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Experimental mixtures of *Phthorimaea operculella granulovirus* isolates provide high biological efficacy on both *Phthorimaea operculella* and *Tecia solanivora* (Lepidoptera: Gelechiidae)

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ABSTRACT

The Guatemalan potato moth *Tecia solanivora* (Povolny) recently invaded part of South America, colonizing zones where *Phthorimaea operculella* (Zeller), another potato moth species belonging to the same group, was previously established. *T. solanivora* is now the major insect pest of potato in this area encompassing Venezuela, Colombia and Ecuador. *P. operculella granulovirus* (PhopGV) (Betabaculovirus) is a bio-control agent to be considered for the simultaneous management of these two potato pests, instead of classical chemical insecticides. In a previous work, five PhopGV isolates were isolated in Colombia from *T. solanivora* and were tested against larvae of the same species showing variable efficacies. Infections with mixtures of different genotypes of Baculoviruses had been carried out in a wide range of species and several showed interesting results. In the present study, the effect of sequential passages of PhopGV in *P. operculella* and *T. solanivora* larvae was analyzed through biological assays. Three different mixtures containing a Peruvian PhopGV isolate (Peru) adapted to *P. operculella* and a Colombian PhopGV isolate (VG003) adapted to *T. solanivora* were tested. A preliminary analysis of the correlation between the geno-typic marker egt gene and the level of pathogenicity after a variable number of replication cycles was made. Mixtures of virus isolates showed a higher efficacy in both hosts compared to individual PhopGV isolates. This higher pathogenicity was maintained through passages. In *P. operculella* the mixtures were between 2.8 and 23.6-fold (from 7.15 OB/mm² to 0.10 OB/mm²) more pathogenic than isolate Peru applied alone. In *T. solanivora* they were between 2.3 and 4.9-fold (from 12.29 OB/mm² to 1.25 OB/mm²) more pathogenic than isolate VG003 alone. Viral biopesticide containing a mixture of selected genotypes active against each hosts seemed suitable for the development of a biopesticide aimed to simultaneously control *P. operculella* and *T. solanivora*.

Keywords:

Potato moth
Baculovirus
Virus isolates
Virus mixture
Successive passages
Efficacy

1. Introduction

Recently, the potato tuber moth *Tecia solanivora* Povolny (Lepidoptera: Gelechiidae), originating from Guatemala, became the major potato insect pest in Central America and in the northwest of South America i.e. Venezuela, Colombia and Ecuador (Barragán et al., 2004). *T. solanivora* entered the north of Colombia in 1985 through infested potato seeds imported from Venezuela and 11 years later, it was found in the southwest of the country, at the border with Ecuador (Puillandre et al., 2007). This species also

invaded the Canary Islands (Spain) during the last decade (Carnero et al., 2008). It was then included by the European and Mediterranean Plant Protection Organization (EPPO) in its A2 action list of pests recommended for regulation as quarantine pest (EPPO/OEPP, 2005). During its invasion process, *T. solanivora* colonized many zones where the related species *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) was already established. The potato tuber moth *P. operculella* is an important pest for potato and is present worldwide under warm climates, both in field and in storage facilities (Sporleder et al., 2005). Nowadays, both species can be found in the same localities at different relative abundance according to local factors (Dangles et al., 2008). No specific chemical insecticides are available for the control of *T. solanivora*. Different molecules had been used but their efficacy has not been precisely

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quantified and they could cause adverse effects on beneficial organisms and environment (Arévalo and Castro, 2003). Furthermore, toxic residues can be found on potatoes (Benavides, 2000) and pesticides could generate resistance in insects. In this context, the use of biological control agents, and particularly insect viruses belonging to the family *Baculoviridae* (Baculoviruses), can be viewed as an interesting option to control these insects and to overcome the problems previously mentioned. *Phthorimaea operculella granulovirus* (PhopGV) (*Betabaculovirus*, Jehle et al., 2006) was first reported from *P. operculella* collected from Sri Lanka (Reed, 1969) but various isolates were found later in *P. operculella* populations from other countries (Vickers et al., 1991; Léry et al., 2005). PhopGV efficiently controls and prevents *P. operculella* proliferation under storage conditions without inducing any harm to beneficial organisms (Kroschel et al., 1996; Vickers et al., 1991). Several geographical *graulovirus* isolates were also found on *T. solanivora*. They appear to be genetically close to PhopGV (Espinel-Correal et al., 2010). Tests of some of these isolates on their original host species showed diverse efficacy levels (Espinel-Correal et al., 2010). Currently, the use of PhopGV-based insecticides is promoted by the International Potato Center (Lima-Peru) (CIP/IPC, 1992). We previously showed that the Colombian isolate VG003 PhopGV exhibited promising biological and technological characteristics for being used in the formulation of a viral biopesticide against *T. solanivora* (Chaparro et al., 2010). Furthermore, a significantly higher pathogenicity was observed in both *P. operculella* and *T. solanivora* when PhopGV isolates corresponding to a natural mixture of viral genotypes were used (Espinel-Correal et al., 2010), when compared to control (unmixed) isolates. Accordingly, virus isolates from either *T. solanivora* or *P. operculella* which exhibited apparent homogeneous genotypes appeared as more adapted to their respective original host as they had a lower efficacy on the other susceptible species (Espinel-Correal et al., 2010).

