

A Novel Binary Mixture of *Helicoverpa armigera* Single Nucleopolyhedrovirus Genotypic Variants Has Improved Insecticidal Characteristics for Control of Cotton Bollworms

Maite Arrizubieta,^a Oihane Simón,^a Trevor Williams,^b Primitivo Caballero^{a,c}

Bioinsecticidas Microbianos, Instituto de Agrobiotecnología, CSIC-UPNA, Gobierno de Navarra, Mutilva Baja, Navarra, Spain^a; Instituto de Ecología AC, Xalapa, Veracruz, Mexico^b; Laboratorio de Entomología Agrícola y Patología de Insectos, Departamento de Producción Agraria, Universidad Pública de Navarra, Pamplona, Navarra, Spain^c

The genotypic diversity of two Spanish isolates of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) was evaluated with the aim of identifying mixtures of genotypes with improved insecticidal characteristics for control of the cotton bollworm. Two genotypic variants, HearSP1A and HearSP1B, were cloned *in vitro* from the most pathogenic wild-type isolate of the Iberian Peninsula, HearSNPV-SP1 (HearSP1-wt). Similarly, six genotypic variants (HearLB1 to -6) were obtained by endpoint dilution from larvae collected from cotton crops in southern Spain that died from virus disease during laboratory rearing. Variants differed significantly in their insecticidal properties, pathogenicity, speed of kill, and occlusion body (OB) production (OBs/larva). HearSP1B was ~3-fold more pathogenic than HearSP1-wt and the other variants. HearLB1, HearLB2, HearLB5, and HearLB6 were the fastest-killing variants. Moreover, although highly virulent, HearLB1, HearLB4, and HearLB5 produced more OBs/larva than did the other variants. The co-occluded HearSP1B:LB6 mixture at a 1:1 proportion was 1.7- to 2.8-fold more pathogenic than any single variant and other mixtures tested and also killed larvae as fast as the most virulent genotypes. Serial passage resulted in modified proportions of the component variants of the HearSP1B:LB6 co-occluded mixture, suggesting that transmissibility could be further improved by this process. We conclude that the improved insecticidal phenotype of the HearSP1B:LB6 co-occluded mixture underlines the utility of the genotypic variant dissection and reassociation approach for the development of effective virus-based insecticides.

Alphabaculoviruses (*Baculoviridae*) are lepidopteran-specific nucleopolyhedroviruses (NPVs) that have been used successfully as biological control agents against several agricultural and forest pests (1). These viruses are characterized by high intraspecific heterogeneity not only between isolates from different geographic regions (2, 3) but also within single isolates that often comprise mixtures of several genotypes present in different proportions (4–8). The molecular heterogeneity of genotypes is often associated with phenotypic differences in pathogenicity, speed of kill, and virus production (8, 9), which are traits of practical importance for the use of these viruses as biological insecticides (10). Infections involving mixtures of genotypes can result in positive (11–14) or negative (15–18) effects on a number of these traits.

As natural alphabaculovirus populations in virus-killed insects comprise mixtures of genotypes, the genotypic interactions that modulate the insecticidal characteristics have attracted the attention of insect pathologists (8, 19–21) and have been used to develop novel products based on unique user-defined genotypic combinations in specific user-defined proportions that result in improved insecticidal properties of the final mixture (22, 23). This has resulted in the generation of a novel paradigm for the development of baculovirus-based insecticides, in which isolates are first dissected into their individual genotypes, which are subjected to a phenotype screening process involving insect bioassay techniques, and then assembled into novel mixtures of genotypes and tested for their insecticidal characteristics against the target pest.

The cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae), is a major polyphagous pest that is distributed across the Old World and Oceania and is in the process of invading South America (24, 25). *H. armigera* single NPV (HearSNPV) (genus *Alphabaculovirus*) has been shown to be an effective agent for the

control of *H. armigera* (26–31). Several isolates of HearSNPV have been characterized at the molecular and phenotypic levels (32–35), including some isolates from the Iberian Peninsula (27, 36; M. Arrizubieta, O. Simón, T. Williams, and P. Caballero, submitted for publication). Genotypic diversity within field isolates of HearSNPV has been demonstrated by cloning the genotypic variants *in vivo* (37), *in vitro* (34), or by using bacterial artificial chromosome (BAC) technology (38). Moreover, various isolates of this virus have been commercialized as the basis for biological insecticides in different parts of the world (39).

A Spanish field isolate of HearSNPV obtained from virus-killed larvae collected at Guadajira, Badajoz, Spain, named HearSNPV-SP1 (HearSP1), showed a suitable insecticidal phenotype, in terms of occlusion body (OB) pathogenicity, speed of kill, and OB productivity, compared with isolates from other parts of the Iberian Peninsula (36). Some of the genotypes present in the HearSP1 isolate were identified as the active ingredient for a potential biological insecticide (40). Additional HearSNPV isolates

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Address correspondence to Primitivo Caballero, pcm_92@unavarra.es.

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were obtained from *H. armigera* larvae that died during rearing of a laboratory colony established from larvae collected from cotton crops in Lebrija, southern Spain; these were named HearSNPV-LB (HearLB) isolates. These isolates were found to differ in their insecticidal characteristics during routine screening.

In the present study, we applied the isolate dissection and genotype reassembly model to the study of the genotypic variants present in the HearSP1 and HearLB isolates. The insecticidal properties of a number of different mixtures of the component genotypes were evaluated in the laboratory. Finally, the genetic and biological stability of the selected genotypic mixture was evaluated following five successive passages *in vivo*.

MATERIALS AND METHODS

Insect rearing, insect cells, and HearSNPV isolates. The *H. armigera* colony was established with pupae received from the Centre for Ecology and Hydrology (NERC-CEH), Oxford, United Kingdom, and was maintained at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with 70% \pm 5% relative humidity and a 16-h/8-h light/dark photoperiod on a semisynthetic diet (41). The HzAM1 cell line (42) was maintained at $28^{\circ}\text{C} \pm 2^{\circ}\text{C}$, using Ex-Cell 420 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Lonza, WA, USA). The HearSNPV-SP1 (HearSP1) isolate was originally obtained from diseased larvae from tomato crops in Guadajira, Badajoz, Spain. HearSP1 was included as a reference isolate in this study because it was previously identified as a highly pathogenic isolate in terms of concentration-mortality metrics (27, 36). From a total of 17 HearSNPV isolates obtained from individual larvae that died of polyhedrosis disease during rearing of *H. armigera* larvae collected from cotton crops in Lebrija, Spain, six different EcoRI restriction endonuclease profiles were identified. These variants were named HearLB1 to HearLB6.

