Research Article

Received: 3 May 2013 Revised: 8 August 2013 Accepted article published: 23 August 2013 Published online in Wiley Online Library: 13 October 2013

(wileyonlinelibrary.com) DOI 10.1002/ps.3637

Selection of a nucleopolyhedrovirus isolate from *Helicoverpa armigera* as the basis for a biological insecticide

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Abstract

BACKGROUND: The cotton bollworm, *Helicoverpa armigera*, is an insect that causes damage in a wide range of crops in Spain. Seven isolates of *H. armigera* single nucleopolyhedrovirus (HearSNPV) from the Iberian Peninsula were subjected to molecular and biological characterization and compared with a Chinese genotype (HearSNPV-G4).

RESULTS: The estimated sizes of the Iberian genomes varied between 116.2 and 132.4 kb, compared to 131.4 kb of the HearSNPV-G4 reference genome. Phylogenetic analysis based on the *lef-8*, *lef-9* and *polh* genes revealed that the Iberian strains were more closely related to one another than to other HearSNPV isolates. Occlusion body (OB) concentration-mortality responses (LC_{50} values) did not differ significantly among Iberian isolates when tested against a *Helicoverpa armigera* colony from Oxford (UK). Despite being the fastest killing isolate, HearSNPV-SP1 was as productive as isolates with lower virulence, with an average yield of 3.1×10^9 OBs $larva^{-1}$. OBs of HearSNPV-SP1 and HearSNPV-G4 were similarly pathogenic against a recently established colony from southern Spain, although HearSNPV-SP1 was faster killing than HearSNPV-G4 against a range of instars.

CONCLUSION: The insecticidal properties of HearSNPV-SP1 mean that this strain is likely to prove useful as the basis for a biological insecticide for control of *Helicoverpa armigera* in Spain.
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Keywords: *Helicoverpa armigera*; baculovirus; REN analysis; physical map; insecticidal properties; ingredient active production

1 INTRODUCTION

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is an important pest of field and glasshouse crops in many parts of the world, except in North and South America.[1] In the Iberian Peninsula (Spain and Portugal), *H. armigera* larvae cause serious damage to field-grown tomatoes, peppers, cotton and maize, and a diversity of glasshouse vegetables and ornamental crops.[2] Environmental and food residue problems associated with chemical control strategies have stimulated the search for sustainable pest control measures.

The occlusion bodies (OBs) of alphabaculoviruses (lepidopteran specific nucleopolyhedrovirus, NPV, Baculoviridae family) have an established record as the basis for effective biological insecticides due to their high specificity, virulence, and compatibility with other beneficial organisms (parasitoids, predators and pathogens). NPV-based products can also be incorporated into integrated programs of pest management, reducing both farmer dependence on chemical products and the likelihood of the development of resistance in the pest population.[3]

Several NPVs have been isolated from *Helicoverpa armigera* larvae in different parts of the world.[4–7] The genomes of four of these isolates, HearSNPV-G4[7] (GeneBank accession number AF271059) and HearSNPV-C1[8] (GeneBank accession number AF303045) from Hubei province, China, HearSNPV-NNg1[9] (GeneBank accession number AP010907) from Kenya, and HearSNPV-Aus (GeneBank accession number JN584482) from Australia have been sequenced completely. HearSNPV-G4 was developed as a biopesticide that has been used extensively on cotton in China,[10] whereas in Thailand a locally-produced HearSNPV-based product is available for control of this pest.[11] However the slow speed of kill of HearSNPV compared to chemical insecticides has hampered the commercialization of this virus as a bioinsecticide product, and genetically modified viruses have been produced to improve this trait, by the deletion of certain genes or the insertion of insect-selective toxin genes.[12–14]

However, the use of recombinant baculoviruses is not presently authorized in most countries, so that the selection of wild-type

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isolates with suitable insecticidal characteristics remains the only viable mechanism of selecting the active ingredient for virus based insecticides. As such, the process of developing an NPV-based insecticide requires a comparative evaluation of local virus strains with strains from elsewhere for two reasons. First, different insect host biotypes can vary in their susceptibility to the OBs of geographically distinct isolates[15–17] and second, under certain circumstances the use of exotic NPV isolates can have a negative impact on the biological activity of native strains.[18] As such, the selection of an isolate as a bioinsecticide requires geographical isolates to be tested against the local pest population.

Seven NPV isolates from the Iberian Peninsula were previously identified and characterized. Of these, HearSNPV-SP1 was selected as the most active isolate.[4] However, subsequent studies indicated that three different isolates, HearSNPV-SP7, HearSNPV-PT1 and HearSNPV-PT2 had more favorable insecticidal characteristics against *Helicoverpa armigera*,[19] although no comparative analyses of HearSNPV-SP1 and HearSNPV-SP7, HearSNPV-PT1 or HearSNPV-PT2 have been performed to date. In the present study, genomic and phenotypic characterization of different HearSNPV isolates from the Iberian Peninsula, including the four previously mentioned, was performed in order to select an isolate with highly insecticidal characteristics against a laboratory colony of *H. armigera*. The biological activity was determined in terms of pathogenicity, speed of kill, and the yield of OBs, which is a key component of virus transmissibility and production costs, was also examined. In addition, patterns of genomic variation, that could account for phenotypic differences in biological activity between HearSNPV-SP1 and HearSNPV-G4, were determined using physical maps. Finally, the insecticidal properties of the most effective Iberian strain were compared with those of the Chinese reference isolate HearSNPV-G4 using a recently-established colony from southern Spain.

