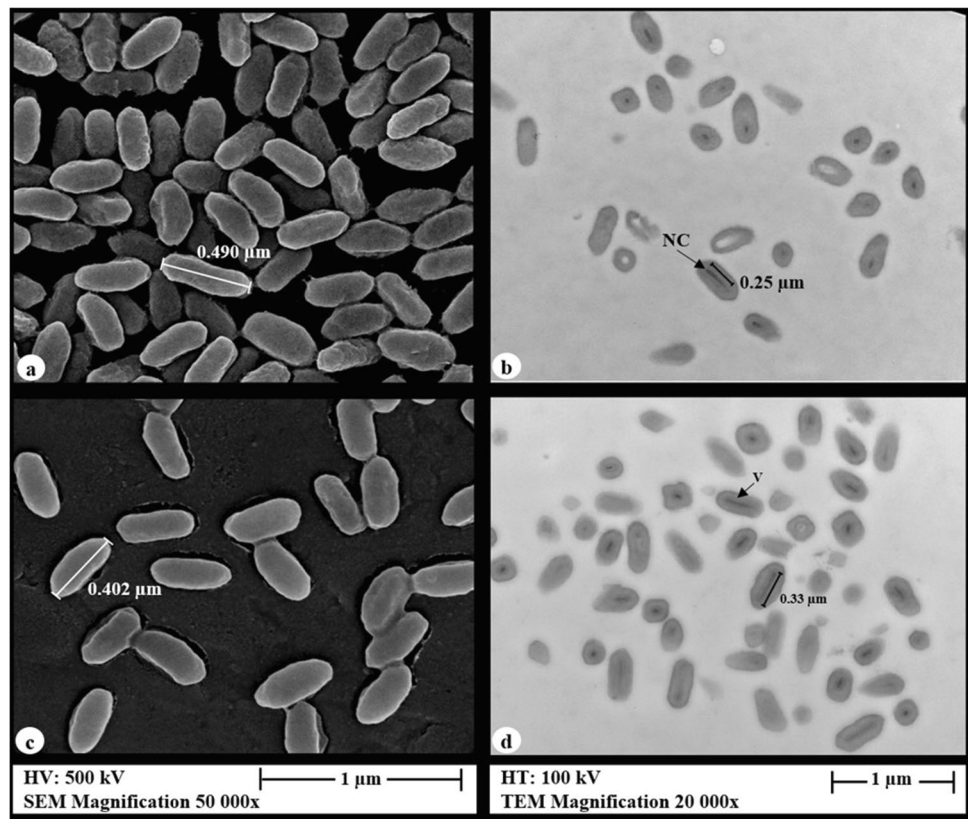
Molecular Characterization

Both isolates showed the same DNA restriction profiles. Thirteen, 14, and 16 restriction fragments were observed with the enzymes *Hind*III, *Bam*HI, and *Pst*I, respectively (Fig. 5). The DNA size for tested isolates was about 126 kb.

Discussion

SfGV-CH13 and SfGV-CH28 isolates started to kill FAW larvae since 12th dpi. Similar times for the onset of death of insects infected by type I GVs (7–14 dpi) have already been reported (Inceoglu et al. 2001). In our study, the larvae died from 12 to 45 dpi, with this time range being higher than that reported for NPVs (3–8 dpi) infecting FAW larvae (Barrera et al. 2011; Ordóñez-García et al. 2020).

The data for insecticidal activity of granulovirus isolates, the morphology, and the symptoms observed in the infected larvae of this study demonstrated that both isolates were type I, causing a monorganotropic infection and a slow kill of the insect. Alletti et al. (2017) also observed similar symptoms in *Agrotis segetum* Schiff. larvae infected with AgseGV. The kinetics of the insecticidal activity is used to determine the type of GVs. If the GV causes death in at least 10 days, or

even 20–30 dpi, it can be considered as type I instead of type II GV (Pidre et al. 2019).

The symptomatic infection caused by the tested isolates was similar to that documented for this type of GVs, where the symptoms differed clearly from those observed in insects infected with NPVs (Barrera et al. 2011; Ordóñez-García et al. 2020; Pidre et al. 2019). The symptoms commonly observed in larvae infected with GVs include the cessation of feeding, larvae swelling, little or no liquefaction of insect body, and darkening of insect body (Sauer et al. 2017; Wang et al. 2008).

The tested GVs did not cause liquefaction of larvae body, probably due to they did not infect the epidermis, with this preventing the deposition of chitinase on the peritrophic membrane (Sciocco-Cap 2001). Rohrmann (2019) observed that some NPVs contained gp37, a chitinase favoring the fusion of virions with cells of the middle intestine.

