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Potential of betabaculoviruses to control the tomato leafminer *Tuta absoluta* (Meyrick)

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Abstract

The tomato leafminer *Tuta absoluta* (Meyrick 1917) (Lepidoptera:Gelechiidae) is a devastating pest, causing losses of up to 100%. An interesting tool for its control is the use of the insect specific viruses of *Baculoviridae* family. Despite its high potential, its use on *T. absoluta* larvae has been poorly studied. In this work, Colombian granuloviruses VG013 and VG003 isolated from *T. absoluta* and *Tecia solanivora* (Lepidoptera:Gelechiidae) larvae sampled in tomato and potato crops, respectively, were morphologically, biologically and molecularly characterized. Occlusion bodies showed ovoid shape containing one nucleocapsid. Restriction endonuclease analysis revealed a pattern similar to *Phthorimaea operculella granulovirus* and bioinformatics studies showed that both isolates are variants of that baculovirus specie. Similar mean lethal concentrations (LC_{50}) on *T. absoluta* larvae were estimated for both viruses, although VG013 exhibited shorter mean time to death than VG003. The latter reached a higher OBs yield in comparison with VG013. These results demonstrated an interesting potential of evaluated betabaculoviruses to control *T. absoluta* populations and pointed key features to its use under field conditions.

KEYWORDS
baculovirus, biological control, characterization, *Phthorimaea operculella*, *Tecia solanivora*, *Tuta absoluta*

1 | INTRODUCTION

The tomato leafminer *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae) is a serious pest native from South America, described first in Peru in 1917 and then in Chile, Brazil, Bolivia, Colombia, Ecuador, Uruguay, Venezuela and Argentina (Souza & Reis, 1986). In 2006, the pest was reported in Spain and rapidly distributed to south Europe, north Africa, Asia and Mediterranean basin (Desneux et al., 2010; Urbaneja, González-Cabrera, Arnó, & Gabarra, 2012). Currently, it is considered the most important tomato pests in the Mediterranean countries, Middle East, eastern, central and western Europe as well as north Africa (Allache, Bouta, & Demnati, 2015).

Particularly in Colombia, *T. absoluta* has been found to cause serious damages in most of greenhouse tomato crops and its control is carried out mainly using chemical methods. However, food residues problems have motivated the development of sustainable pest control measures to mitigate such disadvantages. In this regard, baculoviruses appear as interesting active ingredient candidates for bio-insecticides formulations as evidenced by successful experiences previously reported (Reviewed by Haase, Sciocco-Cap, & Romanowski, 2015).

Baculoviridae family includes a group of arthropod-specific-viruses with large circular double-stranded-DNA genomes that infect insects from Lepidoptera, Hymenoptera and Diptera orders (Rohrman, 2011). Currently, baculoviruses are classified into four genera: *Alphabaculovirus* (lepidopteran nucleopolyhedroviruses); *Betabaculovirus* (lepidopteran granuloviruses); *Gammabaculovirus* (hymenopteran nucleopolyhedroviruses); and *Deltabaculovirus* (dipteran

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nucleopolyhedroviruses) (Jehle et al., 2006). These invertebrate pathogens are characterized by the presence of two different phenotypes during their infection cycle in susceptible hosts: *budded viruses* (BVs) and *occlusion derived viruses* (ODVs), which are enclosed in protein crystalline structures denominated *occlusion bodies* (OBs) exposing a polyhedral or granular shape according to the genus [polyhedroviruses (*alpha*-, *gamma*- and *delta* baculoviruses) or granuloviruses (*beta* baculoviruses), respectively].

Worldwide, a member of the genus *Betabaculovirus*, *Phthorimaea operculella granulovirus* (PhopGV) (King, Lefkowitz, Adams, & Carstens, 2012) has demonstrated its potential for controlling lepidopteran pests from Gelechiidae's family such as *Phthorimaea operculella* (Zeller) and *Tecia solanivora* (Povolny) (Mascarin et al., 2010), due to their high specificity, good levels of pathogenicity, and compatibility with beneficial organisms. Several isolates of PhopGV have been collected worldwide and evaluated in terms of biological activity and molecular profile, finding differences depending on their geographical origin (Carpio et al., 2012; Cortés, León, & Del Rincón-castro, 2013; Vickers, Cory, & Entwistle, 1991). In this sense, some biopesticides based on these variants were developed to control *T. solanivora* and *P. operculella* larvae (Carpio et al., 2012; Chaparro, Espinel-Correal, Cotes, & Villamizar, 2010); however, there are no records of their efficacy use for *T. absoluta* control. Mascarin et al. (2010) demonstrated that a Brazilian PhopGV was able to infect *T. absoluta* delaying larval growth and inhibiting pupation, becoming interesting the possibility to find natural baculoviruses infecting this insect.

A screening campaign was carried out on potato and tomato crops through Gelechiidae distribution areas in Colombia between 2004 and 2011, allowing the isolation of eight granuloviruses (Espinel-Correal et al., 2010; Gómez-valderrama, Herrera, Uribe-vélez, López-ferber, & Villamizar, 2014). In this article, we report the morphological characterization and classification of two Colombian granuloviruses: VG013, isolated from *T. absoluta* and VG003, isolated from *T. solanivora*. Besides, we describe the results of laboratory bioassays to evaluate viral lethal concentrations, mean time to death and OBs yield on *T. absoluta* larvae, as the basis for a microbial pesticide development.