2 EXPERIMENTAL METHODS

2.1 Insect rearing and *Helicoverpa armigera* SNPV strains

Two *Helicoverpa armigera* colonies were maintained in the insectary at the Universidad Pública de Navarra (UPNA) at 25 ± 1 °C, 70 ± 5% relative humidity and 16h:8h day/night photoperiod on a semi-synthetic diet.[20] One colony was started using pupae from a long-established laboratory population maintained at the Center for Ecology and Hydrology (NERC-CEH) in Oxford, UK. We refer to these insects as the Oxford colony. The other colony was started using larvae collected in the glasshouses of Almería, southern Spain, and reared in our insectary for five generations. We refer to these insects as the Almerian colony.

Iberian strains of HearSNPV were initially isolated from diseased *Helicoverpa armigera* larvae collected in Spain (HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7 and HearSNPV-SP8) and Portugal (HearSNPV-PT1 and HearSNPV-PT2).[4,19] Larvae with the typical signs of NPV infection were collected individually in 1999 from maize plants in Olivenza (Portugal) and Toledo (Spain) and from field tomatoes and glasshouse grown tomatoes and sweet peppers in Oeste (Portugal).[19] Each isolate originated from a single larva, representing a single isolate. To purify OBs, each dead larvae was macerated individually in 300 μL distilled water, filtered through muslin and centrifuged at 2500 × *g* for 5 min. Pellets were resuspended in 1 mg mL^{-1} sodium dodecyl sulphate (SDS) and centrifuged for 5 min at 2500 × *g*. The resulting pellets were washed twice in distilled water and finally resuspended in 300 μL distilled water and stored at 4 °C until required.

The Chinese genotype HearSNPV-G4,[21] isolated by *in vivo* cloning, was kindly provided by JM Vlak (Wageningen University, The Netherlands) as OB suspension and was used as a reference isolate.

2.2 OB amplification, purification and DNA extraction

Purified OBs were multiplied in groups of 25 *Helicoverpa armigera* fourth-instars from the Oxford colony. Larvae were reared individually on semi-synthetic diet until death or pupation. Infected larvae were frozen as soon as they died. OBs were purified as described earlier. After each multiplication, the identity of the different strains was confirmed by examining the restriction endonuclease profiles, as described later.

Volumes of 100 μL of purified OB suspensions (10^9 OBs mL^{-1}) were incubated with one volume of 0.5 M Na_2CO_3, 0.5 volumes of 100 mg mL^{-1} SDS and 2.5 volumes of distilled water at 60 °C during 10 min to dissolve the polyhedrin matrix. Undissolved OBs were pelleted at 6000 × *g* for 5 min. The virion-containing supernatant was transferred to sterile 1.5 mL vials and incubated at 50 °C with proteinase K (500 μg) during 1 h. Viral DNA was extracted twice with an equal volume of phenol (pH 7.8) and then at least once with an equal volume of chloroform. DNA was precipitated with 10% (v/v) 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold absolute ethanol at 12,000 × *g* for 10 min. DNA was then washed with 70% cold ethanol and centrifuged for 5 min. DNA pellets were resuspended in 100 μL 0.1% TE (10 mM Tris, 1 mM EDTA) and kept at 4 °C until use.

For restriction endonuclease digestion, 2 μg of viral DNA, quantified in a spectrophotometer (BioPhotometer Plus, Eppendorf, Freiberg, Germany), were incubated with *Bgl*II or *Eco*RI (10 U) (Takara, Japan) at 37 °C for 4 to 12 h. These enzymes were selected as they previously had proved useful in the differentiation of HearSNPV strains.[4,19] Each reaction was stopped by the addition of 1/6 volume of 6× loading buffer (2.5 μg mL^{-1} bromophenol blue, 40 mg mL^{-1} sucrose). Fragments were separated by electrophoresis in gels containing 10 mg mL^{-1} agarose in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer with 0.25 μg mL^{-1} ethidium bromide, at 20 V for 14 h and visualized on a GeneSnap (Syngene). DNA fragment sizes were estimated by comparison to a standard molecular weight marker (HyperLadder I, Bioline, London, UK).