Co-infection or mixed infections caused by different genotypes of a single viral species or even by different species of viruses are frequently observed in insect-pathogen interactions. As a consequence, antagonism or synergism can occur between these genotypes or viral species. These interactions can modify biological parameters such as pathogenicity, virulence or virus yield, among others. In some occasions, the mixture of viral genotypes caused an increase of efficacy (Frank, 2003; López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simon et al., 2005) but in other cases, virulence was reduced (Arends et al., 2005; Muñoz et al., 1998; Muñoz and Caballero, 2000).

Considering these results, our study examines the comparative efficacy of experimental mixtures of two PhopGV isolates (Peru and VG003) adapted to the two hosts *P. operculella* and *T. solanivora*, respectively. We also followed the evolution of pathogenicity through successive passages of various mixture ratios. Previous works (Léry et al., 2005, 2008) showed that some regions of the PhopGV genome exhibited significant sequence variation between isolates. Thus, we analyzed the evolution of the genetic composition of mixed viral populations through replication cycles using the genotypic marker *egt* gene. The correlation between the allelic frequency of this gene and efficacy was examined. The potential of mixture of PhopGV isolates as active ingredients of a biopesticide for the potato moths and others Gelechiidae pests is discussed.

2. Materials and methods

2.1. Insect and viruses

P. operculella larvae were obtained from a laboratory colony maintained during more than 10 years in the research facility of the Institut de Recherche pour le Développement (IRD) in Saint-

Christol-lez-Alès, France. The colony, originated from Egypt, was reared continuously on potato tubers (v. *Agata*) at 27 ± 1 °C, $70 \pm 5\%$ RH and 18L: 6D h photoperiod. *T. solanivora* larvae were multiplied in a laboratory colony of the Center of Biotechnology and Bioindustry of the Colombian Corporation for Agricultural Research (CORPOICA), in Colombia. *T. solanivora* were reared on potato tubers (v. *Parda pastusa*) at 28 ± 2 °C, $70 \pm 5\%$ RH and 16L: 8D h photoperiod. The viruses used in this study were: (A) a Peruvian PhopGV (named Peru) isolated from *P. operculella* (gift from International Potato Center), and molecularly similar to isolate 1346 (data not shown), the completely sequenced PhopGV (Genbank accession: NC 004062; (Croizier et al., 2004) and (B) a Colombian PhopGV (named VG003) isolated from *T. solanivora* larvae. Each virus isolate was amplified in its original host species. The two isolates differed in their restriction patterns and the nucleotide sequence of different variable regions (Espinel-Correal et al., 2010). For experiments, virus occlusion bodies (OBs) of each isolate used in the study were purified on continuous 30–70% (wt/vol) sucrose gradient and quantified as previously described (Zeddiam et al., 2003), using the following formula: $6.8 \times 10^8 \times OD_{450} \times \text{dilution} = \text{number of granules ml}^{-1}$.

2.2. Biological tests and statistical analysis

Biological tests were performed to evaluate the possible changes occurring in sequentially passaged mixtures of the two virus isolates. Bioassays to determine the lethal concentrations (LC₅₀ and LC₉₀) of mixed virus isolates were carried out using neonate larvae (L1) of *P. operculella* and *T. solanivora*. Three experimental populations were constructed by mixing OBs of isolates Peru (A) and VG003 (B) at the following proportions: 10%A + 90%B (Mix 10A:90B), 50%A + 50%B (Mix 50A:50B) and 90%A + 10%B (Mix 90A:10B). Eight concentrations (ranging from 1.5×10^4 to 5×10^9 OBs in 2 ml distilled water) of each A + B mix were applied homogeneously to potato tubers using a nebulization chamber as previously described (Carrera et al., 2008; Espinel-Correal et al., 2010). The calculated OB concentrations laid onto the potato surface were respectively 0.015, 0.05, 0.15, 0.5, 5, 50, 500 and 5 000 OBs/mm². Two potato tubers were used for each virus concentration. Three to five replicates per concentration were set up, each consisting of 10 and 15 neonate larvae per tuber for *T. solanivora* and *P. operculella*, respectively. Infected and dead larvae were collected and mortality data was submitted to Probit analysis using PLO-PLUS 1.0 (LeOra Software, 2002–2010) and Statplus 4.9 (Analystsoft 2007). The first application of the A + B mix (either in *P. operculella* or in *T. solanivora*) was considered as passage zero (P0). OBs from P0-treated larvae were purified and applied to a new batch of larvae (passage P1) of the same species it was purified from. Again, purified OBs from P1 were used in a new bioassay (P2). For *P. operculella*, a third and a fourth passages (P3 and P4) were carried out on 120 larvae. Then, OBs from P4 were purified and used for a last bioassay (P5). For *T. solanivora*, OBs purified from P2 were used in a bioassay (P3) (Fig. 1).