2 | MATERIALS AND METHODS

2.1 | Insect rearing

A *T. absoluta* colony was established from larvae collected in tomato crops located in Cundinamarca and Boyacá (Colombia, 04°00′N, 72 °00′w). Insects were reared at 25 ± 3°C with relative humidity 50 ± 10% and maintained under a photoperiod of 12:12 h (light: dark) on natural diet (tomato plants). *T. solanivora* eggs were obtained from a laboratory colony reared on potato tubers (*Solanum tuberosum*, variety Parda pastusa) at 25 ± 3°C, relative humidity 50 ± 10% and a 12:12 light:dark photoperiod. Both colonies were maintained at the 'Tibaitata' Research Center of Corpoica, Mosquera, Cundinamarca (Colombia).

2.2 | Granulovirus isolate amplifications

Granulovirus from *T. solanivora*, coded as VG003, and granulovirus from *T. absoluta*, coded as VG013, were isolated previously from naturally infected larvae, collected from potato and tomato crops, respectively (Espinel-Correal et al., 2010; Gómez-valderrama et al., 2014). The isolates were amplified on the *T. solanivora* laboratory colony. For that, OBs of each isolate at a concentration of 1×10^8 OBs/ml were sprayed over paper sheets covered with *T. solanivora* eggs (Reed, 1971). Then, eggs were placed over clean potato tubers and incubated in plastic cages (25 ± 3°C, relative humidity 50 ± 10% and 12:12 light:dark photoperiod). Larvae were collected 25 days later, phenotypically tested for virus infection, and those showing symptoms (milky appearance) were collected. Finally, OBs were extracted from dead larvae by homogenizing cadavers in 0.1% sodium dodecyl sulphate (SDS) solution (w/v) and purified by filtration and centrifugation on a 30%/50%/70% sucrose gradient (Espinel-Correal et al., 2010). Viral suspensions were quantified by absorbance measurements at 280 nm (Nanodrop 2000 ThermoFisher) using a standard curve previously calibrated with VG003 OBs stocks counted by optical microscopy and Neubauer chamber. All viral suspensions were stored at 4°C until those were required.

2.3 | Morphological characterization

Shape and size from VG003 and VG013 OBs were observed by transmission electron microscopy (TEM). For this, 100 μL of each OB suspension (1×10^8 OBs/ml) was fixed overnight at 4°C by mixing in a volume of fixative buffer [4% v/v formaldehyde and 1% v/v glutaraldehyde in 0.1M phosphate buffer (pH 7.4)]. Negative staining was made with 1% v/v phosphotungstic acid (pH 6.0), and viral suspension was placed on copper grids for analysis (TEM CM 10 Microscopy).

2.4 | Restriction endonuclease profiles

Viral DNAs were obtained from the OBs previously purified, which were treated using a standardized methodology including proteinase k treatment, phenol extractions and alcohol precipitation (Murhammer, 2010). The genomic analysis using restriction endonuclease (REN) profiles with *Sma*I, *Hind*III, *Bste*II, *BamH*I and *Mlu*I (Promega) was performed according to manufacturer's specifications. A sample of approximately 2 ug of viral DNA was mixed with 10 U of each enzyme. DNA concentration was estimated by spectrophotometric absorbance at 260 nm (ND 1000 – Thermo Scientific). The products of nuclease treatments were then resolved by electrophoresis using horizontal 1% w/v agarose gels in TAE buffer [0.04M Tris–acetate, 0.001M EDTA, (pH 8.0)] at 20 V for 18 h. DNA fragments were stained with SYBR® gold (Invitrogen) and visualized on a UV transilluminator (Chemi-Doc, Bio-Rad Laboratories, CA, USA), and the fragment sizes were estimated using 1 Kb Plus DNA ladder (Invitrogen). Restriction patterns of the viruses were analysed for the presence or absence of specific bands, comparing with the restriction pattern of a *P. operculella* granulovirus from Peru, coded as VG006 (Léry et al., 2008).

2.5 | Phylogenetic analysis

DNAs isolated from VG003 and VG013 were used as templates in PCR assays using a set of primers previously reported with the goal to amplify fragments of *lef-8*, *lef-9* and *granulin* genes (Jehle et al., 2006; Barrera, Cuartas, & Villamizar, 2009). Then, the amplicons were molecularly cloned into pGEM®-T vector (Promega) and subsequently sequenced using universal M13 and T7 primers (Macrogen Company and Corporación Corpogen). Later, nucleotide data were in silico translated and subjected to phylogenetic analysis. For this, the homologous sequences from reported betabaculoviruses (Table 1) were independently aligned using ClustalX program (Thompson, Higgins, & Gibson, 1994) with the following parameters: pairwise alignment (gap open penalty = 10, gap extension penalty = 0.1, protein weight matrix: Blosum 30); multiple alignment (gap open penalty = 10, gap extension penalty = 0.05, protein weight matrix: Blosum series). A concatemer was then generated by adding complete individual alignments, and phylogeny was inferred using MEGA 5 software (Tamura et al., 2011) with the following parameters: UPGMA; bootstrap with 1,000 replicates; gap/missing data = pairwise deletion; model = amino (Dayhoff matrix); patterns among sites = same (homogeneous); rates among sites = different (gamma distributed); gamma parameter = 0.25. Besides, multiple alignments of homologous sequences were done to estimate Kimura 2-parameter distances using MEGA 5 software with the following parameters: scope = pairs of taxa; estimate variance, variance estimation method = bootstrap method; no.° of bootstrap replications = 1000; substitution model, substitution type = nucleotide, model/method = Kimura 2-parameter model, substitutions to include = d: transitions + transversions; rates and patterns, rates among sites = gamma parameter, gamma parameter = 0.25. Pattern among lineages = same (homogeneous); data subset to use, gaps/missing data treatment = pairwise deletion, select codon positions = all+noncoding sites.