2.3 Comparison of HearSNPV-SP1 and HearSNPV-G4 physical maps

The endonuclease *Eco*RI was selected for the construction of physical maps as *Bgl*II digestion resulted in many small restriction fragments[19] compared to *Eco*RI. The genomic library of *Eco*RI fragments generated after digestion of the HearSNPV-SP1 DNA was constructed using the pUC19 plasmid (Promega, Madrid, Spain). The physical map of HearSNPV-SP1 isolate was prepared by ordering the restriction fragments following multiple digestions of the cloned *Eco*RI fragments. All *Eco*RI cloned fragments were double digested with *Bgl*II or *Hin*dIII, electrophoresed and fragment sizes were then compared to that of cloned *Eco*RI fragments. The construction of the physical map was completed by terminal sequencing of *Eco*RI cloned fragments using M13/pUC sequencing primer and M13/pUC reverse sequencing primer (M13 and M13R) (Sistemas Genómicos S.L., Valencia, Spain). Using sequence information of cloned fragments the possible gaps between two fragments were amplified by PCR using a High Fidelity Taq Polymerase (Prime Star HS DNA polymerase, Takara, Japan) and primers designed on each side of the gaps.

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PCR products used for direct sequencing were purified using QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany) and sequenced (Sistemas Genómicos S.L., Valencia, Spain). All the sequences were aligned and searched for the presence of open reading frames (ORFs) by using Clone Manager 9 program (Scientific & Educational Software, Cary, North Carolina). Homology searches were performed at the nucleotide and deduced amino acid levels, using all putative ORFs. Comparisons with entries in the GeneBank/EMBL databases were performed using BLASTn and BLASTx programs.[22,23] Baculovirus sequences used in the comparative analysis were GeneBank (accession numbers included): HearSNPV-G4 (AF271059), HearSNPV-C1 (AF303045), HearSNPV-NNg1 (AP010907) and HearSNPV-Aus (JN584482). As is convention, the first nucleotide of the genome sequence was designated as the A of the initial ATG of the *polyhedrin* (*polh*) gene, determined by sequence information.

2.4 Phylogenetic analysis

Partial sequences of *late expression factor 8* (*lef-8*), *late expression factor 9* (*lef-9*) and *polyhedrin* (*polh*) genes were used for phylogenetic analysis, based on the procedures of Eberle *et al.*[24] for *Cydia pomonella* granulovirus (CpGV) isolates. Partial sequences of *lef-8*, *lef-9* and *polh* genes were amplified by PCR using a High Fidelity Taq Polymerase (Prime Star HS DNA polymerase, Takara, Japan) and DNA from HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1 and HearSNPV-PT2, which were compared with the homologous sequences of HearSNPV available in the NBCI database. The primer pairs used were halef8.1/halef8.2, halef9.1/halef9.2, and hapolh.1/hapolh.2 for *lef-8*, *lef-9* and *polh* amplifications, respectively. PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany) and directly sequenced (Sistemas Genómicos SL, Valencia, Spain). Partial sequences of *lef-8*, *lef-9* and *polh* were concatenated. Concatenated sequences from the same strain were treated as a single sequence; multiple-sequence alignments were done using the Muscle program, and MEGA5 software[25] was used to determine the optimal nucleotide substitution model and to construct the phylogenetic tree. Neighbor-joining, maximum-parsimony, and maximum-likelihood phylogenetic tree of the concatenated data sets were generated using MEGA5 with the optimal model parameters and the option of complete deletion to eliminate positions containing gaps. Confidence levels for the branching points were determined using 1000 bootstrap replicates.

2.5 Insecticidal properties of Iberian strains against a laboratory colony

To determine the most active Iberian strain, OB pathogenicity expressed as 50% lethal concentration (LC_{50}), mean time to death (MTD) and OB production were determined for each of the Iberian strains using the Oxford colony insects by the droplet feeding method.[26] The HearSNPV-G4 genotype was included as a reference strain.

To determine concentration-mortality responses, newly molted second instars that had been starved for 12 h were used. Larvae were allowed to drink OB suspensions containing 100 mg mL^{-1} sucrose and 0.05 mg mL^{-1} Fluorella Blue food dye and one of five OB concentrations (5.7×10^5, 1.9×10^5, 6.3×10^4, 2.1×10^4 and 7.0×10^3 OBs mL^{-1}) which were found to cause between 95% and 5% mortality in preliminary assays. Groups of 25 to 30 larvae were inoculated with each OB concentration. Larvae that drank the suspension in a 10 min period were individually transferred to 24-well plates containing semi-synthetic diet. Control larvae were allowed to drink a sucrose and food dye solution containing no OBs. Larvae were incubated at 25 ± 1 °C and 70 ± 5% relative humidity. Virus mortality was recorded every 24 h during a 10 day period. The experiment was performed on three occasions. Concentration-mortality data were subjected to Probit analysis using the POLO-PC program.[27] The study was performed three times.

Time-mortality responses were determined using second instars that consumed a LC_{90} of OBs. The LC_{90} values, determined in the previous concentration-mortality bioassays, were 1.3×10^5, 6.4×10^5, 4.7×10^5, 7.6×10^5, 3.5×10^5, 2.1×10^5, 1.1×10^5 and 1.0×10^5 OBs mL^{-1}, for HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4, respectively, that resulted in mortalities of 96, 96, 86, 93, 96, 92, 89 and 86%. Groups of 25 to 30 larvae were inoculated with each isolate. After a 10 min period, larvae that drank OB suspensions were individualized in 24 well plates with diet, incubated at 26 ± 1 °C and virus mortality was recorded at 8 h intervals for 10 days. Control larvae that had not consumed OBs were treated identically. Time-mortality results of individuals that died due to lethal polyhedrosis disease were subjected to Weibull analysis using the GLIM program.[28] The validity of the Weibull model was determined using the Kaplan–Meier survival macro present in the GLIM program. Larvae that did not die during the assay were excluded from these analyses. The study was performed three times.