Isolation of HearSNPV genotypic variants. (i) Isolation from the HearSP1 isolate. The isolation of individual genotypes of HearSP1 was performed once by a plaque assay. For this, the hemolymph from 25 *H. armigera* fourth-instar larvae previously infected with 10^9 OBs/ml was taken by bleeding at 48 h postinoculation (p.i.) and filtered through a 0.45- μm -pore-size filter. Six-well tissue culture plates were seeded with 1.5×10^6 HzAM1 cells/well and incubated at 27°C for 3 h with Ex-Cell 420 medium supplemented with 1% penicillin-streptomycin (Lonza). The medium was removed, and 0.1 ml of serial 10-fold dilutions (from 1 to 10^{-5}) of the filtered hemolymph was inoculated onto cells. After 1 h, the inoculum was removed, and 2 ml of Ex-Cell 420 medium supplemented with 10% FBS, 2% (wt/vol) SeaPlaque agarose (Lonza), and antibiotics was added to each well. Once the agarose had solidified, it was overlaid with 1 ml of Ex-Cell 420 medium supplemented with 10% FBS and the antibiotics. After 7 days, well-isolated plaques were examined microscopically for the presence of OBs within the cells. This was necessary as cell adhesion was occasionally poor, requiring each plaque to be confirmed by microscopic inspection. Virus-positive plaques were picked individually with a sterile Pasteur pipette and transferred into a vial containing 0.1 ml of PBS (phosphate-buffered saline). Finally, 10 *H. armigera* fourth-instar larvae were intrahemocoelically injected with 8 μl of each plaque suspension and then reared individually on a semisynthetic diet. Mortality was recorded daily, and virus-killed larvae were individually transferred into a microcentrifuge tube and stored at -20°C until required.

(ii) Isolation from HearLB isolates. Due to difficulties with cell adhesion and the need to check plaques by microscopy, the purification of genotypes from the 17 diseased cadavers from Lebrija was performed by endpoint dilution (EPD). The hemolymph of 25 fourth-instar insects previously infected with 10^9 OBs/ml of each isolate was taken at 48 h postinoculation, filtered through a 0.45- μm -pore-size filter, and serially diluted using 5-fold dilutions (1.0 to 2.56×10^{-6}) in Ex-Cell 420 medium supplemented with 10% FBS and 1% antibiotics. A 1-ml volume of each dilution was mixed with 9 ml of 2×10^5 HzAM1 cells/ml, and a 100- μl volume of the resulting suspension was placed into each well of a 96-well

plate, leaving the last two wells of each row as negative controls, containing cells without virus. Plates were incubated at 28°C . The assay was performed four times to produce a sufficiently large sample size to facilitate detection of possibly rare genotypic variants. Seven days later, wells were observed to determine the presence of infected cells. The supernatants of plates infected with dilutions that produced $\leq 10\%$ infected wells, which meant that almost all wells were infected by a single budded virion, i.e., a single genotype (43), were removed individually by using a sterile Pasteur pipette, and volumes of 8 μl were injected into groups of 10 *H. armigera* fourth-instar insects that were reared individually on a semisynthetic diet until death.

(iii) OB purification. OBs were extracted from virus-killed larvae as described previously (36). Briefly, individual cadavers were homogenized in 300 μl water, filtered through a piece of muslin, washed with 500 μl of 0.1% sodium dodecyl sulfate (SDS), and then washed twice in distilled water. The resulting OBs were resuspended in double-distilled water and stored at -20°C .

Restriction endonuclease analysis of genotypic variants. Genotypic variants were subjected to restriction endonuclease analysis to determine the identity of the different genotypes. For this, DNA was extracted from OBs by incubating 100 μl of 10^9 OBs/ml with a solution containing 100 μl of 0.5 M Na_2CO_3 , 50 μl of 10% SDS, and 250 μl of distilled water at 60°C during 10 min. Debris was pelleted at $6,000 \times g$ for 5 min, and the supernatant was incubated with 25 μl proteinase K (20 mg/ml) at 50°C during 1 h. Viral DNA was extracted twice with phenol (pH 7.8) and once with chloroform, precipitated with a solution containing 10% (vol/vol) 3 M sodium acetate (pH 5.2) and ice-cold ethanol at $12,000 \times g$ for 10 min, and washed with 70% ice-cold ethanol. Finally, DNA was resuspended in 50 μl of 0.1% TE (10 mM Tris, 1 mM EDTA) and stored at 4°C until use.

Viral DNA (2 μg) was incubated with 10 U EcoRI (TaKaRa Bio Inc., Shiga, Japan) at 37°C for 4 h, and fragments were separated by electrophoresis in 1% TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) agarose gels containing 0.25 $\mu\text{g}/\text{ml}$ ethidium bromide and visualized on a GeneSnap transilluminator (Syngene, San Diego, CA, USA). DNA fragment sizes were estimated by comparison to standard molecular weight markers (HyperLadder I [Biolone] or 1 kb [Nippon]).

Biological activity of genotypic variant OBs. The biological activities of genotypic variant OBs were compared in terms of pathogenicity (expressed as OB concentration-mortality metrics [50% lethal concentration $\{LC_{50}\}$], mean time to death (MTD), and OB production (OBs/larva) in *H. armigera* second-instar insects inoculated by using the droplet feeding method (44). The wild-type HearSP1 (HearSP1-wt) isolate was included as a reference isolate.

Concentration-mortality responses were determined by using five OB concentrations of each variant that were estimated to result in a range of mortality rates of between ~ 5 and $\sim 95\%$ (36). Groups of 24 second-instar insects that had molted in the previous 12 h were inoculated with each OB concentration in each replicate; larvae that consumed the inoculum within 10 min were individually transferred into 25-ml cups with diet and reared at $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ in darkness. Control larvae were inoculated with an aqueous solution without OBs. Virus mortality was recorded at 24-h intervals during 10 days. The whole procedure was performed on three different occasions. Concentration-mortality data were subjected to probit analysis using the POLO-PC program (45).

Speed of kill and OB production were determined by using groups of 24 second-instar insects (< 12 h postmolting) inoculated with the corresponding LC_{90} of OBs, previously determined by a bioassay. The experiment was performed on three different occasions. Virus mortality was recorded at 8-h intervals for 10 days. Moribund insects showing signs of the final stages of polyhedrosis disease were individually transferred into microtubes, incubated at 28°C for up to 8 h until death, and subsequently stored at -20°C . Time-mortality results for individuals that died due to lethal polyhedrosis disease were subjected to Weibull survival analysis in GLIM (46). For OB counting, infected cadavers were thawed and individually homogenized in 1 ml of distilled water, and triplicate samples were

TABLE 1 Genotypic mixtures prepared in order to evaluate the influence of genotype-genotype interactions on insecticidal activities of HearSNPV

Mixture	Genotypes	Proportion
1	SP1A + SP1B	1:1
2	SP1A + SP1B	1:2
3	LB1 + LB3	1:1
4	LB3 + LB6	1:1
5	LB1 + LB3 + LB6	1:1:1
6	HearLBmix (LB1 + LB2 + LB3 + LB4 + LB5 + LB6)	4:4:6:1:1:1
7	SP1B + LB1	1:1
8	SP1B + LB6	1:1

counted by using a Neubauer improved hemocytometer. OB production values were normalized by log transformation and subjected to analysis of variance (ANOVA) and a Tukey test by using the SPSS 21.0 program.