The bioassays described above allowed assessing the biological efficacy of successive passages of viral populations in a single host. The fact that both hosts can be present in the same localities in Colombia could be a key point to explain how viral diversity is maintained. In order to know whether efficacy remains stable after host shifts, viral population 50A:50B P0 amplified in *P. operculella* was tested on *T. solanivora*. OBs recuperated at the end of this infection were named “P1 Mix Retro”.

2.3. Relative dosage of PhopGV isolates by semiquantitative PCR

A region located within viral ORF 129 (*egt* gene) was used as the marker which allowed univocal identification of each isolate (either

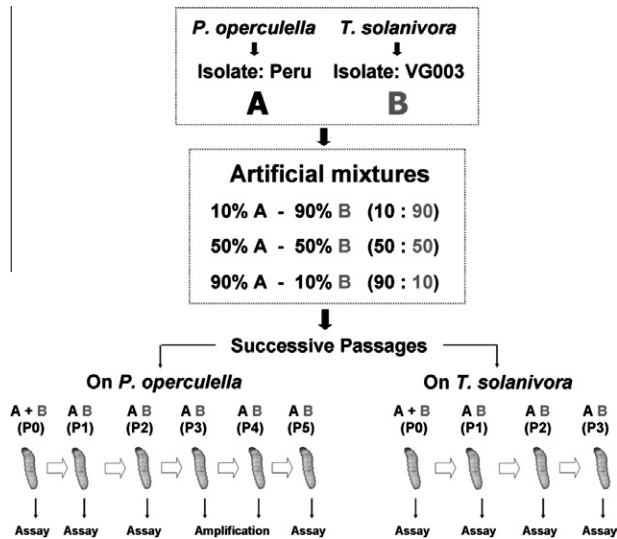


Fig. 1. Diagram of experimental virus mixtures of PhopGV isolates Peru (A) and VG003 (B). The three mixtures (10:90, 50:50 and 90:10) were evaluated by bioassays in neonates. P: Passage number.

Peru or VG003) (Espinel-Correal et al., 2010). Briefly, viral DNA was extracted from larvae applied with virus mixtures having concentrations comprised between 50 OBs/mm² and 5,000 OBs/mm² (equivalent to LC₇₀ – LC₁₀₀). The diagnostic region of the *egt* gene was amplified with the primer pair: PhopGV129-7: 5'-CGAGTC-GAGCCAATTTGTTTGCCTAAT-3' and PhopGV129-2: 5'-TGCCTGCTGTGCTCGACAACAATAGACC-3' (116714 to 116741 and 117401 to 117374, on the complementary strand, respectively). Cycling conditions were 95 °C for 5 min, 20 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1 min, and 73 °C for 5 min. PCR products were separated by electrophoresis at 150 V during 90 min in 2% agarose gel in TAE (0.04 M Tris, 0.1% Acetic acid, 0.001 M EDTA, pH 8.0) buffer. PCR-amplified products (474 and 774 bp for Peru and VG003, respectively) deriving from gene *egt* were visualized and photographed under a UV light after ethidium bromide staining. The relative proportions of PCR products present in progeny virus gathered after mixed infections were then estimated by densitometric analysis using the PhotoCapt MW program, V.10.01 (Vilber Loumat, France). Reactions and measurements of product intensities were performed twice.