2.6 | Biological assays

2.6.1 | Pathogenicity and virulence

Lethal concentrations (LC_{50} and LC_{90}) of VG003 and VG013 were determined on *T. absoluta* larvae. For that, five different virus dilutions from 1×10^4 to 1×10^9 OBs/ml were sprayed on tomato leaves with a nebulizer (Carrera, Zeddam, Pollet, Lery, & López-Ferber, 2008). Inoculated leaves were cut into pieces (approximately 2.5 cm × 2.5 cm), and each one was then placed into plastic cups of 0.5 ounce where two neonate *T. absoluta* larvae were placed using a soft brush. Subsequently, groups of five cups were located inside of plastic containers, which were maintained in a room with controlled conditions (25 ± 3°C, relative humidity 50 ± 10%, 12:12-h light:dark photoperiod). Larval mortality was recorded every day until the pupal stage occurred. Leaves without treatment or treated with water were used as absolute or treated controls, respectively. Experimental design was completely randomized with three replicates per treatment and

TABLE 1 Betabaculoviruses used in bioinformatics studies

Baculovirus	Acc. number	Abbreviation
Adoxophyes orana GV	NC_005038	AdorGV
Adoxophyes orana GV—Strain Miyazaki	KM226332	AdorGV-Miyazaki
Agrotis segetum GV	NC_005839	AgseGV
Agrotis segetum GV—Strain L1	KC994902	AgseGV-L1
Agrotis segetum GV—Strain DA	KR584663	AgseGV-DA
Choristoneura fumiferana GV	NC_008168	ChfuGV
Clostera anachoreta GV—Isolate HBHN	NC_015398	ClanGV
Clostera anastomosis GV—Strain Henan	NC_022646	CalGV
Cryptophlebia leucotreta GV	NC_005068	CrleGV
Cydia pomonella GV	NC_002816	CpGV
Cydia pomonella GV—Isolate E2	KM217577	CpGV-E2
Cydia pomonella GV—Isolate S	KM217573	CpGV-S
Cydia pomonella GV—Isolate I12	KM217576	CpGV-I12
Cydia pomonella GV—Isolate M	KM217575	CpGV-M
Cydia pomonella GV—Isolate I07	KM217574	CpGVI07
Diatraea saccharalis GV—Isolate Parana-2009	NC_028491	DisaGV
Epinotia aporema GV	NC_018875	EpapGV
Erinnyis ello GV—Strain BrS86	NC_025257	ErelGV
Helicoverpa armigera GV	NC_010240	HearGV
Phthorimaea operculella GV	NC_004062	PhopGV
Pieris rapae GV	NC_013797	PiraGV
Pieris rapae GV—Strain-E3	GU111736	PiraGV-E3
Pieris rapae GV—South Korea	JX968491	PiraGV-SK
Plutella xylostella GV	NC_002593	PlxyGV
Pseudaletia unipuncta GV—Strain Hawaiian	NC_013772	PsunGV
Spodoptera frugiperda GV—Isolate VG008	NC_026511	SpfrGV
Spodoptera litura GV—Strain K1	NC_009503	SpliGV
Xestia c nigrum GV	NC_002331	XecnGV

two-time replicates. Results were subjected to probit analysis (Finney, 1971) using the software POLO-PLUS 1.0 [LeOra Software]. For the comparison of LC_{50} values, the parallelism of the calculated lines was checked using the parallel-line assay option using POLO-PLUS 1.0.

Mean time to death (MTD) was determined using the results obtained at the concentration of 1×10^7 OBs/ml. Time-mortality results of individuals that died due to virus were subjected to Weibull survival analysis using the generalized linear interactive modelling (GLIM) program (Crawley, 1993).

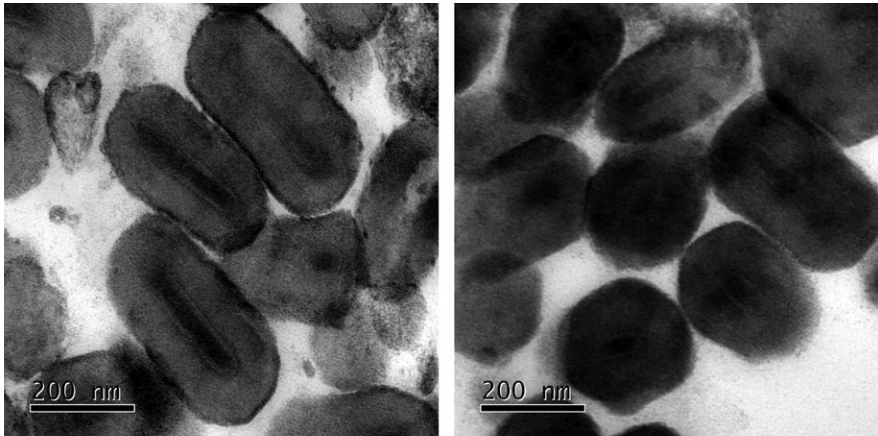

FIGURE 1 Ultra structural morphology of VG003 and VG013. Micrographs of VG003 (left panel) and VG013 (right panel) performed by transmission electron microscopy