Biological activity of genotypic mixtures. The influence of variant interactions on the insecticidal characteristics of mixed-genotype preparations was examined by producing co-occluded mixtures of variants. Co-occlusion of variants involves mixtures of variants within OBs that can be produced according to methods developed by us (20). Briefly, OBs of each genotype were mixed in the desired proportion, and 50 *H. armigera* fourth-instar insects were orally inoculated with an LC₉₀ (2.4×10^6 OBs/ml) by the droplet feeding method. Eight mixtures were prepared with the intention of examining whether particular traits of their component variants would be preserved, improved, or diminished (Table 1). Inoculated larvae were individually maintained on a semisynthetic diet at $25^\circ\text{C} \pm 1^\circ\text{C}$ until death. The resulting OBs were purified from virus-killed insects, and these OBs were considered co-occluded genotype mixtures. Their pathogenicity (LC₅₀), speed of kill (MTD), and OB production (OBs/larva) were determined as described above. All assays were performed three times, and negative controls were included to control for contamination and nonspecific mortality. OBs of each of the single HearSP1A, HearSP1B, HearLB1, HearLB3, and HearLB6 genotypes were also included as reference treatments.

Stability of a co-occluded variant mixture during passage *in vivo*. Based on the results of the study on the biological activity of variant mixtures described above, the co-occluded mixture comprising HearSP1B:LB6 (1:1) was selected and was subjected to successive passages *in vivo* in order to determine its genetic and biological stability. OBs of the initial co-occluded mixture were designated passage zero (P₀) OBs. Groups of 24 fourth-instar insects were allowed to drink a suspension of 1×10^7 OBs/ml of P₀ OBs, which resulted in 96 to 100% mortality. Following incubation and death, virus-killed insects were homogenized, and OBs were extracted and purified. These OBs, pooled among the 23 to 24 larvae that died following inoculation with P₀ OBs, were considered passage 1 (P₁) OBs and were used as the inoculum to infect the subsequent group of larvae (24 for each passage). The virus population was monitored for four additional passages (P₂, P₃, P₄, and P₅). The entire experiment was performed in triplicate. A sample of OBs produced in each replicate at each passage was used to determine the genotypic and phenotypic characteristics, as described below.

The relative frequency of each genotype at each passage was estimated by quantitative PCR (qPCR). Sequence comparison of the HearSP1B and HearLB6 complete genomes (GenBank accession numbers KJ701033 and KJ701031, respectively) (M. Arrizubieta, O. Simón, T. Williams, and P. Caballero, submitted for publication) was used to design the following set of primers and probe specific to HearLB6 in homologous region 1 (hr1): LB6-f (5'-GCACACGGCACTATTCCAA C-3'; nucleotides 22326 to 22345 in the HearLB6 genome), LB6-r (5'-ACGAACACATCCAAGACAG-3'; nucleotides 22414 to 22433), and LB6 double-quenched probe (5'-6-FAM-TAACAAATC-ZEN-ACGC

CACGCCCAAAG-3' Iowa Black FQ-3'; nucleotides 22374 to 22397 [5' 6-FAM, ZEN, and 3' Iowa Black FQ are specific probes used by Integrated DNA Technologies, Coralville, IA, USA]). Due to the unavailability of specific primers for HearSP1B, the following set of primers was designed by using conserved sequences in the *ha29* gene of HearSNPV (47): ha29.1 (5'-CTCGTATCATGCAAAACGCC-3'; nucleotides 25140 to 25159 in the HearLB6 genome and nucleotides 25212 to 25231 in the HearSP1B genome) and ha29.2 (5'-GAATCTGGCTTCGACTGGC-3'; nucleotides 25201 to 25219 in the HearLB6 genome and nucleotides 25273 to 25291 in the HearSP1B genome). The relative frequency of HearSP1B was estimated from the difference between the qPCR quantification obtained for the *ha29* gene and that obtained for HearLB6. All reactions were performed by using 5 μl of Premix (TaKaRa Bio Inc.) and the probe at a 0.2 μM final concentration for the LB6-f and LB6-r primers, 5 μl of SsoAdvanced SYBR green Supermix (Bio-Rad, Berkeley, CA, USA) for the ha29.1 and ha29.2 forward and reverse primers at a 0.2 μM final concentration, and 1 μl of template DNA in a total reaction mixture volume of 10 μl . Nontemplate controls (NTCs), standard curves (3.0×10^{-1} to 1.9×10^{-3} ng/ μl of serial 5-fold dilutions of template DNA from HearLB6), and samples were analyzed twice. The PCRs were performed with a CFX96 Touch real-time PCR detection system (Bio-Rad). The program used was as follows: 2 min 30 s at 95°C and 40 cycles of 98°C for 15 s and 60°C for 30 s. For reactions performed with SYBR green, this program was monitored by using melting curve (60°C to 95°C). Data were analyzed by using Bio-Rad CFX Manager software (Bio-Rad). Results were subjected to repeated-measures ANOVA and a Tukey test ($P < 0.05$) for homogeneous groups by using the SPSS 21.0 program.

In addition, the concentration-mortality response, speed of kill, and OB production of virus OBs sampled at P₀, P₁, and P₅ were determined in recently molted (<12 h) second-instar *H. armigera* larvae, as described above.

Finally, to confirm co-occlusion of the different genotypes within an individual OB, 100 second-instar larvae were allowed to drink from a suspension of 1×10^3 OBs/ml of the selected co-occluded mixture (P₁). These larvae drank <1 μl of the suspension. According to the Poisson distribution, the probability of consuming between 0 and 1 OB at this concentration was 0.74, whereas the probability of consuming >1 OB was 0.26 for larvae that drank 1 μl of the suspension. As larvae drank <1 μl , these probability values are likely to be an overestimate. Therefore, evidence of co-occlusion of those genotypes would be provided if both genotypes were detected in individual larvae at a prevalence of >26% of virus-killed insects. OBs from each dead larva were purified individually, and DNA was extracted and subjected to qPCR analysis, as described above, to determine the relative prevalence of each genotypic variant in each infected insect.

Nucleotide sequence accession numbers. The sequences of the complete genomes of HearSP1B and HearLB6 have been submitted to GenBank under accession numbers KJ701033 and KJ701031, respectively.

RESULTS

New genotypes of HearSNPV. Among the virus-positive plaque picks, two new genotypes were cloned *in vitro* from the wild-type isolate HearSP1, which were distinguished from one another by EcoRI restriction profiles of their genomic DNAs (Fig. 1A) and were named HearSNPV-SP1A (HearSP1A) and HearSNPV-SP1B (HearSP1B). Of the 145 clones obtained, 69% showed the characteristic profile of HearSP1A, while the remaining clones (31%) showed the characteristic HearSP1B variant profile.