3. Results and discussion

3.1. Biological activity of virus mixtures in *P. operculella* and *T. solanivora* larvae

For *P. operculella* (Table 1), the probit analysis indicated a significantly higher pathogenicity of the three experimental populations (10A:90B, 50A:50B and 90A:10B) at passage 0 when compared to isolate Peru ($\chi^2 = 83.9$; df = 6; $P < 0.05$) and VG003 ($\chi^2 = 167$; df = 6; $P < 0.05$), individually applied to the same insect species. The decreases in LC₅₀ of the experimental virus populations were between 2.8 and 23.6-fold compared to the isolate Peru which is considered as well adapted to this host species. Most of the times, a variation of pathogenicity was observed through successive passages of the same experimental population. The more variable was 10A:90B with LC₅₀ being 45-fold higher for P2 than for P3. Meanwhile, maximum variations of LC₅₀ between two passages were around 2.5-fold and 4-fold for 50A:50B and 90A:10B, respectively.

For *T. solanivora* larvae (Table 2), a similar pattern was obtained i.e. the pathogenicity of mixed experimental populations at P0 was

significantly higher than the pathogenicity of the two isolates tested individually, i.e. VG003 ($\chi^2 = 25.5$; df = 6; $P < 0.05$) and Peru ($\chi^2 = 56.6$; df = 6; $P < 0.05$) (Table 2). On the other hand, the efficacies of all experimental populations were lower in this host compared to those obtained in *P. operculella*. This might be related to the smaller size of *P. operculella* larvae. Mix 10A:90B presented the lowest efficacy in first passages, and no significant differences were seen between 50A:50B and 90A:10B (P0: $\chi^2 = 3.94$; df = 2; $P = 0.140$. P1: $\chi^2 = 0.35$; df = 2; $P = 0.841$. P2: $\chi^2 = 4.35$; df = 2; $P = 0.113$. P5: $\chi^2 = 0.67$; df = 2; $P = 0.717$). Changes in LC₅₀ through successive passages differ according to the virus population considered. Indeed, 10A:90B evolved toward a higher efficacy and 90A:10B to a lower efficacy. 50A:50B appeared more stable through passages as it only exhibited limited (inferior to 2-fold) variations. In the perspective to manage simultaneously the two pest species, a higher efficacy of the virus on both hosts is desirable. For 50A:50B at P0, a similar efficacy was found in both hosts ($\chi^2 = 5.11$; df = 2; $P = 0.077$). It was not the case for the two populations (10A:90B: $\chi^2 = 10.40$; df = 2; $P = 0.005$. 90A:10B: $\chi^2 = 9.20$; df = 2; $P = 0.010$).

As a general rule, viral populations comprising a mixture of PhopGV genotypes (Peru and VG003) in different proportions showed an increased efficacy on both hosts compared to isolates isolated from, and multiplied in their respective host. In spite of fluctuations, the general pattern is that this higher pathogenicity of the mixtures is verified whatever the number of passages considered.

In the shift host assay (OBs produced on *P. operculella* and tested on *T. solanivora*), the efficacy of “Mix Retro” was 6.6-fold higher ($\chi^2 = 20.89$; df = 2; $P < 0.05$) than the P1 of 50A:50B on *T. solanivora* (Table 3). This result shows that the pathogenicity of 50A:50B increased independently of the host tested (comparison of lines 1 and 3 from Table 3). Also, the shift to an alternative host did not affect the pathogenicity of the virus (comparison of lines 1 and 4 of Table 3). However, these results should be confirmed by testing the virus mixtures through several amplification cycles on both hosts. The fact that we previously found a higher efficacy of isolates naturally mixed VG001 and VG005 in both hosts (Espinel-Correal et al., 2010), lead us to hypothesize that there could be a correlation between the cyclic host change and the efficacy of isolates. This observation is important to be considered for the viral biopesticide production as it can be envisaged to produce the virus in the more suitable host which might not necessarily be the final target species.

There are several examples of interactions during co-infections among viral genotypes of different species or variants of the same viral species. Genotype interactions may be present throughout the viral cycle, from entry into the larvae to replication within cells. Synergistic factors named “enhancing factors” that improves efficacy in mixture infections (Li et al., 2003) were identified in granuloviruses (GVs), (*Anticarsia gemmatalis* GV (AgGV); *Trichoplusia ni* GV (TnGV); *Xestia c-nigrum* GV (XecnGV)) and some nucleopolyhedroviruses (NPVs) (*Lymantria dispar* MNPV (LdMNPV); *Mamestra configurata* NPV (MacoNPV). Enhancins are present in the OBs and appears to target the digestion of the peritrophic membrane facilitating access of virions to the intestinal cells. Mixture of OBs that contains or not enhancins allows transit of all genotypes. For example, LdMNPV encodes two enhancins, deletion of either results in a 2- to 3-fold reduction in potency, whereas deletion of both causes a 12-fold reduction (Rohrmann, 2008). Synergism phenomena had been observed in *Helicoverpa zea* (Boddie), *Spodoptera exigua* (Hübner) and *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) during a simultaneous infection of a LdMNPV and NPVs from these insects, HzMNPV, SeMNPV and SfMNPV; the potency increased 8.7-, 7.5- and 10.3-fold (Shapiro and Shepard, 2006). In a SfMNPV from Nicaragua, cooperation among different genotypes