FIGURE 2 Restriction endonuclease analyses for VG003 and VG013. The genomes of VG003 (isolated from *T. solanivora*), VG013 (isolated from *T. absoluta*) and VG006 (isolated from *P. operculella*) were isolated and digested with *Hind*III, *Mlu*I, *Bam*HI, *Bst*EII and *Sma*I. Then, fragments were resolved by 1% (w/v) agarose gel electrophoresis, which was stained using SYBR® gold and UV light. The 1 Kb Plus DNA ladder (Invitrogen) was used as reference. The identity of each sample is indicated, and the band differences are highlighted using arrows (in white for unique segments and in grey for those absent)

2.6.2 | Productivity

Dead larvae obtained from a treatment corresponding to 1 × 10^7 OBs/ml of VG003 or VG013 were individually weighed and homogenized with water to isolate viral progeny as previously described. The yield was estimated by absorbance measurements at 280 nm using a standard curve of OBs as reference. Quantifications were performed three times, and the concentration values of OBs/ml were normalized with the larva weight. Results were expressed as OBs/g of larval tissue and analysed using the Kruskal–Wallis nonparametric test (K-W) (α = 0.05) via the Statistix program (version 8.0).

3 | RESULTS

3.1 | Morphological characterization

Baculovirus isolates from *T. solanivora* and *T. absoluta* larvae, coded as VG003 and VG013, respectively, were morphologically analysed by TEM (Figure 1). The micrographs revealed in both cases the presence of granular-shaped OBs (ovoid) enclosing single virion and showing typical sizes for betabaculoviruses (Jehle et al., 2006; Rohrman, 2011). Particularly, VG013 had OBs larger than VG003 (514.4 ± 22 × 249.1 ± 16 nm and 448.6 ± 55 × 252.5 ± 28 nm, respectively).

3.2 | Restriction endonuclease profiles and phylogenetic analysis

With the aim of analysing if these two GV isolates were genotypes of the same baculovirus species, their genomes were purified and characterized by treatment with restriction endonucleases, using as reference the PhopGV VG006 isolated from *P. operculella*, because this pathogen infect Gelechiidae's insects (Figure 2). The fragment patterns resolved by electrophoresis were similar for all tested viruses suggesting that VG003 and VG013 would be variants of PhopGV. In fact, only two differences were detected in VG003 with respect to the other viruses showing a high conservation degree of genomic organization among these baculoviruses: (i) the presence of two *Mlu*I-fragments with lengths between 3-4 kbp; (ii) the absence of one *Sma*I-fragment comprised between 4 and 5 kbp plus the presence of one *Sma*I-fragment of approximately 3 kbp. Moreover, the estimated genome size for VG003 and VG013 was about 120 kbp, similar to the corresponding of PhopGV, which has a genome size of 119,217 pb (GenBank NC_004062).

To confirm the above presumption regarding both VG003 and VG013 were genotypes of PhopGV, the standard methodology currently accepted based on the partial sequencing of *lef-8*, *lef-9* and *granulin* genes was applied (Jehle et al., 2006). Thus, these sequences were obtained (GenBank), and with this data, the *Betabaculovirus* phylogenetic inference was carried out showing that VG003 and VG013 consistently grouped with PhopGV (Figure 3). Besides, the Kimura-2 parameter model was applied confirming that VG003 and VG013 are closely related viruses belonging to PhopGV species (Table 2). This assumption is supported by the study of Jehle et al. (2006) where it was

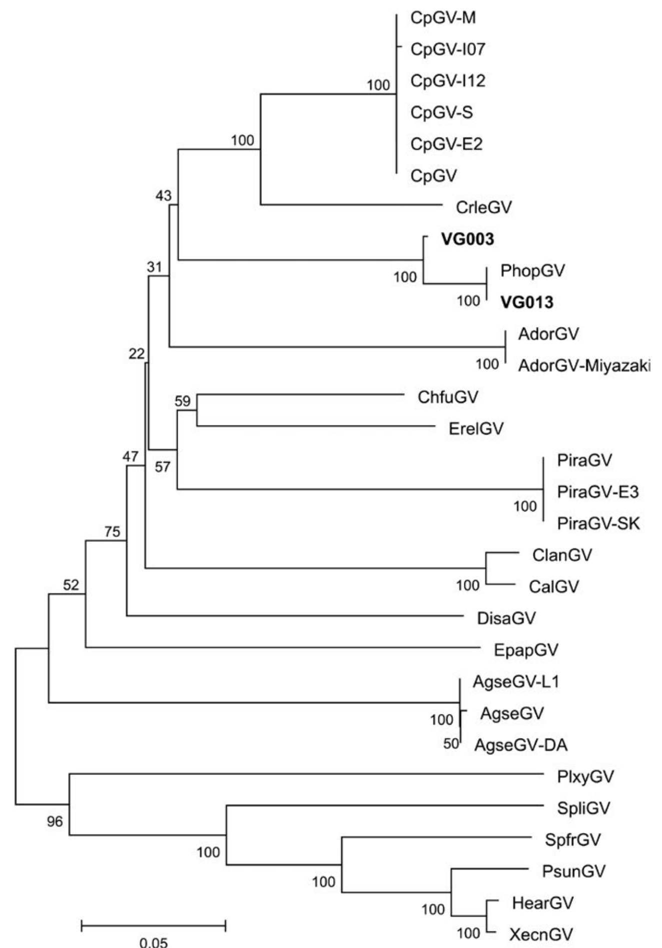

FIGURE 3 Phylogenetic inference for VG003 and VG013. Cladogram based on a concatemer built with a partial sequence of *lef-8*, *lef-9* and *granulin* proteins obtained from 30 betabaculoviral genomes including data from VG003 and VG013. The phylogenetic tree was inferred using the MEGA 5 program, and bootstrap values are indicated

established that distances less than 0.015 between a pair of viruses correspond to genotypes of the same baculoviral species. A similar pattern is observed for *Adoxophyes orana* GV, *Cydia pomonella* GV, *Agrotis segetum* GV and *Pieris rapae* GV isolates.

