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Development of a viral biopesticide for the control of the Guatemala potato tuber moth *Tecia solanivora*

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A B S T R A C T

The Guatemala potato tuber moth *Tecia solanivora* (Povolny) (Lep. Gelechiidae) is an invasive species from Mesoamerica that has considerably extended its distribution area in recent decades. While this spe-cies is considered to be a major potato pest in Venezuela, Colombia, and Ecuador, currently no specific control methods are available for farmers. To address this issue we developed a biopesticide formulation to be used in integrated pest management of *T. solanivora*, following three steps. First, search for entomo-pathogenic viruses were carried out through extensive bioprospections in 12 countries worldwide. As a result, new *Phthorimaea operculella granulovirus* (PhopGV) isolates were found in *T. solanivora* and five other gelechid species. Second, twenty PhopGV isolates, including both previously known and newly found isolates, were genetically and/or biologically characterized in order to choose the best candidate for a biopesticide formulation. Sequence data were obtained for the ecdysteroid UDP-glucosyltransferase (*egt*) gene, a single copy gene known to play a role in pathogenicity. Three different sizes (1086, 1305 and 1353 bp) of *egt* were found among the virus isolates analyzed. Unexpectedly, no obvious correlation between *egt* size and pathogenicity was found. Bioassays on *T. solanivora* neonates showed a maximum of a 14-fold difference in pathogenicity among the eight PhopGV isolates tested. The most pathogenic PhopGV isolate, JLZ9f, had a medium lethal concentration (LC_{50}) of 10 viral occlusion bodies per square mm of consumed tuber skin. Third, we tested biopesticide dust formulations by mixing a dry carrier (cal-cium carbonate) with different adjuvants (magnesium chloride or an optical brightener or soya lecithin) and different specific amounts of JLZ9f. During laboratory experiments, satisfactory control of the pest (>98% larva mortality compared to untreated control) was achieved with a formulation containing 10 macerated JLZ9f-dead *T. solanivora* larvae per kg of calcium carbonate mixed with 50 mL/kg of soya lec-ithin. The final product provides an interesting alternative to chemical pesticides for Andean farmers affected by this potato pest.

Keywords:
Insect virus
Biological control
Pathogenicity
Viral variant
Egt gene

1. Introduction

For centuries, potato has been the staple food of Andean populations and is still an essential part of their diet (Horton, 1987). Potato production is negatively affected by many abiotic (drought, frost among others) or biotic (diseases, pests) factors (Wale et al., 2008). Species belonging to the potato tuber moth (Lepidoptera: Gelechiidae) complex, or PTMs, are among the main insect pests of the crop worldwide (Raman, 1988a; Barragán et al., 2005; Rondon, 2010). Their larvae cause losses by mining tubers, mainly during storage (Raman, 1988a,b). For decades, *Phthorimaea operculella* (Zeller) was the only PTM species identified as a serious pest in South America. In the 1980s and 1990s, *Symmestrichema tangolias* (Gyen), also received pest status in some Andean countries (Barragán et al., 2005). Recently, *Tecia solanivora* (Povolny) successively invaded several countries from its native Central America (Puillandre et al., 2008). Use of chemical pesticides is generally the farmers' first choice to control PTMs. However, serious side effects of these products combined with a rapid build-up of pesticide resis-

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tance by pest populations (Kay and Collins, 1987; El-Kady, 2011) call for alternative methods of control.

Two decades ago, the International Potato Center (CIP) and collaborating institutions initiated an integrated pest management (IPM) program for the control of *P. operculella* (Raman, 1994). About a dozen different components were included in the IPM, the most important being a viral biopesticide (Kroschel et al., 1996). The virus used was *Phthorimaea operculella granulovirus*, PhopGV (Reed, 1969; Fauquet et al., 2005), a member of the genus *Betabaculovirus* (formerly, *Granulovirus*) belonging to the family *Baculoviridae*. Considering the success of the IPM approach for the control of *P. operculella*, we carried out similar studies in Ecuador on *T. solanivora,* as the two species are phylogenetically and ecologically closely related. As the viral biopesticide used against *P. operculella* was not efficient enough against *T. solanivora* (J. Suquillo, Instituto Nacional Autónomo de Investigaciones Agropecuarias – INIAP, pers. comm.), it was thus necessary to develop another biopesticide formulation. In this paper, we report on the field collection of new granulovirus isolates found in populations of different PTM species and on their genetic characterization on the basis of the ecdysteroid UDP-glucosyltransferase (*egt*) gene, which has been reported to play a role in pathogenicity in *Baculoviridae* (Cory et al., 2004). We also evaluated the efficiency of different formulations of the most pathogenic of virus isolates tested for the control of *T. solanivora* neonates.

2. Materials and methods

2.1. Bioprospections and origin of virus samples

Bioprospections were carried out in traditional potato storage from where thousands of larvae were gathered and visually assessed. Twenty larvae of the Gelechiidae *P. operculella, T. solanivora, S. tangolias, Tuta absoluta* (Meyrick), *Eurysacca quinoae* (Povolny) and *Paraschema detectendum* (Povolny) presenting typical symptoms of granulovirus infection (e. g., a whitish body; Reed, 1969; Briese, 1981) were collected by different institutions (for details see Table 1) and kept at −20 °C for further studies. Only larvae unequivocally identified by their taxonomic characteristics (Barragán et al., 2005) were used in subsequent works. Presumably virus-infected samples originated from 12 countries representing 5 continents (Table 1).