Due to the problems associated with the plaque assay technique, endpoint dilution was used for the isolation of HearLB genotypes. Endpoint dilution of HearLB isolates indicated that each of the 17 larvae analyzed was infected by a single genotypic

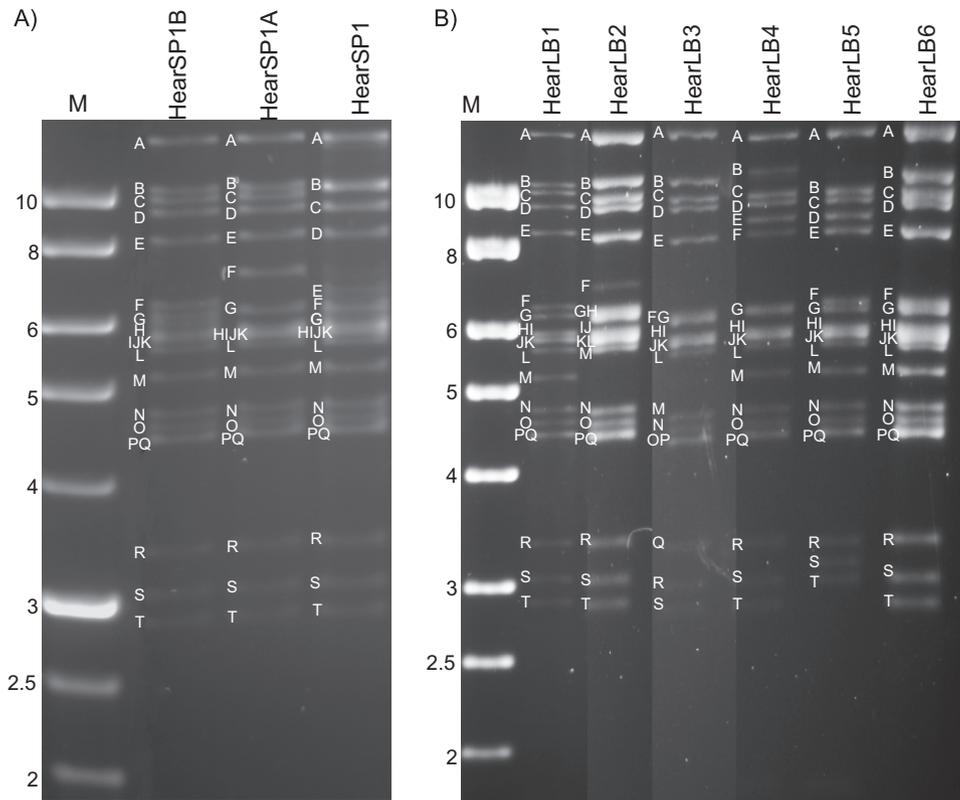


FIG 1 (A) EcoRI profiles of the genomic DNAs of wild-type HearSP1 and the HearSP1A and HearSP1B genotypes. A 1-kb DNA ladder (Nippon) was used as a molecular size marker (M), and fragment sizes are indicated at the left (in kilobases). (B) EcoRI restriction endonuclease profiles of the genomic DNAs of the HearLB1, HearLB2, HearLB3, HearLB4, HearLB5, and HearLB6 genotypes. DNA HyperLadder I (Bioline) was used as a molecular size marker (M), and fragment sizes are indicated at the left (in kilobases). Fragments were named alphabetically by size.

variant. In total, six distinct EcoRI profiles were identified from the 17 larvae (Fig. 1B); these isolates were designated HearSNPV-LB1, HearSNPV-LB2, HearSNPV-LB3, HearSNPV-LB4, HearSNPV-LB5, and HearSNPV-LB6 (HearLB1, HearLB2, HearLB3, HearLB4, HearLB5, and HearLB6, respectively). HearLB3 was the most frequent variant and was isolated from 6 larvae (35.3%), followed by HearLB1 from 4 larvae (23.5%) and HearLB2 also from 4 larvae (23.5%). The HearLB4, HearLB5, and HearLB6 genotypes were each isolated from a single larva (5.9% each).

EcoRI digestion of variant DNAs revealed the presence of polymorphic fragments that could be used as markers (Fig. 1). The EcoRI-B fragment of HearLB4 (11.0 kb) was larger than those of HearLB2, HearLB3, and HearLB6 (10.5 kb); HearSP1A and HearSP1B (10.18 kb); and HearLB1 (10.15 kb) but was absent in the HearLB5 profile. HearLB1 (EcoRI-D), HearSP1A (EcoRI-D), and HearSP1B (EcoRI-D) showed a common fragment of 9.2 kb, whereas in HearLB1 (EcoRI-D), HearLB2 (EcoRI-D), HearLB3 (EcoRI-D), HearLB4 (EcoRI-D), HearLB5 (EcoRI-C), and HearLB6 (EcoRI-D), this fragment was slightly larger (9.38 kb). The EcoRI-E (8.7-kb) fragment of HearLB4 is present only in this profile and the HearLB5 (EcoRI-D) profile. HearSP1A and HearLB2 showed a common fragment of 7.16 kb (EcoRI-F), whereas the EcoRI-M fragment of HearSP1A (5.26 kb) was absent in HearLB2 and HearLB3. Finally, HearLB5 showed a unique fragment of 3.10 kb (EcoRI-S), whereas this variant lacked a 2.83-kb fragment that was present in all the other variant profiles. In ad-

dition, the EcoRI profile of HearSP1A and HearSP1B showed a fragment of 9.73 kb that was absent in the HearSP1 profile.

Some of the bands found in the restriction endonuclease analysis (REN) profiles, even those characterized as polymorphic fragments, appeared at a lower intensity. This might be due to the fact that they are single bands and not double bands, as observed for band L of HearLB1 or HearLB3, which, being a single band, appeared at a low intensity, in comparison with those formed by EcoRI-J and EcoRI-K fragments in the same genotypes (Fig. 1B).

Biological activity of individual variants and mixtures. (i) **Biological activity of HearSP1 variants.** Insect bioassays indicated that HearSP1B variant OBs (1.3×10^4 OBs/ml) were 2.8-fold more pathogenic, in terms of concentration-mortality metrics, than the wild-type isolate HearSP1 (2.4×10^4 OBs/ml), whereas the pathogenicity of HearSP1A OBs was intermediate between these values (Table 2). No significant differences in mean time to death were observed; second-instar insects infected by the HearSP1A (99.6 h p.i.) and HearSP1B (98.3 h p.i.) variants died at mean times similar to those for insects infected by wild-type HearSP1 (102.8 h p.i.) (Table 2). OB production values were similar for variants and slightly lower (~25% reduction) than those observed for the wild-type isolate, although this difference was not significant ($F_{2,6} = 0.46$; $P > 0.05$) (Fig. 2A).