The creamy-yellow appearance observed on larvae infected with the tested GV isolates can be attributed to the accumulation of high quantities of OBs on the fatty body tissues of larvae (Sciocco-Cap 2001). This fatty tissue completely detached from the rest of the larva body in 8% of the larvae infected with the tested GVs. Pidre et al. (2019) also observed severe lesions in the last abdominal segments (fatty tissue) of FAW larvae infected with a granulovirus isolate from Argentina. Cuartas et al. (2014) reported LC_{50}

Fig. 5 DNA restriction profiles of SfGV-CH13 and SfGV-CH28 isolates on a 1% agarose gel (7 h/25 V). First column shows the molecular marker size. Second to fourth columns show the restriction profiles of the SfGV-CH13 isolate generated by *Hind*III, *BamH*I, and *Pst*I enzymes, respectively. Fifth to seventh columns show the restriction profiles of the SfGV-CH28 isolate generated by *Hind*III, *BamH*I, and *Pst*I enzymes, respectively
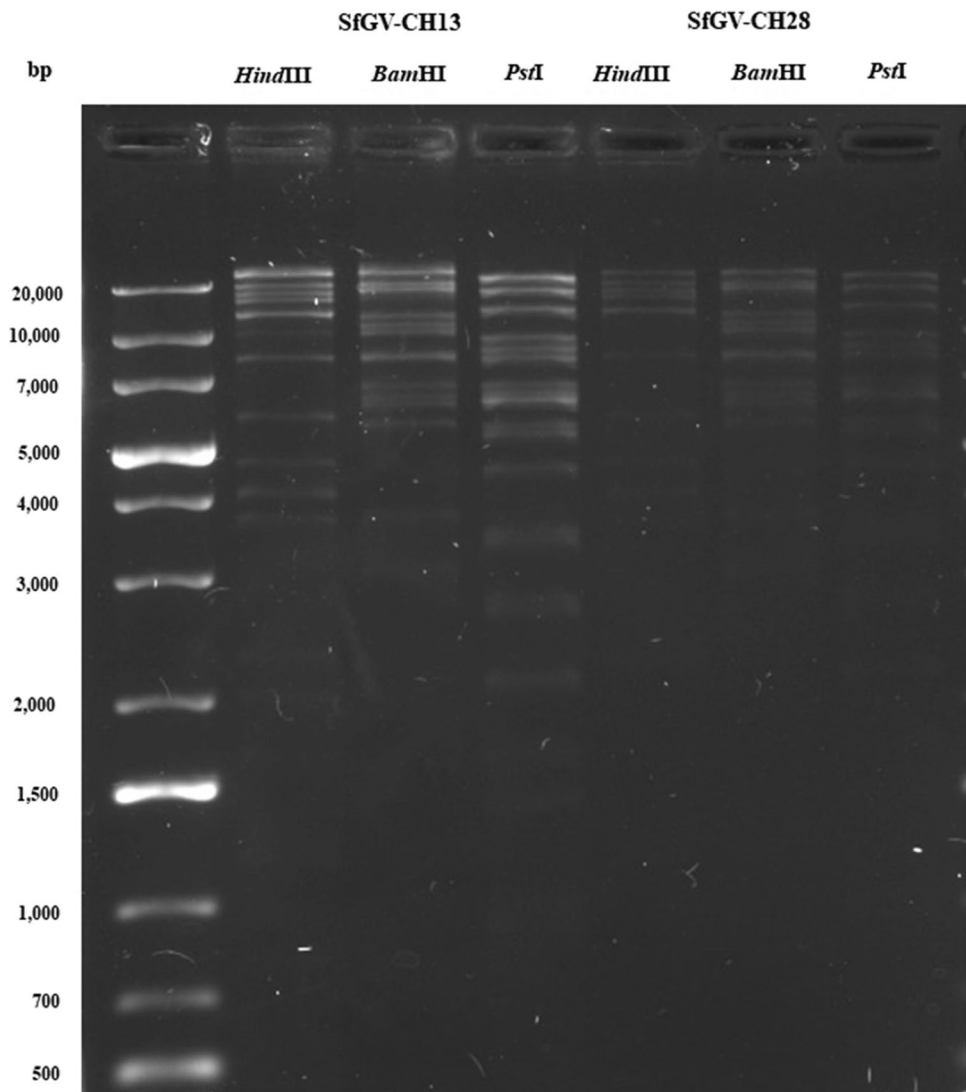
of 4.5×10^5 and 1.6×10^5 OBs/ml for Brazilian (VG008) and Colombian (VG014) GVs isolates from *S. frugiperda* with mean times to death (MTD) of 29 (= 694 h) and 33 days (= 792 h), respectively. Although the results obtained by Cuartas et al. (2014) in terms of LC_{50} are not comparable with ours based on LD_{50}, they coincide in terms of the longer time required by this type of GVs to kill *S. frugiperda* larvae. Hackett et al. (2000) observed shorter survival times (367–439 h) for *Helicoverpa armigera* larvae infected with a *H. armigera* type I granulovirus (HearGV) than those observed in our study. The differences observed between these investigations and the results obtained in this study could be attributed to the larval instar used since they used first and second instar larvae, which makes them more susceptible.