2.2. Virus purification and quantification

The presence of granulovirus occlusion bodies (OBs) in the twelve biological samples was confirmed by transmission electron microscopy (TEM) of negatively (phosphotungstic acid) stained larva homogenate (Brenner and Horne, 1959) using a Zeiss EM10 electron microscope. Each virus isolate was then multiplied in the laboratory by contaminating virus-free *T. solanivora* neonates using potato tubers previously submersed in a homogenate of virus-containing larvae (for details see Zeddam et al., 1999). The identity of the virus progeny collected after mass multiplication to the virus inoculum was determined by PCR amplification and sequencing of the *egt* gene (see below) and/or RFLP analysis (Zeddam et al., 1999).

Batches of dead larvae previously infected by each virus isolate were homogenized separately in Tris-EDTA (TE) buffer (10 mM Tris, 1 mM EDTA, pH 7.2). Virus OBs were purified by centrifuging the cheesecloth-filtered homogenate for 30 min at 12,000 g on a sucrose gradient (15–30–45% w/v). OB concentrations were estimated by measuring the optical density (OD) at the 450 nm wavelength (Helios Alpha UV–Vis spectrophotometer; Thermo Scientific) of the purified virus suspensions considering that 1 OD corresponds to $679.9 \pm 53.9 \times 10^6$ OB (Espinel-Correal et al., 2010).

2.3. Genetic characterization of virus isolates

In a preliminary step, DNAs extracted from new virus isolates were digested with endonucleases (Zeddam et al., 1999) and the restriction profiles obtained were compared to the established PhopGV reference isolate 1346 (GenBank AF499596). Genetic characterization was then carried out for establishing the taxonomic status of presumed GVs isolated from PTM larvae. For this purpose, two genes were sequenced: the one encoding for granulin (the main structural protein of virus OB) and the pathogenicity-related gene *egt*. In a second step, *egt* sequences of different GV variants were compared to determine whether this gene could be usable to select highly pathogenic GV isolates for biopesticide formulation instead of carrying tedious and time-consuming bioassays.

2.3.1. PCR amplification of virus sequences

For DNA extraction, OBs were dissolved in an equal volume of sodium carbonate solution (1 M, pH 11) for 30 min and then centrifuged for 1 h at 75,000 g. The pellet (virions) was dispersed in

Table 1
Host list and geographic origins of putative *Phthorimaea operculella granulovirus* isolates used in the study. All larvae were collected from potato tubers (*Solanum tuberosum*) except those of *E. quinoae* (from quinoa, *Chenopodium quinoa*) and *Tuta absoluta* (from tomato leaves, *Lycopersicum esculentum*). CIP: International Potato Center, Lima, Peru. INIA: Instituto Nacional de Investigaciones Agrícolas, Venezuela.

Isolate name	Original host	Geographic origin	GenBank No.	*Egt* size (in bp)	Reference
1346	*P. operculella*	Tunisia	AF499596.1	1305	CIP
1390-2	*P. operculella*	Peru	HQ317419	1305	Vickers et al. (1991)
YEM	*P. operculella*	Yemen	HQ317411	1086	Kroschel et al. (1996)
INDO	*P. operculella*	Indonesia	HQ317413	1353	Zeddam et al. (1999)
EGY	*P. operculella*	Egypt	HQ317420	1086	This study
KEN	*P. operculella*	Kenya	HQ317414	1353	CIP
TUR	*P. operculella*	Turkey	HQ317404	1086	This study
HUAN	*P. operculella*	Peru	HQ317405	1086	This study
CHI	*P. operculella*	Chile	HQ317406	1353	This study
AUS	*P. operculella*	Australia	HQ317407	1086	Briese (1981)
TESO	*T. solanivora*	Ecuador	HQ317408	1086	This study
JLZ9f	*T. solanivora*	Ecuador	HQ317403	1353	This study
GUA	*T. solanivora*	Guatemala	HQ317410	1086	CIP
GV005	*T. solanivora*	Ecuador	HQ317417	1086	This study
GV006	*T. solanivora*	Ecuador	HQ317418	1086	This study
VZ	*T. solanivora*	Venezuela	HQ317415	1353	INIA
TUTA	*T. absoluta*	Peru	HQ317412	1305	This study
EUQU	*E. quinoae*	Peru	HQ317409	1086	This study
PADE	*P. detectendum*	Bolivia	HQ317416	1086	This study
TANG	*S. tangolias*	Ecuador	JX082398	1086	This study

1 ml dH_20 and successively extracted three times with phenol and twice with chloroform iso–amylic alcohol (24:1). The aqueous phase was collected and precipitated at −20 °C overnight with 2.5 volumes of absolute ethanol in presence of sodium acetate (pH 5.5) at a final concentration of 0.3 M.