(ii) **Biological activity of HearLB variants.** The LC_{50} s of the six HearLB variants were all similar to that of the reference isolate HearSP1, with an LC_{50} of 1.6×10^4 OBs/ml in second-instar in-

TABLE 2 LC₅₀s, relative potencies, and mean time to death values^a

Virus(es)	LC ₅₀ (OBs/ml)	95% fiducial limit for LC ₅₀ (OBs/ml)		Mean slope ± SE	Relative potency	MTD (h p.i.)	95% fiducial limit for MTD (h p.i.)	
		Low	High				Low	High
HearSP1 variants								
HearSP1	3.6 × 10 ⁴	2.3 × 10 ⁴	6.1 × 10 ⁴	0.99 ± 0.16	1	102.8a	100.0	105.7
HearSP1A	2.4 × 10 ⁴	1.7 × 10 ⁴	3.4 × 10 ⁴	1.45 ± 0.18	1.5	99.6a	96.5	102.8
HearSP1B	1.3 × 10 ⁴	0.9 × 10 ⁴	1.8 × 10 ⁴	1.46 ± 0.18	2.8	98.3a	95.3	101.4
HearLB variants								
HearSP1	1.6 × 10 ⁴	0.9 × 10 ⁴	2.4 × 10 ⁴	1.15 ± 0.16	1	114.5b	112.6	116.4
HearLB1	1.2 × 10 ⁴	0.7 × 10 ⁴	1.8 × 10 ⁴	1.16 ± 0.16	1.3	109.8a	108.1	111.5
HearLB2	1.6 × 10 ⁴	0.8 × 10 ⁴	2.5 × 10 ⁴	1.32 ± 0.16	1.0	108.0a	106.4	109.7
HearLB3	1.5 × 10 ⁴	0.7 × 10 ⁴	2.7 × 10 ⁴	1.05 ± 0.15	1.0	116.3bc	114.5	118.2
HearLB4	1.6 × 10 ⁴	0.9 × 10 ⁴	2.6 × 10 ⁴	0.96 ± 0.14	1.0	118.4c	116.9	119.9
HearLB5	1.4 × 10 ⁴	0.8 × 10 ⁴	2.2 × 10 ⁴	1.28 ± 0.17	1.1	109.1a	107.3	110.9
HearLB6	1.3 × 10 ⁴	0.8 × 10 ⁴	2.0 × 10 ⁴	1.49 ± 0.20	1.2	108.9a	107.4	110.6
Variant mixtures and individual variants								
HearLB3:LB6 (1:1)	2.1 × 10 ⁴	1.5 × 10 ⁴	2.9 × 10 ⁴	1.36 ± 0.15	1	114.1b	112.8	115.5
HearLB1:LB3 (1:1)	1.6 × 10 ⁴	0.9 × 10 ⁴	2.7 × 10 ⁴	1.47 ± 0.16	1.3	115.8b	114.3	117.3
HearLB1:LB3:LB6 (1:1:1)	1.1 × 10 ⁴	0.8 × 10 ⁴	1.4 × 10 ⁴	1.36 ± 0.15	1.9	108.7ab	107.3	110.1
HearLBmix (4:4:6:1:1:1)	1.4 × 10 ⁴	0.9 × 10 ⁴	2.0 × 10 ⁴	1.38 ± 0.19	1.5	115.3b	113.6	117.1
HearSP1A:SP1B (1:1)	1.7 × 10 ⁴	0.9 × 10 ⁴	3.0 × 10 ⁴	1.64 ± 0.16	1.2	108.2a	106.0	110.5
HearSP1A:SP1B (1:2)	1.2 × 10 ⁴	0.8 × 10 ⁴	2.3 × 10 ⁴	1.18 ± 0.13	1.7	110.9ab	108.6	113.2
HearSP1B:LB1 (1:1)	9.8 × 10 ³	0.8 × 10 ⁴	1.2 × 10 ⁴	1.65 ± 0.16	2.1	112.8b	110.6	115.3
HearSP1B:LB6 (1:1)	5.7 × 10 ³	0.4 × 10 ⁴	0.8 × 10 ⁴	1.36 ± 0.15	3.6	108.8ab	106.5	111.1
HearSP1A	1.6 × 10 ⁴	1.2 × 10 ⁴	2.1 × 10 ⁴	1.26 ± 0.13	1.3	108.1a	105.7	110.4
HearSP1B	1.1 × 10 ⁴	0.8 × 10 ⁴	1.5 × 10 ⁴	1.48 ± 0.15	1.9	112.4ab	109.9	114.9
HearLB1	1.6 × 10 ⁴	0.9 × 10 ⁴	2.9 × 10 ⁴	1.35 ± 0.14	1.3	112.3b	110.8	113.8
HearLB3	1.5 × 10 ⁴	0.9 × 10 ⁴	2.7 × 10 ⁴	1.05 ± 0.15	1.4	113.5b	112.0	115.0
HearLB6	1.3 × 10 ⁴	0.8 × 10 ⁴	2.6 × 10 ⁴	1.15 ± 0.13	1.6	109.5ab	107.8	111.3

^a Shown are values for the HearSP1 wild-type isolate and single HearSP1A and HearSP1B genotypes; the HearSP1 isolate and HearLB1, HearLB2, HearLB3, HearLB4, HearLB5, and HearLB6 genotypes; and mixtures of genotypes and individual genotypes in second-instar insects. Probit regressions were fitted with POLO-PC. A test for parallelism was not significant ($\chi^2 = 5.57$, $df = 2$, and $P = 0.06$ for HearSP1 genotypes; $\chi^2 = 6.93$, $df = 6$, and $P = 0.33$ for HearLB genotypes; and $\chi^2 = 16.84$, $df = 12$, and $P = 0.16$ for mixtures of genotypes and individual genotypes). Relative potencies were calculated as the ratio of LC₅₀s relative to those of the HearSP1 wild type and HearLB3:LB6 (1:1 mixture). MTD values were estimated by Weibull analysis (46). Values followed by different letters are significantly different for comparisons among values within each of the three sections of the table ($P < 0.05$ by *t* test).

sects (Table 2). However, the variants could be classified into two groups according to their speed of kill. The fastest-killing variants, HearLB1, HearLB2, HearLB5, and HearLB6, had MTD values of between 108.0 and 109.8 h p.i. (Table 2). A second group consisted of the slowest-killing variants, HearLB3 and HearLB4 (116.3 to 118.4 h p.i.), whereas wild-type HearSP1 had an intermediate speed of kill (Table 2). Variants differed significantly in OB production ($F_{6,14} = 12.18$; $P < 0.001$) (Fig. 2B). HearLB1 and HearLB4 were the most productive variants, whereas wild-type HearSP1 was the least productive, producing approximately half the number of OBs per larva in insects infected by HearLB1 ($P > 0.05$ by Tukey test). HearLB2, HearLB3, HearLB5, and HearLB6 produced intermediate numbers of OBs per larva (Fig. 2B).

Biological activity of the genotypic mixtures. The co-occluded HearSP1B:LB6 OB mixture was between 1.7- and 3.7-fold more pathogenic than the OBs of any of the co-occluded mixtures of variants that were evaluated in this study (Table 2). The co-occluded HearSP1B:LB6 mixture (1:1) was also between 1.9- and 2.8-fold more pathogenic than any of the individual variants, including its individual components, HearSP1B and HearLB6 (Table 2). Moreover, the co-occluded mixture of HearSP1B:LB6 was

between 1.7- and 3.7-fold more pathogenic than any of the co-occluded mixtures of variants that were evaluated in this study (Table 2).