3.3 | Biological assays

Adaptation to a new host might not result in changes visible at the RFLP level, but it might produce important changes in the biological properties; for this reason, VG003 and VG013 were evaluated as biocides on *T. absoluta* larvae (Table 3). The two viral isolates produced typical symptoms of disease, characterized by loss of movement, decreased feed rate and change in colour from green to bright white followed by sluggishness and flaccidity as previously described by Lacey, Hoffmann, and Federici (2011) (Figure 4). In addition, individuals changed their behaviour getting out of the mines and dying adhered to the surface of the leaves. It can be related to the fact that the baculovirus alter the behaviour in lepidopteran larvae, inducing

TABLE 2 Kimura 2-parameter distances among betabaculoviruses. Shaded cells indicate genotype of the same baculoviral species

	AdorGV-Miyazaki	AgseGV	AgseGV-L1	AgseGV-DA	ChfuGV	ClanGV	CalGV	CrleGV	CpGV	CpGV-E2	CpGV-S	CpGV-I12	CpGV-M
AdorGV	0.0006	0.4224	0.4212	0.4212	0.3242	0.3799	0.3906	0.3461	0.3539	0.3516	0.3529	0.3527	0.3539
AdorGV-Miyazaki		0.4210	0.4198	0.4198	0.3230	0.3787	0.3893	0.3473	0.3552	0.3529	0.3541	0.3539	0.3552
AgseGV			0.0006	0.0006	0.3973	0.4488	0.4609	0.3920	0.4194	0.4182	0.4182	0.4180	0.4194
AgseGV-L1				0.0000	0.3961	0.4490	0.4612	0.3908	0.4182	0.4170	0.4170	0.4168	0.4182
AgseGV-DA					0.3961	0.4490	0.4612	0.3908	0.4182	0.4170	0.4170	0.4168	0.4182
ChfuGV						0.3779	0.3952	0.2912	0.3496	0.3481	0.3494	0.3483	0.3496
ClanGV							0.0706	0.3976	0.3595	0.3559	0.3571	0.3582	0.3595
CalGV								0.4093	0.3628	0.3592	0.3617	0.3641	0.3628
CrleGV									0.2735	0.2733	0.2745	0.2724	0.2735
CpGV										0.0024	0.0024	0.0006	0.0000
CpGV-E2											0.0012	0.0030	0.0024
CpGV-S												0.0030	0.0024
CpGV-I12													0.0006
CpGV-M													
CpGVI07													
DisaGV													
EpapGV													
ErelGV													
HearGV													
PhopGV													
PiraGV													
PiraGV-E3													
PiraGV-SK													
PlxyGV													
PsunGV													
SpfrGV													
SpliGV													
VG003													
VG013													

hyperactive behaviour in order to increase virus spread on the foliage of the plant (van Houte, Ros, & van Oers, 2014).

Both viruses revealed similar mean lethal concentrations with values ranging from 1.6×10^4 to 5.1×10^4 OBs/ml. The fit of the transformed data was acceptable using the chi-squared test (Table 3). It is important to mention that the natural mortality in the absolute and treated controls oscillated between 3% and 12% showing values lower than those reported by Mascarin et al. (2010) (27.8 ± 10.1%), indicating in consequence that a more reliable bioassay method was carried out in our study.

Mean time to death (MTD) values were estimated for virus concentrations that resulted in ~90% larval mortality (1×10^7 OBs/ml). Granulovirus VG003 presented an MTD of 14.0 days (confidence limits: 12.3 and 15.7) and VG013 presented an MTD of 8.6 days (confidence limits: 7.3 and 9.8), values that were significantly different ($\chi^2 = 32.05$; $df = 2$; $P = .0003$).

Regarding virus yield, in both cases productivities overall 10^{10} OBs/g were obtained (Table 4). However, yield was significantly higher with VG003 than the obtained for VG013 (K–W: $\chi^2 = 8.9192$; $df = 59$; $P = .0028$). Larval weight was also higher with the viral isolate VG003 from *T. solanivora* (K–W: $\chi^2 = 7.1880$; $df = 59$; $P = .0073$).