GV isolates were genetically characterized using the genes *granulin* and *egt*. *Granulin* is located between nt 1 and nt 747 on the direct strand and *egt* between nt 116,818 and nt 118,122 on the complementary strand of the PhopGV genome, isolate 1346. Specific PCR primers pairs GranTotF (5′-TTTCGCGATGTTT-CAAAATG-3′)/GranTotR (5′-CATGATTTCTTCGATTTCGG) and egtF (5′-TTTTTTCGAGTCGAGCCAA-3′)/egtR (5′-CAACGATGATCTCATA-TATGAG-3′) were used with either *Pfu* (Promega, USA) or *Pfx* (Invitrogen, USA) DNA polymerases for the amplification of the full sequence of either *granulin* or *egt*. Reactions were carried out in a PTC-100 thermocycler (MJ Research Inc., USA) using the following cycles and temperatures: denaturation at 94 °C for 2 min, 35 cycles at 95 °C for 45 s, 50 °C for 45 s, 72 °C for 1.5 min, followed by final extension at 72 °C for 10 min. Amplicons were directly sequenced using the dideoxynucleotide chain termination method in an ABI 377 automated DNA sequencer.

2.3.2. Phylogenetic analysis of virus sequences

Granulin and *egt* sequences from our virus isolates were aligned using the MultAlin algorithm (Corpet, 1988), and checked visually. *Egt* alignment was used for phylogenetic analysis (for *granulin*, see Results section). For comparison purpose, this alignment also included published *egt* sequences belonging to other granuloviruses (namely, *Pieris rapae granulovirus* (PrGV; GenBank Accession Number: GQ884143.1), *Cydia pomonella granulovirus* (CpGV; GenBank Accession Number: U53466.2) and *Choristoneura occidentalis granulovirus* (CoGV; GenBank Accession Number: DQ333351.1). The phylogenetic model was optimized by maximum likelihood using the software PhyML (Guindon and Gascuel, 2003). The model of nucleotide substitution was optimized among 56 models using the Akaike criterion in the program Modelgenerator Version 0.85 (Keane et al., 2006), assuming four categories of Gamma distribution substitution rates. One hundred bootstrap replicates were performed assuming four categories of substitution rates. The transition/transversion ratio, the proportion of invariable sites and the gamma distribution parameter were optimized with the phylogeny. The nucleotide sequences of the gene *egt* from the different PhopGV isolates were deposited in GenBank (see Table 1 for Accession numbers).

2.4. Comparative pathogenicity of granulovirus isolates against *T. solanivora* neonates

2.4.1. Experimental design

Eight granulovirus isolates were selected among the twenty considered for the genetic comparison. The selection was made on the basis of the host origin (four were isolated from *P. operculella*, two from *T. solanivora*, one from *P. detectendum* and one from *E. quinoae*) and the *egt* group the isolates belong to (egt-I, egt-II and egt-III were all represented at least once). The respective pathogenicity of these eight granulovirus isolates was determined. The purified OBs were applied to potato tubers using a specially designed device previously described in detail in Carrera et al. (2008). Briefly, a medical compressor/nebulizer (De Vilbiss Pulmo-Aid Sunrise® Compressor/Nebulizer, model 5650, Sunrise Medical, USA) creates an aerosol of OB-containing liquid particles which are guided to the surface of the tuber skin by coupled 12-cm diameter PVC tubes. It was previously established that the system allowed an homogenous quantity of OB to be deposited per surface unit proportionally to the initial OB concentration of the virus suspension. The system was calibrated to obtain deposits of 0, 1.5, 10, 50, 100, and 1000 OB mm^{-2}, respectively at the surface of two potato tubers (each weighing about 50 g) of the variety Superchola. Five replicates (of two tubers each) were carried out for each virus concentration. Then, 25 *T. solanivora* eggs were deposited onto each tuber, which was placed individually in a 105 mm-diameter circular plastic box and maintained in darkness at 18° C to mimic environmental conditions commonly found in potato storages in the Andes. After 5 days, when needed, unhatched eggs were replaced by newly hatched neonates to maintain an even number of individuals in each of the 5 replicates. After 6 weeks, dead and live larvae were counted.

2.5. Statistical analysis

Medium lethal concentrations (LC_{50}) were determined as previously described (Espinel-Correal et al., 2010) by probit analysis (Finney, 1952) using POLO PC software (LeOra, 1987). The software was used to estimate the concentration killing 50% of larvae tested (LC_{50}) and the 95% fiducial limits of the LC_{50}. Overall, 240 experimental units (8 viruses × 6 dosis × 5 replicates) were installed and monitored to determine the LC_{50} for the eight virus isolates considered (listed in Table 2). Although medium lethal time (LT_{50}) is also an important parameter of the virulence of pathogens, only LC_{50} was considered in this study. As the larvae remain inside the tubers during their development, we were unable to monitor the exact time of their death. POLO PC was also used to calculate the relative LC_{50} ratios between virus isolates. Ratios with 95% Confidence intervals which include 1.0 indicate no significant difference from JLZ9f ($P \leqslant 0.05$; Robertson and Preisler, 1992).