To determine OB production, overnight-starved fourth instars were allowed to drink from an OB suspension containing 1×10^6 OBs mL^{-1}, that resulted in 84, 82, 88, 80, 82, 86, 92 and 90% mortality for HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4, respectively. Groups of 25 to 30 larvae were inoculated with each OB concentration. Control larvae that had not consumed OBs were treated identically. Inoculated larvae were reared individually in plastic pots containing diet and inspected daily. Insects showing signs of the final stages of polyhedrosis disease were individually transferred to microtubes, incubated at 28 °C until death and subsequently stored at −20 °C. For OB counting, cadavers were thawed, individually homogenized in 1 mL of distilled water and OBs were counted in triplicate using a Neubauer improved hemocytometer (Hawksley, Lancing, UK). OB production results were normalized by log transformation and subjected to analysis of variance (ANOVA) using the SPSS 15.0 program. The study was performed three times.

2.6 Insecticidal properties of the most active HearSNPV Iberian strain against an Almerian field colony

To determine the susceptibility of Almerian colony insects to the most active HearSNPV Iberian strain, OB pathogenicity and speed of kill were determined in second, third, fourth and fifth instars, whereas OB production was determined in fourth and fifth instars and compared with that of HearSNPV-G4 genotype. These assays were performed as described for experiments using insects from the Oxford colony. To determine concentration-mortality responses, five different OB concentrations were used (5.7×10^5, 1.9×10^5, 6.3×10^4, 2.1×10^4 and 7.0×10^3 OBs mL^{-1} for second and third instars; 1.7×10^6, 5.7×10^5, 1.9×10^5, 6.3×10^4 and 2.1×10^4 OBs mL^{-1} for fourth instar; and 5.1×10^7, 1.7×10^6, 5.7×10^5, 1.9×10^5 and 6.3×10^4 OBs mL^{-1} for fifth instar) which resulted in ~95% to 5% mortality in preliminary assays. Statistical

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analyses were performed as described for experiments using insects from the Oxford colony.

To determine virulence and OB production in Almerian colony insects, each instar was inoculated with the corresponding estimated LC_{90}. For HearSNPV-SP1 these concentrations were 1.3×10^5, 6.1×10^5, 2.4×10^6 and 2.5×10^7 OBs mL^{-1} for second, third, fourth and fifth instars, respectively, that resulted in mortalities of 87, 91, 88 and 83%. For HearSNPV-G4 these values were 3.8×10^5, 3.6×10^5, 1.2×10^6 and 1.4×10^7 OBs mL^{-1}, that resulted in 87, 87, 85 and 85% mortality, respectively. Statistical analyses were performed as described for experiments using insects from the Oxford colony.

3 RESULTS

3.1 Molecular identification by REN of Iberian HearSNPV isolates

*Eco*RI profiles of the seven Iberian strains comprised 22 to 24 visible fragments (Fig. 1), of which 16 were present in the profiles of all seven strains. The *Eco*RI profile of the HearSNPV-G4 genotype differed from those of the Iberian strains.

The size estimates for the HearSNPV *Eco*RI-REN fragments and the estimated total genome size are given in Table 1. The genomes of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4 were estimated to be 132.4, 129.8, 125.3, 124.2, 116.2, 125.1, 131.0 and 129.6 kb, respectively, and were calculated from the sum of the estimated lengths of the restriction fragments, generated by single or double enzyme digestions. However, according to the sequence of HearSNPV-G4, the genome was 131.4 kb,[8] 1.8 kb larger than the estimated size. This difference could be explained because the smallest fragments were not visible in the REN profile, as reported for the HearSNPV-SP1 strain. Presumably, the other Iberian strains might also have these small fragments.

3.2 Physical maps

In order to determine changes at genome level that could account for differences in the biological activity, the *Eco*RI physical map of HearSNPV-SP1 was built in comparison with the HearSNPV-G4 map obtained using Clone Manager 9 (Scientific & Educational Software). This enzyme was selected as it resulted in polymorphic fragments that allowed clear discrimination among HearSNPV isolates.[4,19] The cloned *Eco*RI fragments were digested with *Bgl*II or *Hin*dIII to confirm fragment sizes. The map of HearSNPV-SP1 strain was confirmed and completed using the terminal sequence information from a total of 22 cloned fragments. Two of the largest fragments (*Eco*RI-A and *Eco*RI-D) could not be cloned. However, the sizes of these fragments were similar to the gaps presented between *Eco*RI-H and *Eco*RI-P, and *Eco*RI-U and *Eco*RI-H, respectively, in the HearSNPV-SP1 physical map compared to that of HearSNPV-G4. Moreover, sequencing revealed that some small fragments might have been missed as some ORFs were not identified between two cloned fragments. To confirm the presence of these small fragments, primers were designed on each side of cloned fragments (Table 2). Six *Eco*RI small fragments were amplified by PCR and sequenced (Table 2). After sequence alignment, a total of 19 putative ORFs were identified in the HearSNPV-SP1 genome (Table 3). The gene content and order were similar to those found in the previously sequenced HearSNPV isolates, with ORF similarity values between 98 and 100%. The HearSNPV-SP1 isolate displayed the largest sequence similarity (99.5%) with the African HearSNPV-NNg1 isolate, whereas HearSNPV-G4, HearSNPV-C1 and HearSNPV-Aus displayed sequence similarities of 99.4, 99.4 and 99.3% with the HearSNPV-SP1 genome, respectively.