4 | DISCUSSION

Worldwide, biopesticides based on PhopGV are used mainly to control *P. operculella* larvae during potato tubers storage. As the

CpGVI07	DisaGV	EpapGV	ErelGV	HearGV	PhopGV	PiraGV	PiraGV-E3	PiraGV-SK	PlxyGV	PsunGV	SpfrGV	SpliGV	VG003	VG013	XecnGV
0.3552	0.3931	0.3749	0.3339	0.4209	0.3522	0.3466	0.3466	0.3501	0.4112	0.4211	0.4182	0.4440	0.3521	0.3521	0.4213
0.3564	0.3918	0.3736	0.3327	0.4195	0.3510	0.3454	0.3454	0.3489	0.4099	0.4225	0.4168	0.4426	0.3509	0.3509	0.4199
0.4180	0.4184	0.4085	0.3986	0.4260	0.4087	0.4255	0.4241	0.4282	0.4395	0.4480	0.4503	0.4369	0.4085	0.4085	0.4260
0.4168	0.4172	0.4074	0.3988	0.4262	0.4075	0.4243	0.4229	0.4269	0.4383	0.4483	0.4505	0.4356	0.4073	0.4073	0.4262
0.4168	0.4172	0.4074	0.3988	0.4262	0.4075	0.4243	0.4229	0.4269	0.4383	0.4483	0.4505	0.4356	0.4073	0.4073	0.4262
0.3483	0.3839	0.3828	0.2754	0.4081	0.3353	0.3196	0.3207	0.3209	0.4211	0.4285	0.4393	0.4260	0.3376	0.3376	0.4164
0.3569	0.4131	0.3891	0.3671	0.4540	0.3973	0.3976	0.3962	0.4042	0.4465	0.4703	0.4224	0.4604	0.3957	0.3957	0.4545
0.3654	0.4239	0.3892	0.3734	0.4493	0.4107	0.4255	0.4240	0.4267	0.4442	0.4615	0.4274	0.4657	0.4092	0.4092	0.4571
0.2713	0.3713	0.3793	0.3168	0.4292	0.3474	0.3224	0.3224	0.3202	0.4201	0.4371	0.4457	0.4201	0.3489	0.3489	0.4221
0.0024	0.3615	0.3373	0.2963	0.4099	0.3123	0.3493	0.3480	0.3504	0.4105	0.4282	0.3676	0.4317	0.3101	0.3101	0.4113
0.0037	0.3625	0.3387	0.2953	0.4101	0.3110	0.3482	0.3470	0.3493	0.4093	0.4294	0.3652	0.4300	0.3088	0.3088	0.4087
0.0037	0.3639	0.3375	0.2977	0.4087	0.3110	0.3457	0.3444	0.3467	0.4080	0.4308	0.3677	0.4329	0.3088	0.3088	0.4101
0.0018	0.3628	0.3360	0.2952	0.4085	0.3111	0.3480	0.3467	0.3491	0.4091	0.4267	0.3663	0.4332	0.3090	0.3090	0.4099
0.0024	0.3615	0.3373	0.2963	0.4099	0.3123	0.3493	0.3480	0.3504	0.4105	0.4282	0.3676	0.4317	0.3101	0.3101	0.4113
	0.3654	0.3373	0.2986	0.4113	0.3100	0.3480	0.3467	0.3491	0.4105	0.4296	0.3663	0.4361	0.3078	0.3078	0.4127
		0.4389	0.3774	0.4607	0.3832	0.3793	0.3767	0.3822	0.4580	0.4494	0.5179	0.4354	0.3817	0.3817	0.4597
			0.3651	0.4026	0.3739	0.3680	0.3706	0.3717	0.3618	0.4244	0.3931	0.4232	0.3737	0.3737	0.4049
				0.4046	0.3163	0.3167	0.3179	0.3177	0.4375	0.4397	0.4113	0.4379	0.3174	0.3174	0.4050
					0.3892	0.4176	0.4190	0.4137	0.3989	0.1472	0.2548	0.3399	0.3907	0.3907	0.0155
						0.3381	0.3381	0.3367	0.4099	0.4073	0.4267	0.4449	0.0012	0.0012	0.3985
							0.0012	0.0067	0.4420	0.4471	0.4629	0.4318	0.3403	0.3403	0.4214
								0.0080	0.4405	0.4499	0.4629	0.4346	0.3403	0.3403	0.4228
									0.4461	0.4430	0.4657	0.4292	0.3390	0.3390	0.4174
										0.4304	0.3936	0.4541	0.4125	0.4125	0.4033
											0.2680	0.3382	0.4075	0.4075	0.1470
												0.3720	0.4269	0.4269	0.2498
													0.4465	0.4465	0.3362
														0.0000	0.4000
															0.4000

progressive dispersion of *T. solanivora* to new regions, many studies comparing the biological activity of PhopGV isolates against *P. operculella* and *T. solanivora* have been reported (Espinel-Correal et al., 2012; Gómez-Bonilla, López-Ferber, Caballero, Léry, & Muñoz, 2011; Mascarin et al., 2010; Zeddam et al., 2013). The recent expansion of *T. absoluta* distribution area overlapping those of the two precedent species raised the question of the possible adaptation of virus isolates to this new host, providing a tool for its control. However, until now there are not studies of different PhopGV isolates used against *T. absoluta*. In this sense, the present work is the first report where two natural granulovirus strains (one isolated from *T. solanivora* and the other one from *T. absoluta* larvae) belonging to PhopGV species, as determined by physical maps and *lef-8*/*lef-9*/*granulin* sequence analyses, were used to propose a biological control strategy for *T. absoluta*.