2.6. Biopesticide formulation bioassays

2.6.1. Biopesticide formulation and experimental design

Biopesticide formulations were prepared as detailed elsewhere (Alcazar et al., 1992; Alcazar and Raman, 1992; Kroschel et al., 1996) by mixing of a dry carrier (calcium carbonate = powdered limestone) with different adjuvants (either soya lecithin (50 mL/kg of formulation) or Ciba's optical brightener Tinopal DMA-X (10 g/kg) or magnesium chloride (20.3 g/kg) and specified amounts of the isolate JLZ9f of PhopGV (corresponding respectively to 0, 5, 10 or 15 virus-dead larvae/kg of formulation. Refer to Fig. 3). The same JLZ9f virus stock-suspension was used throughout the study. It was obtained by macerating the last instars of virus-dead *T. solanivora* larvae in distilled water (dH_2O). Dust formulations were applied to 95 ± 5 g tubers (variety Superchola) and then 25 eggs were placed on each tuber. Each experimental unit was kept individually in a 12-cm-diameter plastic box placed in an experimental facility. As above, unhatched eggs were replaced. After 6 weeks, tubers were cut into four slices and photographed. The percentage of damage was determined using image analysis software (see below). Although the intensity of damage was much more pertinent from an agricultural perspective, the numbers of dead and live larvae were also recorded. All the experiments were carried out under conditions similar to those found in traditional (i.e. Andean farmers') potato storage and the temperature was maintained at 14.3 ± 2.8 °C and humidity at 68.8 ± 10.5% throughout the experimental periods.

2.6.2. Damage evaluation and statistical analysis

Because the insect larvae live inside the tubers until pupation, both mortality and damage levels could only be evaluated at the end of the experiments. Damage to tubers is a more relevant parameter as it considers the food consumed by larvae not killed by the virus (surviving individuals) as well as the food consumed by the virus-infected larvae before they die. Also, it is more meaningful from the farmers' point of view. For both reasons, it ap-

Table 2
Medium lethal concentrations (LC_{50}) of PhopGV isolates bio-assayed against *T. solanivora* neonates. (1) LC_{50} are expressed in concentration of viral occlusion bodies deposited per mm^2 of tuber skin. (2) Values followed by the same letter are not significantly different, as determined using POLO·PC software ($P \leqslant 0.05$; Robertson and Preisler, 1992). (3) Chi-square goodness-of-fit statistics as determined using POLO·PC software (LeOra Software, 1987). df:degree of freedom. (4) Relative ratios with 95% confidence intervals indicating the fold-difference for each virus isolate in comparison with the most pathogenic PhopGV isolate (JLZ9f). (d) 95% confidence intervals which include 1.0 indicate no significant difference from JLZ9f ($P \leqslant 0.05$; Robertson and Preisler, 1992).

PhopGV isolate	LC_{50}[1,2]	95% Confidence limits for LC_{50}	Chi-square (df)[3]	RR50[4]	95% confidence limits for RR50
JLZ9f	10.0[a]	3.3–24.0	3.26 (3)	–	–
EUQU	13.1[a,b]	6.6–42.4	8.0 (6)	0.76	0.36–1.63
PADE	13.6[a,b,c]	7.2–27.0	1.8 (2)	0.70	0.33–1.51
1390-2	24.4[b,c,d]	5.3–53.8	0.39 (1)	0.41	0.18–0.97
GV006	31.3[d]	21.2–45.4	1.7 (2)	0.33	0.18–0.60
1346	35.2[d]	20.6–64.6	4.1 (5)	0.23	0.11–0.48
TESO	136.6[e]	82.4–241.1	3.6 (5)	0.07	0.04–0.15
GV005	138.0[e]	65.4–385.8	1.5 (2)	0.06	0.02–0.13

peared to be the best parameter to consider. Parts of the potato damaged by *T. solanivora* larvae were evaluated as recently described (Carpio et al., 2010) with the Scion Image software Scion Image 4.0.2 for Windows software (National Institutes of Health, Bethesda, MD, www.scioncorp.com/pages/scion_image_windows.htm) that was previously used for estimating leaf damage caused by phytophagous insects (O'Neal et al., 2002) or fungal pathogens (Wijekoon et al., 2008). Briefly, at the end of the experiments, tubers were cut into four 1.5 cm-thick slices parallel to the longer axis and photographed from a height of 21 cm with a digital camera (Canon Power Shot S40, Canon, Tokyo, Japan). Each photo included the same calibration sheet for subsequent calculation of area (through the determination of the number of pixels corresponding to 1 square cm). Photos were exported to a computer, transformed into TIFF format and their brightness standardized with the freeware GIMP 2.6.4 (www.gimp.org). The damaged and undamaged areas of potato slices were determined using Scion Image set to a threshold value of 155.

For statistical comparisons of adjuvants and virus concentrations, damage percentages were transformed (arcsin of the square-root), and then subjected to a one-way analysis of variance (ANOVA) using a completely randomized design. Tukey's HSD test was performed as a post hoc test to compare pairwise differences. Box and whisker plots were drawn using Grapher 4 (Golden Software, CO, USA).

3. Results

3.1. Bioprospections and origin of virus samples

Bioprospections showed that different non-occluded viruses were present in populations of *T. solanivora* (Zeddam et al., in preparation) but only one occluded virus was found, namely PhopGV, a recognized member of the genus *Betabaculovirus* (Family *Baculoviridae*). The majority of non-occluded viruses were either not sufficiently pathogenic or rapidly inactivated, and consequently, only PhopGV isolates were used for further analyses. During this study, a total of twelve new PhopGV isolates were identified mainly in the larvae of *T. solanivora* (7 isolates) but also in larvae of five other related gelechid species: the *P. operculella* (4 isolates), *S. tangolias* (1), *T. absoluta* (1), *E. quinoae* (1) and *P. detectendum* (1). PhopGV has a very large geographic distribution as, including these new reports, it was now found in samples from 12 countries representing all inhabited continents: South and North Americas, Europe, Asia, Africa, Oceania.