Table 1
Biological activity through sequential passages of experimental mixtures of Peru and VG003 isolates in *P. operculella* larvae.

| Population | No. of OBs/mm ² (95% CI) | | Slope ± SE | χ^2 | df | P |
|---------------------|-------------------------------------|----------------------------|---------------|----------|----|------|
| | LC ₅₀ | LC ₉₀ | | | | |
| Peru | 6.62 (4.5–9.48) | 258.74 (155.05–491.42) | 0.75 ± 0.10 | 3.73 | 3 | 0.80 |
| VG003 | 26.19 (17.2–39.3) | 2558.35 (1317.82–5944.00) | 0.68 ± 0.04 | 1.69 | 5 | 0.89 |
| Mix 10:90 Passage 0 | 2.36 (1.13–4.93) | 3837.79 (1016.30–24413.73) | 0.399 ± 0.03 | 0.86 | 6 | 0.99 |
| Mix 10:90 Passage 1 | 0.57 (0.13–1.19) | 764.25 (148.50–9767.19) | 0.410 ± 0.03 | 1.39 | 4 | 0.84 |
| Mix 10:90 Passage 2 | 7.15 (5.10–18.3) | 8770.16 (2032.04–71614.92) | 0.431 ± 0.04 | 1.17 | 6 | 0.97 |
| Mix 10:90 Passage 5 | 0.16 (0.04–0.45) | 738.62 (120.17–18166.00) | 0.351 ± 0.03 | 2.9 | 6 | 0.82 |
| Mix 50:50 Passage 0 | 1.23 (0.41–2.60) | 164.55 (77.11–510.05) | 0.603 ± 0.05 | 2.53 | 6 | 0.86 |
| Mix 50:50 Passage 1 | 0.53 (0.22–1.17) | 275.37 (80.89–1609.30) | 0.472 ± 0.03 | 5.26 | 6 | 0.51 |
| Mix 50:50 Passage 2 | 0.91 (0.34–2.26) | 999.02 (232.28–8969.22) | 0.422 ± 0.03 | 2.77 | 6 | 0.84 |
| Mix 50:50 Passage 5 | 1.48 (0.82–2.67) | 1447.28 (509.86–5689.75) | 0.429 ± 0.03 | 1.05 | 6 | 0.98 |
| Mix 90:10 Passage 0 | 0.28 (0.12–0.55) | 182.77 (68.32–690.35) | 0.456 ± 0.04 | 0.23 | 6 | 0.99 |
| Mix 90:10 Passage 1 | 0.30 (0.14–0.57) | 164.21 (70.33–492.09) | 0.470 ± 0.031 | 0.36 | 6 | 0.99 |
| Mix 90:10 Passage 2 | 0.10 (0.03–0.24) | 362.57 (93.84–2748.62) | 0.362 ± 0.042 | 0.21 | 5 | 0.99 |
| Mix 90:10 Passage 5 | 0.43 (0.14–1.10) | 12714 (1512.30–466960.00) | 0.346 ± 0.035 | 2.49 | 5 | 0.87 |

OB: occlusion bodies, CI: confidence interval, χ^2 : chi square. df: degrees of freedom.

Table 2
Biological activity through sequential passages of experimental mixtures of Peru and VG003 isolates in *T. solanivora* larvae.