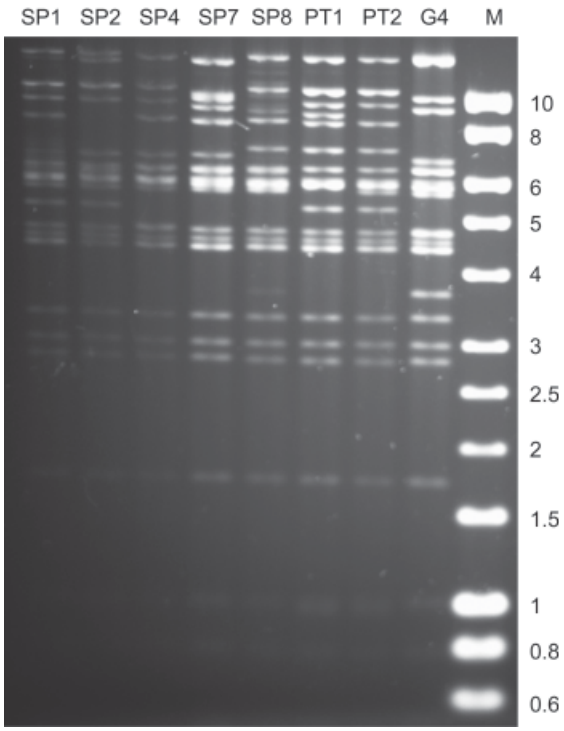

Figure 1. *Eco*RI profiles of the genomic DNAs of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4 isolates. The DNA HyperLadder I (Bioline) was used as a molecular size marker (kb) (M), and fragment sizes are indicated to the right.

The *Eco*RI physical maps of HearSNPV-SP1 and HearSNPV-G4 were closely related with few differences between them (Fig. 2). Both viruses showed differences in *Eco*RI restrictions sites, resulting in different fragments sizes for the same genomic regions. Due to the very similar genome sizes between both viruses, differences in their REN profiles may be due to minor nucleotide polymorphisms that resulted in modification in the restriction sites.

3.3 Phylogenetic analysis of the different HearSNPV Iberian strains

PCR amplification of the partial *lef-8*, *lef-9* and *polh* genes of all isolates resulted in DNA fragments of 490, 495 and 485 bp, respectively. The predicted amino acid sequences were also performed. Sequence information revealed transitions among the gene fragments of the seven isolates. However, there were no deletions or insertions. The transitions among the partial sequences of the Iberian strains compared with that of HearSNPV-G4 genotype are located mostly in the same nucleotides, suggesting that the Iberian strains are the most closely related to one another. The Iberian isolates showed eight nucleotide polymorphisms in the *lef-8* sequence when compared with HearSNPV-G4 genotype, three in the *lef-9* sequence and five in the *polh* sequence. However, the predicted amino acid sequence did not present differences among these strains.

The phylogenetic relationships among Iberian strains and other *Helicoverpa* spp. SNPV were performed by comparing the *lef-8*, *lef-9* and *polh* sequences (Fig. 3). Phylogenetic analysis

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Table 1. Molecular size of *Eco*RI restriction endonuclease fragments of HearSNPV genomic DNAs from Iberian strains and HearSNPV-G4