3.2. Phylogenetic characterization of PhopGV isolates

Restriction endonuclease (REN) analysis of the granulovirus isolates from the different PTM species showed only slight variations in their band patterns in agarose gels (Fig. 1) and consequently, all isolates likely belonged to the same virus species. One of the regions where variability was consistently detected corresponded to the region of the gene *egt* (ORF 129 of PhopGV). No such variability was observed in the granulin region of the virus genome. However, both genes were completely sequenced for all isolates. Sequencing of PCR-amplified products showed that all PhopGV isolates exhibited the same nucleotide sequence, identical to the reference isolate 1346 (GenBank AF499596). Thus, *granulin* gene corroborated the PhopGV identity of virus isolates but was not useful for phylogenetically comparing one of them to the others. Conversely, sequencing of *egt* provided evidence for the existence of two kinds of variations between isolates. First, between nucleotides 1 and 1053, the viral isolates showed only a very limited number (0–4 depending on the isolate) of nucleotide substitutions compared to the consensus sequence. A total of 11 sites were affected: 198G → C, 209G → T, 327G → A, 615G → C, 646G → A, 647C → T, 736G → A, 761A → T, 909C → T, 969C → A, and 1053T → C. These substitutions were silent with the exception of 327G → A, 615G → C, 646G → A and 761A → T which, in these cases, likely resulted in a change in the amino-acid sequence of

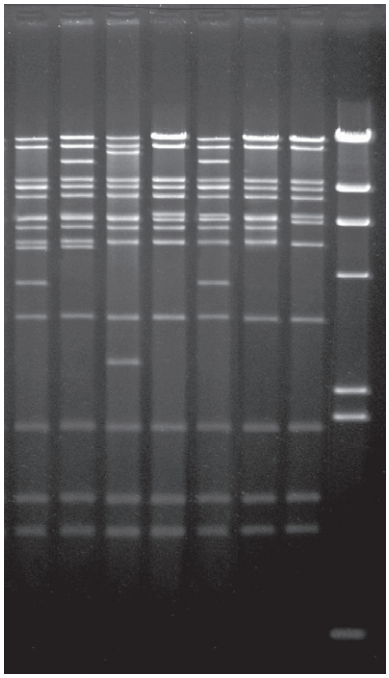

Fig. 1. Gel electrophoresis of *Nde* I-digested genomic DNAs extracted from different PhopGV isolates (Lanes 1–7: Venezuela, Chili, Yemen, 1346, JLZ9f, GV6, 1390-2, respectively. Lane 8: Molecular weight marker with bands of 23 130, 9416, 6557, 4361, 2322, 2027 et 564 pb, respectively).

the translated protein. The maximum number of non-silent mutations in an isolate was two (INDO, KEN and YEM isolates).

More noticeably, major deletions (or insertions) were present in the 3′ moiety of *egt* (beginning at nt 1054). Three size groups were identified for *egt* among the 20 PhopGV isolates analyzed (Fig. 2A). These groups were named egt-I, egt-II and egt-III and, exhibited a gene size of 1305, 1353 and 1086 bp, respectively. PhopGV isolates belonging to egt-III exhibit the shortest coding sequence presently known for baculovirus *egt*. The larger size of egt-I and egt-II compared to egt-III was due to extra sequences (Fig. 2B). PhopGV isolates belonging to egt-III were the most numerous and were found in all (6) gelechid species analyzed. Also, egt-III had the largest distribution area and was present in samples from Central and South America, Australia, Africa and Asia. The two sequences absent from group egt-III but present either in egt-I or in egt-II were used in Blast searches but no significant similarity with sequences deposited in the GenBank was found and so the origin(s) of these extra sequences remain(s) unknown. Available PhopGV *egt* sequences were used for phylogeny purposes. Fig. 2A shows PhopGV *egt* phylogenetic reconstruction based on maximum likelihood, assuming the F81 substitution model (selected by ModelGenerator) in PhyML. The *egt* grouping was supported by strong bootstrap values. No correlation was found between the length of the *egt* sequence and either the host or the geographic origin of the virus.

3.3. Comparative pathogenicity of PhopGV isolates

Marked variations in LC_{50}s were found among the eight PhopGV isolates bioassayed in *T. solanivora* neonates (Table 2). The most virulent isolate, JLZ9f (from *T. solanivora,* Ecuador), had a LC_{50} of 10 OB mm^{-2} which was almost 14 times lower than the LC_{50} of the least efficient isolate, TESO. Very different LC_{50} were observed between PhopGV isolates originating from the same host species and the same country (for example, Ecuadorian *T. solanivora* isolates named JLZ9f and TESO had a LC_{50} of 10 and 144 OB mm^{-2}, respectively). Although JLZ9f, PADE (from *P. detectendum*) and 1390-2 (from *P. operculella*) were isolated from three distinct host species, all three exhibited LC_{50} which were not statistically different ($P \leqslant 0.05$; Robertson and Preisler, 1992) when applied to *T. solanivora* larvae. Then, pathogenicity level did not seem unequivocally linked to the host origin of the PhopGV isolate. Similarly, no correlation was found between *egt* groups and pathogenicity to *T. solanivora* neonates. Indeed, JLZ9f (belonging to egt-I group), EUQU and PADE (both from group egt-III) did not show statistically different LC_{50}s (10.0, 13.1 and 13.6 OB mm^{-2}, respectively). At the contrary, EUQU, GV006, 1346, TESO and GV005, which all belong to egt-III, had very distinct LC_{50} encompassing all the range of values found in this study (13.1, 31.3, 35.2, 136.6 and 138.0, respectively). Due to its higher pathogenicity and to the fact that it was initially isolated from *T. solanivora*, JLZ9f was used in subsequent work on viral formulations.