Of the individual genotypes, the fastest-killing variant was HearSP1A (MTD, 108.1 h), and the slowest-killing variant was HearLB3 (MTD, 113.5 h). The co-occluded HearSP1B:LB6, HearSP1A:SP1B (1:1 and 1:2 proportions), and HearLB1:LB3:LB6 (range of MTD values of 108.2 to 110.9 h) mixtures maintained the speed of kill of the fast-killing HearSP1A, HearSP1B, and HearLB6 genotypes (range of MTD values of 108.1 to 112.4 h). In contrast, the co-occluded mixtures of HearLB1:LB3, HearLB3:LB6, HearLBmix, and HearSP1B:LB1 (range of MTD values of 112.8 to 115.8 h) were as virulent as the slowest-killing variants, HearLB1 and HearLB3 (112.3 and 113.5 h, respectively) (Table 2).

Individual variants and variant mixtures differed significantly in the level of OB production per insect ($F_{12,26} = 13.26$; $P < 0.001$). Of the individual variants, the most productive was HearLB3, whereas the least productive was HearSP1, which produced 9-fold fewer OBs per insect (Fig. 2C). The remaining variants had intermediate levels of production of OBs per larva, although they differed significantly from one another ($P < 0.05$ by

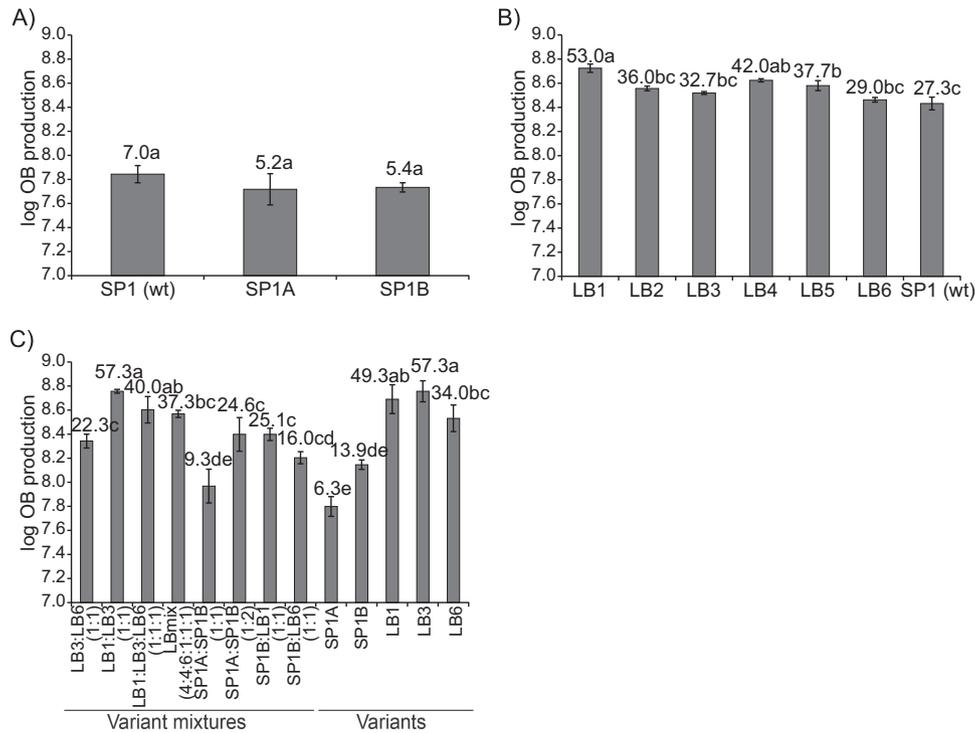


FIG 2 Mean level of OB production from *H. armigera* second-instar insects that died from virus disease following infection with the LC₉₀ of wild-type HearSP1 (from a total of 68 larvae) and the HearSP1A (65 larvae) and HearSP1B (65 larvae) genotypes (A); the HearLB1 (66 larvae), HearLB2 (64 larvae), HearLB3 (63 larvae), HearLB4 (68 larvae), HearLB5 (66 larvae), and HearLB6 (65 larvae) genotypes and the HearSP1 strain (64 larvae) (B); and the HearSP1A (68 larvae), HearSP1B (64 larvae), HearLB1 (62 larvae), HearLB3 (67 larvae), and HearLB6 (65 larvae) genotypes and the HearSP1A:SP1B (1:1 mixture) (66 larvae), HearSP1A:SP1B (1:2) (66 larvae), HearLB1:LB3 (64 larvae), HearLB3:LB6 (68 larvae), HearLB1:LB3:LB6 (63 larvae), HearLBmix (65 larvae), HearSP1B:LB1 (64 larvae), and HearSP1B:LB6 (64 larvae) genotypic mixtures (C). Vertical lines indicate standard errors. Values above bars represent mean OB production values (10⁷ OBs/larva), and values followed by identical letters did not differ significantly by ANOVA and Tukey test ($P < 0.05$).

Tukey test). In the present experiment, HearLB3 was as productive as HearLB1 (Fig. 2C), which was the most productive genotype in the experiment that compared individual genotypes (Fig. 2B). Of the co-occluded variant mixtures, HearLB1:LB3 (1:1) was the most productive, reflecting the high OB production values of its component variants (Fig. 2C). The least productive mixture was HearSP1A:SP1B (1:1), which produced 6-fold fewer OBs per insect than did the HearLB1:LB3 mixture ($P < 0.05$ by Tukey test). The level of OB production by HearLBmix (Table 1), the OB mixture which reflected the relative frequencies of the six variants in the infected insects from Lebrija, was intermediate, as were the OB production values of the remaining mixtures, although they also differed significantly from one another ($P < 0.05$ by Tukey test).

Stability of a co-occluded variant mixture during passage *in vivo*. Following examination of the results for the biological characteristics described above, the HearSP1B:LB6 co-occluded mixture was selected as the mixture with improved insecticidal characteristics compared with single variants, other co-occluded variant mixtures, or the wild-type isolates.

The relative frequencies of HearSP1B and HearLB6 varied significantly throughout the five successive passages ($F_{1,2} = 51.85$ and $P < 0.02$ for HearLB6; $F_{1,2} = 171.49$ and $P < 0.006$ for HearSP1B) (Fig. 3). The frequency of HearSP1B in the mixed-variant population increased from 47.7% at P₁ to 84.6% at P₅. Correspondingly, the relative frequency of HearLB6 decreased from 52.3% at P₁ to 15.4% at P₅.

The pathogenicity of the co-occluded HearSP1B:LB6 mixture at P₀ did not change significantly at P₁ but subsequently increased slightly and was 1.7-fold greater at P₅ (Table 3). The mean time to death decreased significantly between P₀ and P₁ and again between P₁ and P₅ (Table 3). The decrease in the MTD value was reflected in significantly decreased levels of OB production per larva between P₀ and P₅, whereas the P₁ value was intermediate ($P < 0.05$ by Tukey test) (Fig. 4).