	HearSNPV isolates								
Fragment size (kb)	SP1	SP2	SP4	SP7	SP8	PT1	PT2	G4	G4[c]
A	13.4	13.4	13.4	13.2	13.4	13.4	13.4	14.3	14.13
B	10.7[a]	13.2	10.7	10.0	10.7	10.7	10.7	13.4	13.45
C	9.3[a]	10.7	9.3	9.3	9.0	9.3	9.3	10.1	10.15
D	9.2	9.3	9.2	9.0	8.2	9.0	9.2	9.0	9.05
E	8.2[a]	9.2	8.2	8.2	7.5	8.2	8.2	6.6	6.64
F	7.1[a]	7.1	7.1	7.1	6.3	7.5	7.5	6.4	6.36
G	6.3[a]	6.3	6.3	6.3	6.0	6.3	6.3	6.3	6.29
H	6.0[a]	6.0	6.0	6.0	5.9	6.0	6.0	6.0	5.99
I	5.9[a]	5.9	5.9	5.9	5.8	5.9	5.9	5.8	5.84
J	5.8[a]	5.8	5.8	5.8	5.8	5.8	5.8	5.8	5.84
K	5.8[a]	5.7	5.8	5.8	5.7	5.8	5.8	5.7	5.67
L	5.7[a]	5.3	5.7	5.7	4.9	5.3	5.7	4.8	4.75
M	5.3[a]	4.9	4.9	4.9	4.6	4.9	5.3	4.6	4.58
N	4.9[a]	4.6	4.6	4.6	4.4	4.6	4.9	4.4	4.42
O	4.6[a]	4.4	4.4	4.4	4.4	4.4	4.6	4.4	4.4
P	4.4[a]	4.4	4.4	4.4	3.3	4.4	4.4	4.1	4.14
Q	4.4[a]	3.3	3.3	3.3	3.0	3.3	4.4	3.7	3.68
R	3.3[a]	3.0	3.0	3.0	2.8	3.0	3.3	3.4	3.36
S	3.0[a]	2.8	2.8	2.8	1.7	2.8	3.0	3.0	3.0
T	2.8[a]	1.7	1.7	1.7	1.0	1.7	2.8	2.8	2.83
U	1.7[a]	1.0	1.0	1.0	1.0	1.0	1.7	1.7	1.74
V	1.0[a]	1.0	1.0	1.0	0.8	1.0	1.0	1.5	1.48
X	1.0[a]	0.8	0.8	0.8	—	0.8	1.0	1.0	1.0
Y	0.8[a]	—	—	—	—	—	0.8	0.8	0.78
Z	0.5[b]	—	—	—	—	—	—	—	0.48
a	0.4[b]	—	—	—	—	—	—	—	0.45
b	0.4[b]	—	—	—	—	—	—	—	0.41
c	0.3[b]	—	—	—	—	—	—	—	0.31
d	0.18[b]	—	—	—	—	—	—	—	0.18
e	0.02[b]	—	—	—	—	—	—	—	0.02
Total	132.4	129.8	125.3	124.2	116.2	125.1	131.0	129.6	131.4

Note: DNA fragments are named alphabetically, starting with A for the largest fragment and their sizes are given in kb.
[a] Fragments cloned into pUC19 vector and showed in REN profiles.
[b] Fragments generated by PCR amplification using primers on each side of the gaps (Table 2) and not visible by REN.
[c] HearSNPV-G4 *Eco*RI fragments generated *in silico* using Clone Manager 9 (Scientific & Educational Software).

suggested that HearSNPV-SP4 was the least related to the other Iberian strains. In turn, the Iberian strains were more closely related to the isolate from Kenya (HearSNPV-NNg1) than to the isolates from China (HearSNPV-C1 and HearSNPV-G4) or Australia (HearSNPV-Aus), or HzSNPV.[29]

3.4 OB pathogenicity, virulence and productivity of the Iberian HearSNPV strains

OB pathogenicity values of each of the Iberian strains were compared with that of HearSNPV-G4 OBs in second instars from the Oxford colony. LC_{50} values ranged from 1.5×10^4 OBs mL^{-1} for HearSNPV-G4 to 3.4×10^4 OBs mL^{-1} for HearSNPV-SP2. The 95% fiducial limits of the relative potency values, representing the ratio of LC_{50} values,[30] overlapped broadly in the eight viruses, indicating no significant strain-specific differences in the LC_{50} values of these strains in the Oxford colony insects (Table 4).

MTD of the different isolates in second instars was in the range 108–138 hours post-inoculation (hpi). All died larvae showed the typical signs of lethal polyhedrosis disease. The HearSNPV strains could be classified into three groups according to their speed of kill (Table 4). HearSNPV-SP1, with a MTD of 108.4 hpi, was the fastest killing isolate followed by HearSNPV-G4, HearSNPV-SP2, HearSNPV-PT1 and HearSNPV-PT2 with MTD values between 126.1 and 129.6 hpi, while HearSNPV-SP4, HearSNPV-SP7 and HearSNPV-SP8 with MTD values in the range 133.0–137.7 hpi, were the slower killing isolates.

The OB production values in fourth instars differed significantly among strains ($F_{7,16} = 5.88$, $P = 0.002$). Larvae infected with HearSNPV-SP1, HearSNPV-SP4 and HearSNPV-SP7 produced the highest number of OBs with values between 3.1×10^9 and 4.3×10^9 OBs $larva^{-1}$ (Fig. 4). HearSNPV-SP2, HearSNPV-SP8 and HearSNPV-G4 showed intermediate levels of OB production with 2.0×10^9 to 3.0×10^9 OBs $larva^{-1}$, while HearSNPV-PT1 and HearSNPV-PT2 were the least productive isolates with 1.7×10^9 OBs $larva^{-1}$.