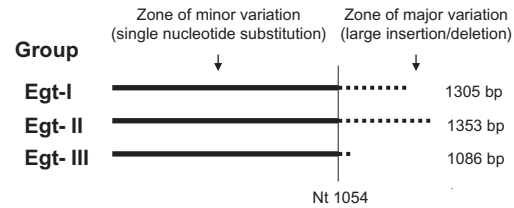

Fig. 2b. Comparative organization of *egt* gene in the 3 PhopGV groups found during the study.

3.4. Bioassays of virus formulations

Neonates were used in independent bioassays to evaluate both levels of damage and mortalities that occurred when different types of adjuvants and virus concentrations were used in a dust formulation applied to potato tubers.

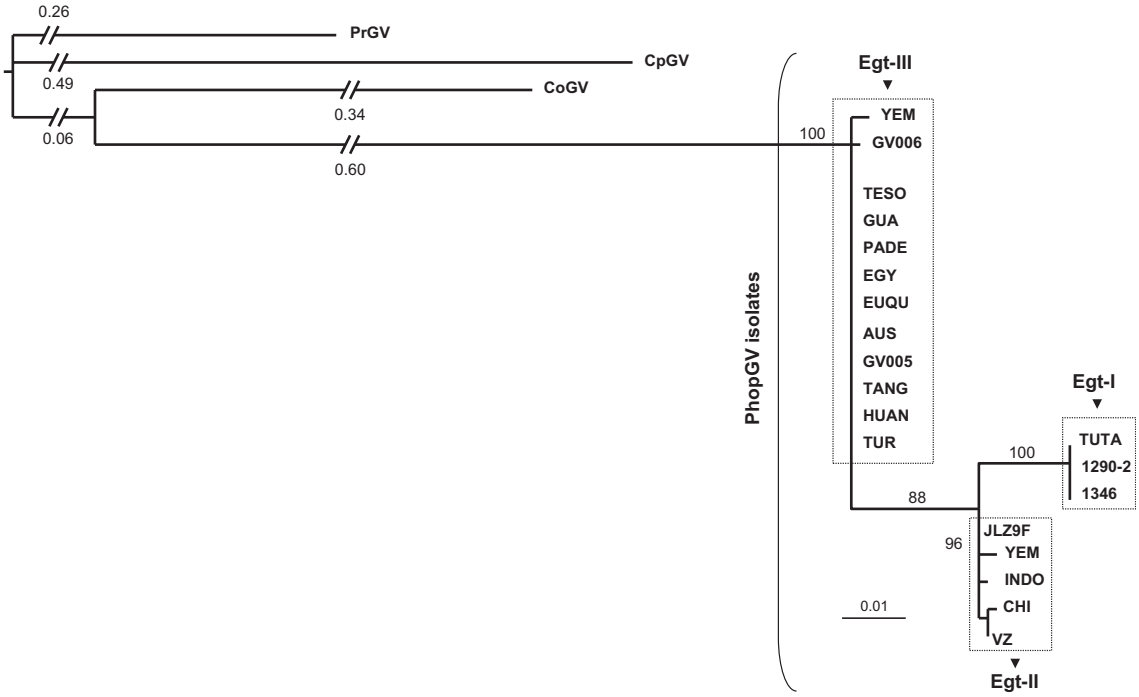

Fig. 2a. Phylogenetic reconstruction based on maximum likelihood for *egt* nucleotide sequence from different viral isolates (see Table 1 for detailed isolate data). Bootstrap values are given at the nodes. PhopGV: Refer to Table 1 for origins and GenBank Accession Numbers. *Pieris rapae granulovirus* (PrGV; GenBank Accession Number: GQ884143.1), *Cydia pomonella granulovirus* (CpGV; GenBank Accession Number: U53466.2) and *Choristoneura occidentalis granulovirus* (CoGV; GenBank Accession Number: DQ333351.1).