| Population | No. of OBs/mm ² (95% CI) | | Slope ± SE | χ^2 | df | P |
|---------------------|-------------------------------------|-----------------------------|---------------|----------|----|------|
| | LC ₅₀ | LC ₉₀ | | | | |
| Peru | 30.11 (18.66–50.28) | 13806.38 (4950.04–54299.40) | 0.482 ± 0.039 | 3.73 | 6 | 0.71 |
| VG003 | 6.93 (4.89–9.80) | 423.64 (236.25–883.72) | 0.718 ± 0.049 | 1.53 | 6 | 0.96 |
| Mix 10:90 Passage 0 | 5 (3.36–7.38) | 600.70 (237.88–2278.64) | 0.616 ± 0.065 | 0.70 | 6 | 0.99 |
| Mix 10:90 Passage 1 | 12.29 (6.32–26.49) | 1080.20 (304.72–9288.60) | 0.712 ± 0.086 | 0.50 | 4 | 0.99 |
| Mix 10:90 Passage 2 | 5.82 (3.82–8.87) | 118.02 (62.95–285.05) | 0.989 ± 0.097 | 0.59 | 6 | 0.99 |
| Mix 10:90 Passage 3 | 2.19 (0.73–5.38) | 93.77 (28.28–1042.90) | 0.799 ± 0.087 | 4.4 | 6 | 0.62 |
| Mix 50:50 Passage 0 | 2.66 (1.86–3.70) | 136.36 (79.03–273.23) | 0.750 ± 0.078 | 0.89 | 6 | 0.98 |
| Mix 50:50 Passage 1 | 4.13 (1.97–8.44) | 431.23 (129.03–3498.10) | 0.672 ± 0.089 | 0.54 | 5 | 0.99 |
| Mix 50:50 Passage 2 | 3.66 (2.27–5.85) | 159.94 (74.08–487.90) | 0.799 ± 0.080 | 0.24 | 5 | 0.99 |
| Mix 50:50 Passage 3 | 4.72 (2.93–7.61) | 216.09 (98.22–677.14) | 0.793 ± 0.078 | 1.54 | 5 | 0.96 |
| Mix 90:10 Passage 0 | 1.25 (0.77–1.90) | 155.03 (82.52–353.92) | 0.624 ± 0.072 | 0.22 | 6 | 0.99 |
| Mix 90:10 Passage 1 | 3.18 (1.43–6.60) | 446.23 (126.94–4127.60) | 0.637 ± 0.085 | 0.35 | 6 | 0.99 |
| Mix 90:10 Passage 2 | 3.65 (2.50–5.31) | 60.10 (33.85–133.54) | 1.056 ± 0.104 | 0.55 | 6 | 0.99 |
| Mix 90:10 Passage 3 | 6.17 (3.86–9.99) | 257.86 (116.33–816.36) | 0.813 ± 0.079 | 0.62 | 6 | 0.99 |

OB: occlusion bodies, CI: confidence interval, χ^2 : chi square. CI, confidence interval, df: degree of freedom.

Table 3
Biological activity of “Mix Retro” in *T. solanivora* larvae compared to efficacies of Mix 50:50 in *P. operculella* (at the first and second passages P0 and P1) and in *T. solanivora* (at the second passage P1) larvae.

| Population | No. of OBs/mm ² (95% CI) | | Slope ± SE | χ^2 | df | P |
|-------------------------------------|-------------------------------------|-------------------------|---------------|----------|----|------|
| | LC ₅₀ | LC ₉₀ | | | | |
| Mix Retro in <i>T. solanivora</i> | 0.62 (0.29–1.08) | 41.55 (19.44–132.73) | 0.701 ± 0.09 | 1.91 | 5 | 0.85 |
| 50:50 (P0) in <i>P. operculella</i> | 1.23 (0.41–2.60) | 164.55 (77.11–510.05) | 0.603 ± 0.05 | 2.53 | 6 | 0.86 |
| 50:50 (P1) in <i>P. operculella</i> | 0.53 (0.22–1.17) | 275.37 (80.89–1609.30) | 0.472 ± 0.03 | 5.26 | 6 | 0.51 |
| 50:50 (P1) in <i>T. solanivora</i> | 4.13 (1.97–8.44) | 431.23 (129.03–3498.10) | 0.672 ± 0.089 | 0.54 | 5 | 0.99 |

OB: occlusion bodies, CI: confidence interval. χ^2 : chi square. df: degree of freedom.

was proved (Frank, 2003). The efficacy of the viral population composed of at least nine genotypes was higher than those of each isolated genotype. Thus, the infection of pure genotype SfNIC-B corresponded to 30% of efficacy of the isolate of origin. Co-infection (multiplication in the same cell) with SfNIC-C genotype leads to a higher efficacy reaching the level of the original population (López-Ferber et al., 2003; Simon et al., 2005). However, genotype mixtures did not guarantee a higher efficacy. In the population there may exist “parasitic” variants (defective genotypes) that often use gene products from the complete genotype for its replication, as it was found in a SeMNPV isolate (Muñoz et al., 1998; Muñoz and Caballero, 2000). Arends et al. (2005) found that infections caused by a mixture of *Cydia pomonella granulovirus* (CpGV) genotypes, had a reduced pathogenicity in its natural host compared to some isolated genotypes.

3.2. Influence of the proportion of alleles

One of the polymorphic markers differentiating the two virus isolates is located in PhopGV ORF 129 that correspond to the *egt* gene (Croizier et al., 2004). The *egt* gene (ecdysteroid glucosyltransferase) encodes an enzyme responsible for the inactivation of the ecdysone in the insect, which induces a cessation of metamorphosis, keeps the individual in its larval state and increases the yield of the progeny virus (O’Reilly, 1999; Piñedo et al., 2003). The *egt* gene deletion or inactivation leads to an increased virulence in other baculoviruses (Bonning and Hammock, 1996; O’Reilly and Miller, 1991; O’Reilly, 1995). Accordingly, we have explored if a relationship could be established between the changes in the marker frequency and the efficacy of the experimental populations.