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Table 2. Primers used in the amplification of the gaps and designed on each side of the specific cloned *Eco*RI fragments of HearSNPV-SP1 genome for the construction of physical map, and primers used in the amplification of the partial *lef-8*, *lef-9* and *polh* genes of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1 and HearSNPV-PT2 for phylogenetic analysis

Purpose	Primer	Direction	Nucleotide position in HearSNPV-G4	Amplified fragment/gene
Physical map	5´-CAGTTTTGACAGTGGCAA-3´	Forward	8,443–8,460	*Eco*RI-a
	5´-GTGTTGGCAAATGTGTAC-3´	Reverse	9,085–9,102	
	5´-GTTACACGACACAGAACA-3´	Forward	37,578–37,595	*Eco*RI-Z
	5´-CCGCTTTAAACATTCAGCA-3´	Reverse	38,320–38,338	
	5´-CCTCCTGGTCATATCATGTG-3´	Forward	40,796–40,815	*Eco*RI-d
	5´-ACTTTGACCGCTGATCA-3´	Reverse	41,544–41,560	
	5´-GTCAAGATGGTTGCGTAAAG-3´	Forward	55,244–55,263	*Eco*RI-b/e
	5´-GCGTTTCATTCCTTTCTCTG-3´	Reverse	56,006–56,025	
	5´-GCCAACAGTATTTACGGA-3´	Forward	61,455–61,472	*Eco*RI-c
	5´-CAATGAGCACATACGGAA-3´	Reverse	62,188–62,205	
Phylogenetic analysis	5´-TACTCGTATGCGGTGAGCAG-3´	Forward	33,255–33,274	*lef-8*
	5´-CACCATGCGTCAAGATATGC-3´	Reverse	33,739–33,758	
	5´-CATCGTTCTATGCCAACGTG-3´	Forward	45,122–45,141	*lef-9*
	5´-GAGAGCACAATCGCGTAACA-3´	Reverse	45,620–45,639	
	5´-CAAATACTTGGTGGCGGAAG-3´	Forward	156–175	*polh*
	5´-TCCTCTTCTTCGGCAGAATC-3´	Reverse	640–659	

Table 3. Position and orientation of the 19 putative ORFs identified in the HearSNPV-SP1 genome by terminal sequencing of the cloned *Eco*RI fragments and complete sequencing of fragments amplified by PCR

				HearSNPV isolates **ORF**/% Identity (Similarity)			
Fragment	Gene family	Dir.[a]	Estimated size (aa) in this study	NNg1	G4	C1	Aus
*Eco*RI-R/a/B	*p49*	>	468	**9**/99 (100)	**9**/99 (100)	**9**/99 (100)	**9**/99 (100)
*Eco*RI-B	*p10*	<	87	**20**/100 (100)	**21**/100 (100)	**21**/100 (100)	**20**/100 (100)
*Eco*RI-B/F	*p26*	<	267	**21**/98 (98)	**22**/98 (98)	**22**/98 (98)	**21**/97 (98)
*Eco*RI-F	ubiquitin	>	83	**28**/98 (98)	**28**/98 (98)	**28**/98 (98)	**29**/98 (98)
*Eco*RI-F	unknown	>	168	**29**/100 (100)	**29**/99 (99)	**29**/99 (99)	**28**/99 (99)
*Eco*RI-G	unknown	>	80	**37**/97 (98)	**37**/98 (99)	**37**/98 (99)	**36**/98 (99)
*Eco*RI-L/Z	unknown	<	181	**42**/100 (100)	**42**/99 (100)	**42**/99 (100)	**41**/99 (100)
*Eco*RI-Z/S	unknown	>	136	**43**/100 (100)	**43**/100 (100)	**43**/99 (100)	**42**/100 (100)
*Eco*RI-S/d	unknown	>	68	**48**/100 (100)	**48**/100 (100)	**48**/100 (100)	**47**/100 (100)
*Eco*RI-d/N	unknown	>	64	**49**/100 (100)	**49**/100 (100)	**49**/100 (100)	**48**/98 (98)
*Eco*RI-b/e/J	unknown	<	133	**66**/100 (100)	**64**/100 (100)	**64**/100 (100)	**62**/99 (99)
*Eco*RI-T/M	*vlf-1*	<	415	**73**/99 (100)	**71**/98 (98)	**71**/99 (99)	**69**/99 (99)
*Eco*RI-X/Q	*cg30*	<	283	**79**/99 (99)	**77**/99 (99)	**77**/99 (99)	**75**/99 (99)
*Eco*RI-O/Y	unknown	>	173	**87**/100 (100)	**85**/99 (99)	**85**/99 (99)	**83**/100 (100)
*Eco*RI-Y/V	unknown	<	321	**88**/99 (99)	**86**/99 (99)	**86**/99 (99)	**84**/99 (99)
*Eco*RI-V/K	*lef-5*	>	315	**89**/99 (100)	**87**/100 (100)	**87**/100 (100)	**85**/100 (100)
*Eco*RI-K	*p6.9*	<	109	**90**/98 (99)	**88**/98 (99)	**88**/98 (99)	**86**/98 (99)
*Eco*RI-K	unknown	>	58	**95**/100 (100)	**93**/98 (100)	**93**/98 (100)	**91**/98 (100)
*Eco*RI-E	unknown	<	181	**142**/99 (100)	**135**/100 (100)	**134**/99 (99)	**133**/99 (99)

Note: The percentage of amino acid sequence identity and similarity to homologous ORFs of other HearSNPV isolates is shown.
[a] Direction of transcription in the same (>) or opposite (<) sense of *polyhedrin* gene.