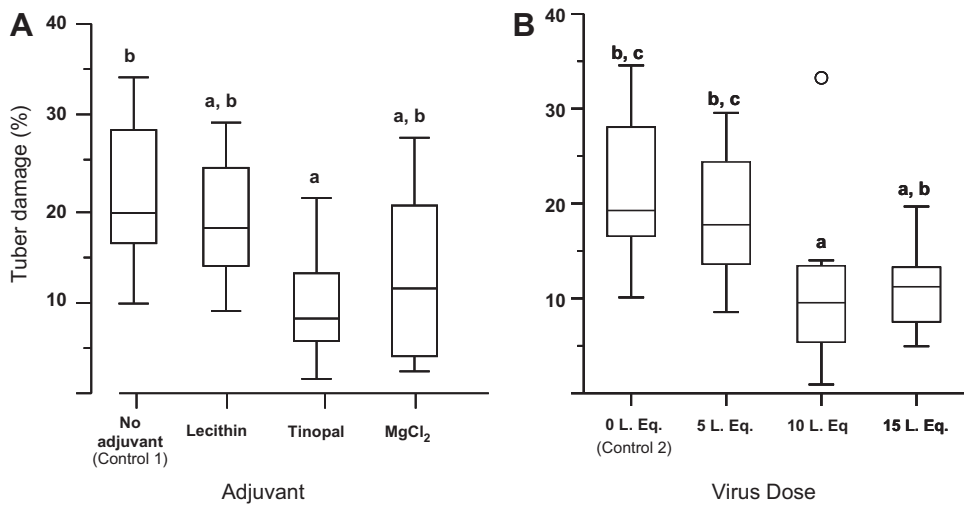

Fig. 3. Box-whisker plots showing levels of tuber damage by *T. solanivora* larvae when using different adjuvants (3A) and different virus doses (3B) in the virus formulation. In 2A, all treatments were with a virus concentration of 5 larval-equivalents/kg of formulation. In 2B, all treatments had a soya lecithin (=adjuvant) concentration of 50 mL/kg (Treatments with the same letter were not significantly different after Tukey's HSD test. Damage is expressed as the percentage of the potato surface evaluated (Refer to Materials and Methods). Sample size: 50 neonates/replicate and 10 replicates/virus dose. Outliers are represented by °).

3.4.1. Effect of adjuvants on biopesticide efficacy

Damage caused by larvae was estimated after the application to tubers of different formulations containing the same quantity of carrier (Ca_2CO_3) and virus (JLZ9f) but a different adjuvant (Fig 3A). The adjuvant was either soya lecithin (Exp1) or the optical brightener Tinopal DMA-X (Exp2) or $MgCl_2$ (Exp3). ANOVA ($F_{3,36}$ = 4.468; P = 0.009) showed a significant difference in potato damage observed depending on the adjuvant used in the virus formulation. Tukey's HSD test ($p < 0.05$) permitted to separate treatment with no adjuvant (Control 1) and treatment with Tinopal. Conversely, treatments containing adjuvants did not segregate into distinct groups. Visual assessment showed that after a certain period of storage, the dust formulation containing Tinopal (a stilbene derivative) adhered less well to tubers. This probably led to a reduced barrier effect of dust, which could explain the lower larval mortality rate observed. Re-infestation of potato by the pest during storage is a common phenomenon and a stable dust cover is thus desired. For this reason as well as for cost-benefit reasons (at the time of the study, local prices were 2.75, 46.7 and 9.4 US $ per kg for soya lecithin, Tinopal and $MgCl_2$, respectively), soya lecithin rather than the two others was the adjuvant used in the following virus-concentration experiments (see below).

3.4.2. Effect of virus concentrations on biopesticide efficacy

Four virus concentrations were compared: 0 (Control 2), 5 (Exp1), 10 (Exp4) and 15 (Exp5) virus-dead larva (or larva equivalents)/kg of biopesticide (=L eq kg^{-1}), respectively. ANOVA ($F_{3,36}$ = 5.242; P = 0.004) established that damage to tubers varied significantly depending on the concentration of virus in the formulation (Fig. 3B). HSD Tukey's ($p < 0.05$) test revealed that increasing the virus concentration in the formulation from 5 to 10 L eq kg^{-1} significantly ($p < 0.05$) reduced damage to the potato caused by larvae. Conversely, no significant difference in damage was observed between 10 and 15 L eq kg^{-1}. Mortalities of exposed neonates were 88.4%, 98.2% and 99.1% for 5, 10 and 15 L eq kg^{-1}, respectively.

4. Discussion

The aim of this study was to isolate, characterize and evaluate viruses amenable to a biopesticide formulation that could be used by resource-poor Andean potato growers, who generally only rely on chemical pesticides for the control of *T. solanivora*. Below, we discuss the different steps that led to the development of this formulation.

4.1. Bioprospections and origin of virus samples

PhopGV was initially reported, in *P. operculella* (Reed, 1969) and later in *T. solanivora* (Zeddam et al., 2008; Espinel-Correal et al., 2010). During the present study, we found PhopGV in six different gelechiid species, namely *P. operculella, T. solanivora, S. tangolias, T. absoluta, E. quinoae* and *P. detectendum*. To our knowledge, the latter four species are new reports of natural hosts for PhopGV which is then the granulovirus with the highest number of natural hosts. This was an unexpected result as GV are generally considered to have a rather narrow host range (Fauquet et al., 2005). One explanation could be that all reported host species are phylogenetically close (Povolny, 1967), providing a rather similar physiological environment that is suitable for PhopGV multiplication. Moreover, this pathogen could be easily transmitted from one host species to the other because their larvae feed on the same host plant (Povolny, 1967). In certain cases, the reduced host range reported for members of the genus *Betabaculovirus* could therefore be due to a lack of systematic screening of the susceptibility of related host species to the same GV.