The qPCR analysis of OBs obtained from larvae that died after

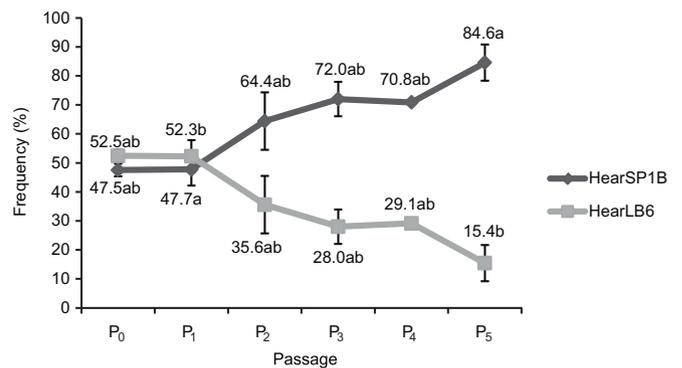


FIG 3 Relative frequencies of HearSP1B and HearLB6 in the HearSP1B:LB6 genotypic mixture throughout five serial passages *in vivo* (P₀ to P₅). Vertical lines indicate standard errors. Values followed by identical letters did not differ significantly by ANOVA and Tukey test ($P < 0.05$).

TABLE 3 LC₅₀s, relative potencies, and mean time to death values of HearSP1B:LB6 (1:1 mixture) of at different passages in second-instar *H. armigera* insects^a

Passage	LC ₅₀ (OBs/ml)	Mean slope ± SE	Relative potency	95% fiducial limit for relative potency		MTD (h p.i.)	95% fiducial limit for MTD (h p.i.)	
				Low	High		Low	High
P ₀	3.3 × 10 ⁴	1.49 ± 0.15	1			119.9a	117.9	122.1
P ₁	4.1 × 10 ⁴	1.07 ± 0.13	0.8	0.5	1.3	112.4b	110.4	114.5
P ₅	1.9 × 10 ⁴	1.35 ± 0.14	1.7	1.1	2.6	106.5c	104.2	108.8

^a Probit regressions were fitted with POLO-PC. A test for parallelism was not significant ($\chi^2 = 4.80$; $df = 2$; $P = 0.09$). Relative potencies were calculated as the ratio of LC₅₀s relative to the value at passage zero (P₀). MTD values were estimated by Weibull analysis (46). Values followed by different letters differed significantly ($P < 0.05$ by *t* test).

having consumed a single OB confirmed that the HearSP1B and HearLB6 genotypes were co-occluded in the same OB. A total of 12 polyhedrosis-killed larvae were analyzed individually by qPCR, whereas 85 larvae reached the pupal stage, and 4 larvae died due to unknown causes. HearSP1B and HearLB6 were both amplified from all OB samples obtained from each virus-killed larva, indicating that both genotypes were present in the same OB inoculum (Fig. 5). The frequency of HearLB6 in each single larva ranged from 20 to 81%, and correspondingly, the frequency of HearSP1B was between 19 and 80% (Fig. 5). Interestingly, the mean frequencies of both genotypes obtained from these OB samples (50.8% HearSP1B and 49.2% HearLB6) were similar to those in which they were inoculated (47.7% HearSP1B and 52.3% HearLB6).

DISCUSSION

In this study, we examined the genotypic composition of two HearSNPV isolates from different parts of southern Spain and were able to select a mixture of two variants with insecticidal properties better than those of the wild isolates, any of the component variants, or any other variant mixtures that we evaluated. The two Spanish HearSNPV isolates are heterogeneous populations consisting of a number of genotypic variants present in different relative proportions. This type of heterogeneity is very common in natural populations of alphabaculoviruses that have been analyzed previously (8, 9, 48).

Only two variants, HearSP1A and HearSP1B, were isolated from HearSP1-wt. However, restriction endonuclease analysis is not the most sensitive method for identifying novel genotypic

variants, and additional variants that could not be detected by using this technique may have been present. This was the case with a *Chrysodeixis chalcites* SNPV (ChchSNPV) isolate from the Canary Islands, in which the wild-type isolate showed the same profile as the genotype that comprised just 36% of the population of genotypes in the isolate (20). Therefore, other genotypes that were not amenable to replication in cell culture may have been present. The presence of helper genotypes has also been reported for alphabaculoviruses (12, 19). For instance, by using an *in vivo* cloning technique, Muñoz et al. (18) were unable to isolate two of the genotypes from a *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) wild-type isolate, as these genotypes required the presence of complementary genotypes to replicate in the insect host. Sun et al. (37) isolated seven HearSNPV genotypes from a HearSNPV wild-type strain by using *in vivo* techniques. Similarly, Wang et al. (38) isolated 10 HearSNPV genotypes from a single wild-type strain by using BAC cloning. In contrast, Ogembo et al. (34) identified between one and five genotypes from different HearSNPV strains by a plaque assay, supporting the idea that some HearSNPV genotypes replicate poorly in cell culture. Unexpectedly, given previous results in this field, the 17 HearLB-killed larvae that we analyzed were each infected by a single genotypic variant. These could be classified into six different variants by their EcoRI restriction profiles, which had not been observed in previously characterized HearSNPV variants (27, 29, 34, 37, 49).

The variants cloned from wild-type isolates differed minimally in their restriction profiles, suggesting that these differences may be due to small changes in their nucleotide sequence.

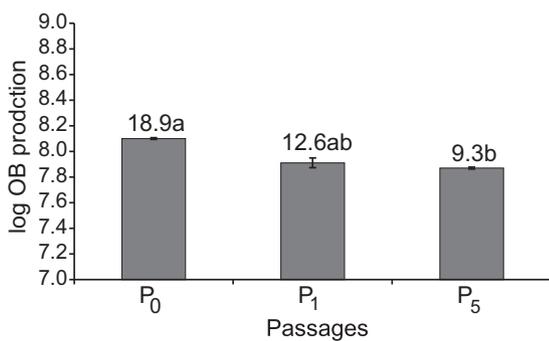


FIG 4 Mean OB production of *H. armigera* second-instar insects that died from virus disease following infection with the LC₅₀ at P₀ (from a total of 66 larvae), P₁ (62 larvae), and P₅ (64 larvae) of HearSP1B:LB6 (1:1). Vertical lines indicate standard errors. Values above bars represent mean OB production values (10⁷ OBs/larva), and values followed by identical letters did not differ significantly by ANOVA and Tukey test ($P < 0.05$).