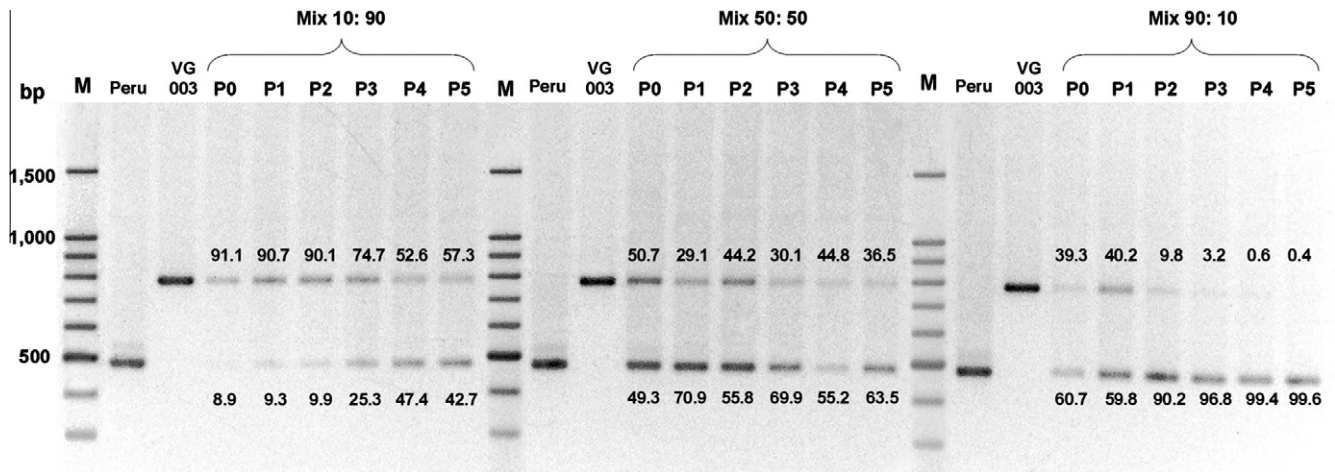


Fig. 2. Semi-quantitative PCR of PhopGV *egt* gene after sequential passages of experimental mixtures of Peru and VG003 viral isolates on *P. operculella* larvae. Proportions (in%) of *egt* alleles are indicated in their respective columns. M: Marker in base pairs. P: Passage number.