4.2. Phylogenetic characterization of PhopGV isolates

The present characterization of the collected PhopGV isolates sheds light on the intraspecific variability of the virus both at sequence and pathogenicity levels. Different genes involved in pathogenicity were identified among members of the *Baculoviridae* and the ecdysteroid UDP-glucosyltransferase (*egt*) gene being one of the best studied as both recombinant and naturally occurring viruses deficient in *egt* are more pathogenic than their wild-type counterparts (Cory et al., 2004; Behle and Popham, 2012). Therefore, it was hypothesized that PhopGV variants with significant size variations in the *egt* 3′ moiety (Espinel-Correal et al., 2010 and this study) could exhibit different levels of pathogenicity. However, data obtained in this study did not enable to directly link the pathogenicity of the PhopGV isolates with particular sequences of *egt*. For example, LC_{50} values of PADE and JLZ9f (Table 2) are in the same range while their respective *egt* sequences are quite different (the *egt* sequence in PADE is about one third shorter than in JLZ9f). This could be due to the fact that in PhopGV, other viral

gene(s) (also) contribute to the difference in virulence among isolates. The gene *egt* exhibited significant variation in size between PhopGV isolates from the same host and region. Three very distinct size groups were identified for *egt* during the study. All three groups were found in Latin America and so, it is more likely that the modifications in the gene *egt* appeared in the genome of a PhopGV variant infecting the gelechid populations of this region. Later, the different *egt* groups were probably dispersed overseas along with their hosts as the latter originated from Central or South America. Hence, it can be assumed that sequence modifications in *egt* are ancient and were maintained unchanged since then. In addition, the fact that all *egt* groups were found in more than one gelechid species strongly supports the hypothesis of the circulation of the PhopGV between these different species. To our knowledge, such variations in *egt* gene have never previously been reported either in other members of *Betabaculovirus*. Surprisingly, aside from these rather large deletions or insertions, only very limited sequence variation was observed even among strains isolated from different hosts and different geographic locations. This could be due to the fact that nucleotide substitutions in the 5′ moiety of the gene have a much more drastic effect on the fitness of the virus.

4.3. Comparative pathogenicity of PhopGV isolates

Most studies on the pathogenicity of species variants in the family *Baculoviridae* focused on the genus *Alphabaculovirus* and far less is known about the genus *Betabaculovirus*. For *Alphabaculovirus*, there is a report of LD_{50} values varying from 1 to more than 10 between different isolates of a same virus species (Muñoz et al., 1999). In the case of PhopGV, the ratio between the more and the less virulent isolates tested reached about 14. Considering the limited number of strains bioassayed, it is highly probable that isolates with more extreme LC_{50} remain to be identified in either *T. solanivora* or in other gelechid larvae. For example, in a recent work (Espinel-Correal et al., 2010), a PhopGV isolate was reported to have a LC_{50} as low as 1.16 OBs mm^{-2} (95% CI: 0.34–2.78 OBs mm^{-2}). Previous works reported similar findings when bioassaying the virus against *P. operculella* neonates (Sporleder et al., 2005, 2008). But major difference in the methodology and in the viral strains used, do not permit to compare the relative efficiency of PhopGV in the two species.

There is a practical consequence of the variability of pathogenicity, an essential phenotypic trait in term of biocontrol. Preliminary works showed unsatisfactory control of *T. solanivora* when a Peruvian PhopGV isolated from *P. operculella* was used, even though this particular isolate was successfully used to control the latter species (Alcazar and Raman, 1992; Sporleder and Kroschel, 2009; Arthurs et al., 2008; Lacey et al., 2010). Conversely, JLZ9f caused high mortality rates in *T. solanivora* neonates even at relatively low concentrations. Thus, mining the intraspecific virus biodiversity provided a solution for biopesticide development when the insect species targeted shifted from one potato moth species to another one.

4.4. Bioassays of virus formulations

Biopesticide formulations in developing countries are designed to be as simple and cheap as possible by combining an inexpensive carrier (calcium carbonate), an adjuvant and a defined amount of virus (Kroschel et al., 1996; Sporleder and Kroschel, 2009). Hereafter, we discuss each of these elements.

We observed the carrier greatly contributed to the overall efficiency of the biopesticide as it significantly reduced the level of damage even when applied alone (Fig. 2). These results obtained with *T. solanivora* confirmed results reported in the case of *P. operculella* (Alcazar et al., 1992): the carrier acts as a mechanical barrier and might also negatively affect larva physiology when ingested and/or deposited on the surface of their body.

Several brighteners have been reported to enhance (sometimes by more than two orders of magnitude) the efficiency of baculoviruses in lepidopteran larvae while others had little effect or even reduced virus-induced mortality (Martínez et al., 2003). In our experiments, the brightener Tinopal DMA-X at 10 mL kg^{-1} of biopesticide only caused a limited reduction in the damage caused by *T. solanivora* larvae. This result should be compared with the 31.4-fold increase in pathogenicity of *Helicoverpa armigera nucleopolyhedrovirus* to its host larvae when 1% Tinopal UNPA-GX was added to OBs (Ibargutxi et al., 2008). A limited positive effect (if any) was associated with the adjunction of $MgCl_2$ or soya lecithin. One hypothesis regarding the fact that the addition of these three products to the PhopGV formulation did not significantly decreased damage by larvae would be that calcium carbonate could counteract some effects the adjuvants would otherwise have. Either of the last two adjuvants could however be added to the formulation as both are cheap and easily available in Latin America.

This viral biopesticide (which was granted an Ecuadorian national patent in 2010) proved to be a useful tool for the control of *T. solanivora* when used as one of the components of an IPM strategy including other practices (e.g. crop rotation, tuber selection, storage cleaning). Potato growers in the Andean region have now an alternative to protect their stored potatoes without using chemicals whose negative effects on the health of Ecuadorian potato growers are well documented (Crissman et al., 1998). Thus, a low-cost PhopGV-based biopesticide would be suitable for the control of *T. solanivora* larvae in Andean farmers' potato stores in replacement of less environmentally-friendly products.

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