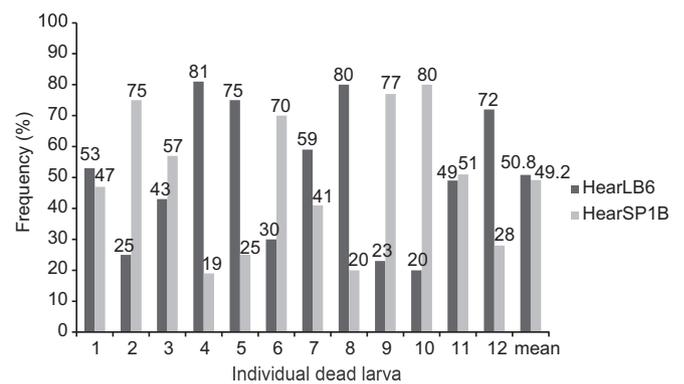


FIG 5 Relative frequencies of HearSP1B and HearLB6 variants in OBs obtained from larvae that died after consumption of a single OB. The presence of both genotypes in larvae infected by a single OB corroborated the co-occlusion of both genotypes within the same OB.

However, as occurs among other genotypes of many alphabaculoviruses, small changes in the genome can result in important differences in biological activity (16, 20, 34). OBs of HearSP1B were ~3-fold more pathogenic than those of HearSP1-wt, although its prevalence in the population was estimated to be 31%. Alternatively, interactions between genotypes might decrease the pathogenicity of the mixture, as previously observed for a SeMNPV population (18). Moreover, additional genotypes may be present in the HearSP1-wt population, thus attenuating the influence of HearSP1B on HearSP1-wt transmissibility, as occurs with certain genotypic variants in wild-type isolates of SeMNPV (18) and *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) (16). In contrast, among the different variants isolated from larvae that died during laboratory rearing, no marked differences in pathogenicity were detected, but differences in speed of kill were observed, with HearLB6 being among the fastest-killing variants, even more so than HearSP1-wt, which was previously selected as one of the fastest-killing isolates from diseased larvae collected in the Iberian Peninsula (36). These results are in line with data from previous studies performed with different variants of HearSNPV, in which variants of similar pathogenicities presented marked differences in speed of kill (36, 50). Generally, the fastest-killing variants tend to produce lower numbers of OBs in each infected insect, because insects die faster and feed and grow less during the incubation period of infection, and the virus has less time to replicate (50–53). However, we observed that HearLB1 was highly productive, despite being one of the fastest-killing variants. This fact has also been observed for other alphabaculovirus species, such as SeMNPV, in which a highly productive genotype was also among the fastest-killing genotypes present in the population (54). Curiously, MTD values for HearLB1 and HearLB3, and OB productivity, differed in different experiments, reflecting natural variation in insect batches and associated experimental variables. However, the tendency was the same: HearLB1 tended to kill faster than did HearLB3, although the difference was significant in one experiment, the one comparing individual genotypes, but not in the other, which compared genotypic mixtures. This is why statistical comparisons among treatments within an experiment and not between different experiments were performed (Table 2).

The genetic structure of natural populations of alphabaculoviruses is adapted to improve the survival of the virus, which depends on a combination of persistence in the environment, the capacity of OBs to infect the host, and the number of OBs produced in each infected insect. The relative importance of each of these components is likely to differ according to the environmental conditions of the habitat in which the host insect lives and feeds.

For the development of baculovirus-based insecticides, understanding the influence of genotypic interactions on the insecticidal properties of the variants present in the virus population has proven to be of great value for the development of several novel virus insecticides (22, 23, 40). However, our current understanding of these interactions at the molecular level is poor, and it is not possible at present to predict the outcome of an interaction between two genotypically distinct variants on the virus phenotype. Consequently, the virus phenotype arising from each interaction or set of interactions has to be determined empirically. Therefore, in the present study, interactions among the different genotypes were studied by constructing different genotypic mixtures, with

the aim of selecting a genotypic mixture with improved insecticidal activities. Six of the mixtures showed the expected biological properties. However, the HearLB3:LB6 co-occluded mixture did not maintain the productivity of HearLB6, and surprisingly, the HearSP1B:LB6 co-occluded mixture was even more pathogenic than HearSP1B, the most pathogenic genotype present within HearSP1-wt. The HearSP1B:LB6 co-occluded mixture also showed a speed of kill similar to that of HearLB6 but with a lower level of OB productivity. These characteristics might favor its use in the field, as the increased pathogenicity and speed of kill could favor the rapid suppression of the pest using a minimum amount of OBs.

Co-occlusion of HearSP1B and HearLB6 in the same OB was confirmed. The co-occluded mixture at P_1 was as pathogenic and productive as the OB mixture (P_0) but, interestingly, was faster killing. This effect has been observed for ChchSNPV, in which co-occluded mixtures of variants were faster killing than were mixtures of OBs of each variant (20). Bernal et al. (20) suggested that the improved speed of kill of the co-occluded mixture of variants in comparison with the mixtures of OBs of each variant could be due to the physical proximity of variants within occlusion-derived virions of the different variants in the co-occluded mixtures. This physical association could increase the likelihood that mixtures of variants infect individual midgut cells during the primary infection process, in the frequencies in which they are present in the inoculum. This is less likely to occur when mixtures of OBs of different variants are inoculated, given that occlusion-derived virions (ODVs) comprise each variant alone when the different variants are segregated in different OBs.

The genotypic frequencies of the HearSP1B:LB6 mixture varied through five successive passages. The frequency of the HearSP1B variant increased and reached ~85% after five passages. The high pathogenicity of HearSP1B was likely responsible for its increased transmission capacity compared to that of HearLB6, resulting in its progressive increase in prevalence during serial passage. In fact, the OBs obtained after five passages were more pathogenic and virulent than the OB mixture or P_1 mixture, which might suggest that the co-occluded mixture of 85% HearSP1B and 15% HearLB6 obtained after five passages *in vivo* is the most suitable active ingredient for a biopesticide product. This process represents a unique mechanism for the selection of biological materials for use in biological insecticides, in which genotypic variants are co-occluded and then subjected to selection for transmissibility by serial passage in the laboratory. Previous studies found that experimental genotypic mixtures of SfMNPV that were subjected to successive passages *in vivo* rapidly converged to the frequencies present in the wild-type isolate (19, 55). However, the HearSP1B and HearLB6 genotypes were isolated from different wild-type isolates from geographically distinct localities, and this genotypic mixture reached genotypic frequencies that apparently favored improved transmission.

In conclusion, the genotypic HearSP1B:LB6 mixture was selected to be developed as a bioinsecticide for the control *H. armigera* larvae, as it showed highly suitable insecticidal characteristics. Moreover, before marketing such a bioinsecticide, it would be necessary to determine the most suitable conditions for the efficient production of this virus and also to perform field assays to determine the effectiveness of the HearSP1B:LB6 mixture as a biocontrol agent under natural conditions. In the present study, we

demonstrated that a unique binary mixture of HearSNPV genotypic variants selected from populations present in different isolates interacted to produce improved insecticidal characteristics. An important finding of the present study is that transmissibility may be further improved by applying serial passage techniques to select for the optimum proportions of each component variant in the mixture. This study underlines the utility of the genotypic variant dissection and reassociation approach for the development of effective virus-based insecticides.

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