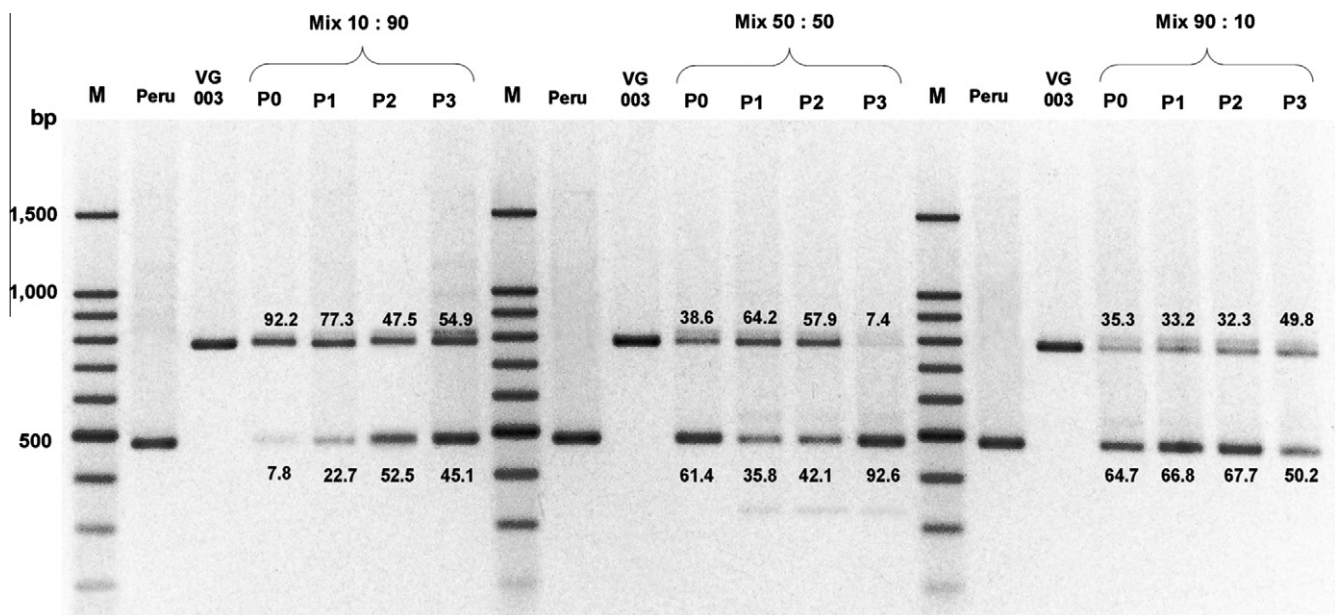


Fig. 3. Semi-quantitative PCR of PhopGV *egt* gene after sequential passages of experimental mixtures of Peru and VG003 viral isolates on *T. solanivora* larvae. Proportions (in%) of *egt* alleles are indicated in their respective columns. M: Marker in base pair. P: Passage number.

The changes on the relative proportions of each allele (corresponding to amplified bands of 474 and 774 bp, respectively) of the *egt* gene, was assessed by semi-quantitative PCR (Figs. 2 and 3). No general pattern was identified in this evolution in regard to the host, to the passage number or to the starting frequencies. In *P. operculella*, 10A:90B exhibited a progressive and marked decline of the more concentrated component (corresponding to the *egt* sequence from Colombian VG003). At the contrary, with 90A:10B, there was a strong reduction of the Peruvian component at the first passage but, after the third one, it largely overwhelmed the other component. Finally, in 50A:50B, the proportions of the two components were somewhat equilibrated over passages. In *T. solanivora*, the results were very different. In 10A:90B, there was a progressive decrease of the Colombian component and the population almost reached a 50–50 equilibrium at the third and fourth passages. With 50A:50B, there was a very drastic drop in the relative proportion of the Colombian component at the fourth passage. Conversely, with 90A:10B, there was a 3-fold increase of the Colombian component at the first passage and then, the

relative proportions of the two components stayed more or less constant around a 50–50 equilibrium.

Baculoviruses are known for their high recombination potential. In mixed infections, like those under study, multiple recombinations are likely to occur at each replication cycle, making it difficult to find a RFLP marker linked to the increasing efficiency. Only the analysis of the complete sequences of various genotypes at each passage might give information on genomic changes underlying the increase in efficacy.

The incidence of genotype mixture in pathogen fitness is determined by the interactions of pathogen genotype × host genotype × environment affected by the competition for host resources or immune response (Seppälä et al., 2009). The efficacy could be increased in mixed genotype infections (Hodgson et al., 2004). In general, the virulence or pathogenicity has a key position as an explanation of the variable response in host–pathogen theoretical models. The classical theory predicts that the competition among isolates (genotypes), will generally favor the selection of isolates of higher virulence (Balmer et al., 2009). Moreover, the conventional

theory of adaptive virulence says that an infection caused by mixed genotypes has a high impact on host reproductive efficacy (e.g. pathogenicity) and increases the rate of use of host resources (e.g. higher productivity/time). However, in infections for which host death is essential for transmission, as it is the case for baculoviruses, the productivity has to be maximized. This is an example in which the trade-off between exploitation rate and virulence is not valid (Hodgson et al., 2004).

The study of host-pathogen interactions based on the model PhopGV-potato moths is of high importance, from both an economic and an evolutionary point of view. PhopGV interacts with a moth species complex representing a challenge for viral adaptive conditions. Genotype flexibility of these isolates for adaptation to alternative hosts could permit to envisage its possible use against other pests of the same family (Gelechiidae). The tomato moth *Tuta absoluta* (Povolny), a native insect of Central and South America, but also present as an invasive pest in Mediterranean Countries of Europe and Africa could be one of these targets as it is also susceptible to PhopGV (Zeddám, pers. comm.). This pest is recommended for regulation in the list A1 by the European and Mediterranean Plant Protection Organization (EPPO) (Germain et al., 2009).

4. Conclusions

Based on biological assays, we conclude that the genotypic heterogeneity in PhopGV experimentally mixed isolates determined their efficacy. Moreover, the three experimental populations showed a higher pathogenicity than isolates tested separately. Apparently, virus passages did not seem to affect the higher efficacy of mixtures over time, at least in the first 3 (*T. solanivora*) or 5 (*P. operculella*) passages. The possibility relying on a viral population active against both insect hosts increases the options for the development of biopesticides to control these pests. This approach might be useful for other related pests. Obviously, other factors have to be analyzed in order to ensure the viability of an industrial production. In particular, virus yield obtained in each potential host will be a key point to assess.

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