The low rate of larval death could be related to the tropism of type I GVs, which only infect the midgut and fatty body of the host insect, as compared to type II GVs and the

NPVs that infect many tissues and cause a rapid death of the host (Sciocco-Cap 2001). Kumar et al. (2017) pointed out that type I GVs kill slowly their hosts compared to type II GVs, and suggested the need for higher LD_{50} (up to 100 times more) of type I GVs. The difference in speed of action of GVs may lie in the range of larval tissues infected by a virus with higher virulence. Some insects can also delay the infection of type 1 GVs as a first defense mechanism, blocking viral replication in cells during the early stages of infection (Hinsberger et al. 2019; Pauli et al. 2018; Wang et al. 2008).

The larvae infected with the SfGV-CH28 isolate required more time to reach the pupal stage and showed a slower weight gain rate than SfGV-CH13 and the control group. Wang et al. (2008) evaluated *Spodoptera litura* granulovirus (SlGV) in *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) larvae observed that infected larvae can live more and be bigger than uninfected larvae. However, larvae

infected with LD_{90} of both isolates showed a lower weight at 15 dpi than control larvae, with this indicating that a lower viral dose (LD_{50}) can increase the survival time and body weight prior to death as compared to the highest viral dose (LD_{90}).

Machado et al. (2020) found that the infection-mediated lengthening of the developmental process of larvae, involving more time to reach pupal or adulthood stages, is favorable for the management of insect pests on field, because the larvae remain exposed to their natural enemies for a longer time, increasing the chances of being parasitized, depredated, or infected by microbial biocontrol agents. This gives an advantage to the use of type I GVs. Additionally, larvae infected by this type of GVs stop feeding and, consequently, they no longer cause any more damage to the crop but produce a large number of new infective OBs ready to infect new healthy larvae. These effects are similar to NPVs, especially in *S. frugiperda*, which is characterized by overlapping generations (e.g., larvae of all stages), at the same phenological stage of the crop. Furthermore, it has been demonstrated that SfGVs enhance infection caused by SfNPVs (Cuartas-Otálora et al. 2019; Ferrelli et al. 2018; Hussain et al. 2021).

The ovoid shape, size, and the number of virions observed for OBs were similar to those observed for other OBs of lepidopteran GVs (Barrera et al. 2014; Ikeda et al. 2015; Luque et al. 2001; Moscardi 1999). The size of the tested GVs was into the range (0.3×0.5 μm) observed by Ikeda et al. (2015). It was also similar to that (0.43 μm) reported by Cuartas et al. (2014) for *S. frugiperda* granulovirus (SfGV).

Restriction enzyme analysis allowed the molecular identification of the tested isolates, which showed same number and positions of the fragments after digestion with the enzymes *Hind*III, *Bam*HI, and *Pst*I (Fig. 5). This similarity might be related to the geographical origin of the isolates, which was similar for them. Barrera et al. (2014) did not observe differences in the restriction profiles of three granulovirus isolates due to they were genotypic variants of the same viral strain. On the other hand, Cuartas et al. (2014) found differences in the restriction fragments of *S. frugiperda* VG008 and VG014 GVs from Colombia and Brazil. Ordóñez-García et al. (2020) also observed small differences in the restriction patterns (*Hind*III and *Bam*HI enzymes) for the SfCH15 and SfCH32 NPV isolates, both obtained from nearby areas in Mexico. Ali et al. (2018) stated that the genotypes of viruses differ in dose response and time required to kill the host (biological activity).

Tested isolates showed significant differences in mortality percentages at some doses (Fig. 1), with the SfGV-CH13 isolate being more virulent than the SfGV-CH28 isolate. Significant differences were also detected in terms of the days necessary to cause larval death and the weight reached by larvae prior to pupation. The SfGV-CH13 isolate showed

the lowest LT_{50} with the three doses tested against *S. frugiperda* larvae. However, no significant differences were observed in LD_{50} and LD_{90} between both isolates.

The genome sizes for tested isolates were about ~126 kb. This size was smaller to those estimated by Cuartas et al. (2014) for VG014 (132.6 kb) and Cuartas et al. (2015) for VG008 isolate (140.9 kb). Pidre et al. (2019) estimated a size of at least 135 kb for an Argentinian isolate of *S. frugiperda* granulovirus. However, complete genome sequencing of GV isolates is necessary to obtain more accurate values.

In conclusion, based on insecticidal activity, the two *S. frugiperda* granulovirus isolates against FAW larvae and symptoms induced in infected larvae, both isolates belong to type I GVs. They killed more than 90% of *S. frugiperda* larvae at 45 dpi at a dose of 1.0×10^5 OBs/larva. Both isolates were genetically identical, according to their DNA restriction profiles. The LD_{90} extended two times the larval development time of *S. frugiperda*. The LD_{50} values obtained with both GVs were similar to those reported for NPVs; however, their LT_{50} were lower than those previously reported for other *S. frugiperda* granuloviruses and SfNPVs. The results suggest that both granulovirus isolates might be considered for use as biocontrol agents against *S. frugiperda* despite their slow insecticidal activity.

Declarations

Competing Interests The authors declare no competing interests.

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