The ability of the original PhopGV isolates adapted to *P. operculella* to control *T. solanivora* was very limited. The LC_{50} and LC_{90} obtained for these isolates were high (up to $LC_{50} = 4 \times 10^8$ OBs/ml), motivating the early assumption that PhopGV could not be used to control *T. solanivora* (Rebaudo, Dangles, Léry, Lopez-Ferber, & Zeddam, 2006). However, viral isolates collected from *T. solanivora* larvae soon after colonization seemed to be adapted to this new host showing an efficient larvae control, for example the Colombian isolate VG003 ($LC_{50} = 3.6 \times 10^6$ (OBs/ml) (Espinel-Correal et al., 2010). The more recent invasion of *T. absoluta* allowed us to compare a virus isolate adapted to *T. solanivora*, (VG003), and a virus isolate collected from this new host (VG013).

TABLE 3 Lethal concentrations (OBs/ml) of VG003 and VG013 isolates on *T. absoluta* larvae under laboratory conditions

		CI (95%) for LC_{50} (OBs/ml)			Probit line			
Viral isolate	LC_{50} (OBs/mL)	Lower	Upper	LC_{90} (OBs/ml)	Slope ± SE	χ^2	*df*	*P*
VG003	5.1×10^4	2.2×10^4	1.1×10^5	1.6×10^7	0.515 ± 0.068	0.626	3	0.877
VG013	1.6×10^4	4.8×10^3	3.7×10^4	7.0×10^6	0.490 ± 0.086	0.513	3	0.986

OBs, occlusion bodies; CI, confidence interval; χ^2, chi-square; df, degrees of freedom.

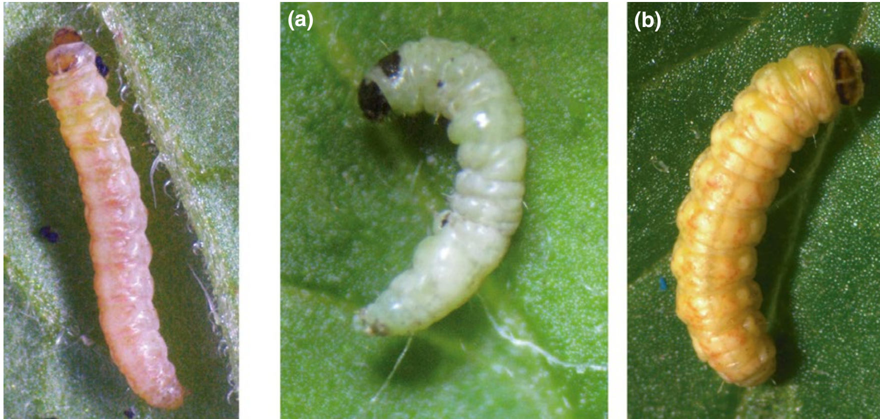

FIGURE 4 Healthy (left panel) and infected (right panel) *Tuta absoluta* larvae. Right panel: (a) larvae infected with VG003 and (b) larvae infected with VG013

TABLE 4 OBs Production of VG003 and VG013 on *Tuta absoluta* larvae

Viral isolate	OBs ($\times 10^{10}$) per gram of larval tissue	Larval weight (g)
VG003	4.5 ± 2.6a	0.0035 ± 0.0021a
VG013	2.5 ± 0.9b	0.0022 ± 0.0012b

Values represent means ± Standard deviation. The values followed by different letters are significantly different according to Kruskal–Wallis (95%). The statistical analysis was carried out independently for each variable.

The LC_{50} and LC_{90} values obtained in the present study showed that VG013 and VG003 are highly pathogenic to *T. absoluta*. Based on obtained lethal concentrations, the possible field dose for both evaluated viruses should be around 10^{12} OBs per hectare (theoretically calculated with the LC_{90} and an application volume of 400L/ha). Estimated dose is lower than the doses used by Kroschel, Kaack, Fritsch, and Huber (1996) and Arthurs, Lacey, Pruneda, and Rondon (2008) for PhopGV isolates used to control *P. operculella* in potato fields, with 10^{13} OBs/ha sprayed three or four times during a crop cycle; however, this dose is not economically viable for mass production and application. A lower field dose may increase the economic and technical feasibility of a biopesticide based on these kinds of viruses, considering the high labour required for in vivo viral production (Ruiz, Gómez-Valderrama, Chaparro, Sotelo, & Villamizar, 2015). However, further studies in tomato fields are needed in order to select the adequate application doses and frequencies to be used with the granuloviruses evaluated in the present work for *T. absoluta* control.

According to our results, there are no differences in pathogenicity between both evaluated betabaculoviruses over insects from the same *T. absoluta* laboratory colony, suggesting that they are capable of infecting alternative hosts with the same insecticidal activity. It was unexpected, because in previous studies it was found that a virus that is able to infect alternative hosts has the higher activity on the specie where it has evolved (Espinel-Correal et al., 2010, 2012). However, it is well known that PhopGV variants have high flexibility and are able to adapt to alternative hosts from Gelechiidae's family due to the presence of genotypes mixtures or particular genes that regulate this adaptation (Hitchman et al., 2007; Léry, Abol-Ela, & Gianotti, 1998), favouring in consequence the selection of virus populations or genotypic variants able to replicate in all hosts.

It is important to note that although *T. solanivora* and *T. absoluta* larvae can feed on the same host plant (potato), they do not have the same feeding habits. In fact, *T. solanivora* larvae only feed on tubers meanwhile *T. absoluta* larvae feed on aerial parts of the plants (e.g., leaves or stems). Moreover, *P. operculella* shares feeding habits with the before mentioned insects, so it is possible that VG003 and VG013 have been originated from a PhopGV infecting *P. operculella* larvae that were in contact with *T. solanivora* and *T. absoluta* generating the virus adaptation to these new hosts. In 1997 in Colombia, an isolate of PhopGV from Peru (coded as VG006) was introduced and applied massively in order to respond to a sanitary emergency caused by *T. solanivora* in potato crops in Cundinamarca and Boyacá regions (Villamizar, Zeddam, Espinel, & Cotes, 2005). This virus could have been in contact with different species of Gelechiidae reinforcing the hypothesis of the PhopGV circulation among them.

In a previous work, 20 betabaculoviruses isolated from different hosts of Gelechiidae's family and from diverse geographical regions were evaluated on neonate *T. solanivora* larvae finding important differences in LC_{50} values (Carpio et al., 2012). Notably, the level of

pathogenicity was linked to the host origin of the PhopGV isolate, suggesting that biological activity of PhopGV depends on factors other than the host. Furthermore, all host species are phylogenetically close (Povolny 1975) providing a similar and appropriate physiological environment for viral multiplication. So that, the influence of the environment could be one important factor to determine the pathogenicity of a specific viral isolate (Cory, Green, Paul, & Hunter-Fujita, 2005). This influence results in a high degree of intraspecific diversity, which makes PhopGV to be adaptable to different habitats.

On the other hand, the fastest isolate to kill the *T. absoluta* larvae was the one originally isolated from the same host (VG013), requiring 6 days less to kill the larvae in comparison with virus VG003 isolated from *T. solanivora*. In this case, the virus isolated originally from *T. absoluta* larvae demonstrated a fitness advantage in terms of lethal time, indicating that pathogenicity is not the only factor that determines the adaptation of a viral isolate to an insect host. According to Ángeles Rivas and Alcázar (1996), a PhopGV isolated from Peru has a mean lethal time of approximately 15 days against *P. operculella*, similar than obtained in the present work over *T. solanivora* and higher than the time needed for kill *T. absoluta* larvae with its original virus. In general, PhopGV is a 'slow-killing virus', and this knowledge is crucial for the correct use of this biocontrol agent. In our case, isolate VG013 could help to reduce pest population faster than VG003, preventing losses on foliage and hence plants photosynthetically active surface. Furthermore, *T. absoluta* also can cause high economic losses in tomato due to infestation of the fruits, and these losses are generally dependent on pest control efficacy on early stages of the crop (Desneux et al., 2010). In this sense, the use of a more persistent PhopGV for *T. absoluta* control at early stages of tomato crops could be efficient to reduce population when fruits are forming. Viruses could persist in field populations of *T. absoluta* eventually leading to a long-term significant reduction of pest density and their persistence could be related with their OBs production. Productivity (OBs/g) estimated in the present work and larval weight were higher when larvae were inoculated with VG003, indicating a higher production of viral particles. Previous works demonstrated that VG003 is the most adapted variant to *T. solanivora* (Espinel-Correal et al., 2010), suggesting that when it is in contact with an alternative host as *T. absoluta*, productivity should be maximized. This phenomenon was demonstrated by Cuartas, Villamizar, Espinel-Correal, and Cotes (2009), who evaluated different viral isolates obtained from *P. operculella* and *T. solanivora* over these two hosts. The granulovirus isolated from *P. operculella* produced more viral particles on its alternative host *T. solanivora* and vice versa, indicating that an isolate less adapted to a host needs to produce more viral progeny in order to ensure dispersion and persistence in the new environment.

Probably, this property is linked to the occurrence of sequence variants of the *ecdysteroid UDP-glucosyltranferase* (*egt*) gene (Cory & Myers, 2003; Pinedo, Moscardi, Luque, Olszewski, & Ribeiro, 2003). PhopGV isolates from different hosts presented variations in *egt* gene (Carpio et al., 2012; Zeddam et al., 2013). Although not demonstrated, changes in this or other encoding sequences would lead to an increased larval size to achieve a greater amount of viral progeny virus and ensure high horizontal transmission, or can induce changes in insect behaviour to achieve the same outcome (Cory, 2015). The differences in the genome restriction analysis could provide a genetic evidence of this phenotypic change.

Higher viral production ensures a higher transmission rate, so 'fast-killing' viruses may controls pests more efficiently in the short term than 'slow-killing' ones, but a 'slow-killing' virus controls pests more effectively in the long term because of its higher transmission efficiency (Takahashi et al., 2015).

Both isolates evaluated in the present work, VG003 and VG013, demonstrated high potential to be used as active ingredient for a new viral biopesticide to control *T. absoluta*. These viruses could be used independently or in an artificial mixture, in order to take advantage of the differences in pathogenicity, virulence and productivity of OBs, allowing to design a more efficient tool to control the pest throughout the crop cycle. Besides, other authors previously have demonstrated that isolate VG003 exhibits biological and technological characteristics for being used as a viral pesticide against *T. solanivora* and *P. operculella* (Chaparro et al., 2010; Quiroga, Gómez, & Villamizar, 2011), what could broaden the spectrum of action of this biocontrol strategy, to control populations of the three pest species, *T. absoluta*, *T. solanivora* and *P. operculella*, in different crops where these pests occur in a mixed infestation.

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AUTHOR CONTRIBUTION

ML-F and LV conceived the original research idea. JG-V and LV designed and performed the bioassays. JG-V, LV and ML-F analysed and interpreted bioassays data. GB, PDG and MB analysed and interpreted the bioinformatics data. JG-V, GB, ML-F, MB, PDG and LV wrote the paper.

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