3.5 Susceptibility of the Almerian colony to HearSNPV-SP1 and HearSNPV-G4

To determine host stage susceptibility the most virulent Iberian isolate, HearSNPV-SP1, was assayed against the Almerian colony, and was compared with HearSNPV-G4. LC_{50} values of HearSNPV-SP1 and HearSNPV-G4 were similar for all instars evaluated (Table 5). In addition, LC_{50} values for HearSNPV-SP1 did not vary significantly for second to fourth instars, whereas fifth instars were significantly less susceptible to infection (LC_{50} value of 2.9×10^5 OBs mL^{-1}) than the other younger stages tested. In contrast, HearSNPV-G4 showed similar LC_{50} values in second and third instars (3.3×10^4 and 1.4×10^4 OBs mL^{-1}, respectively) or in fourth

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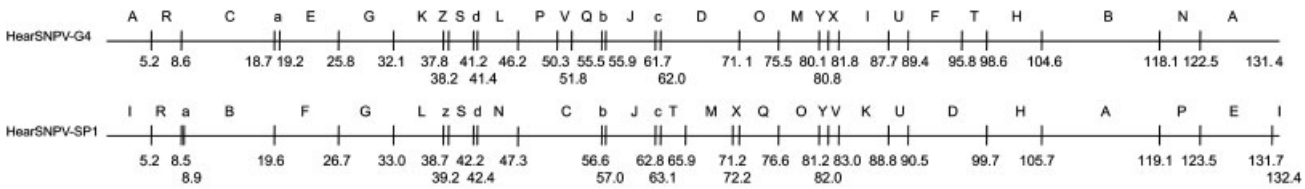

Figure 2. Physical maps based on *Eco*RI for HearSNPV-SP1 and the Chinese genotype HearSNPV-G4. The first nucleotide of the map is the first nucleotide of the *polyhedrin* gene. The circular HearSNPV DNA is represented in linear form. Nucleotide positions representing restriction sites are indicating below the maps.

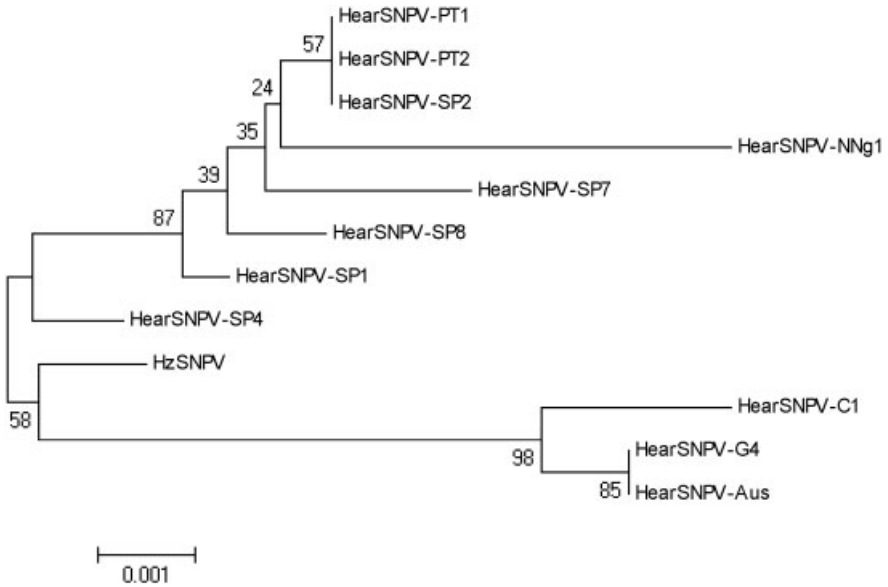

Figure 3. Phylogenetic analysis of *lef-8*, *lef-9* and *polh* partial sequences using MEGA5 software for alignment. Baculovirus sequences used in this phylogenetic analysis were GeneBank (accession numbers indicated in parentheses): *Helicoverpa armigera* (Hear) SNPV-C1 (AF303045), HearSNPV-G4 (AF271059), HearSNPV-NNg1 (AP010907), HearSNPV-Aus (JN584482) and *H. zea* (Hz) SNPV (AF334030).

and fifth instars (5.1×10^4 and 3.4×10^5 OBs mL^{-1}, respectively) (Table 5).

The speed of kill of HearSNPV-SP1 was significantly faster than that of HearSNPV-G4 in all insect stages (Table 5), and was negatively correlated with insect stage in both viruses. MTD values of HearSNPV-SP1 ranged between 134.1 and 144.9 h depending on instar, whereas the corresponding values for HearSNPV-G4 on the different instars were 145.1 to 163.0 h.

Finally, the OB production values for HearSNPV-SP1 and HearSNPV-G4 strains was significantly higher in fifth instar than in fourth instar ($F_{3,8} = 15.46$, $P = 0.001$), showing no significant differences between both strains for fifth instar (Tukey test, $P > 0.05$). However, the OB production value in fourth instar larvae was significantly lower for HearSNPV-SP1 (4.1×10^9 OBs $larva^{-1}$) than the production for HearSNPV-G4 (6.1×10^9 OBs $larva^{-1}$) (Tukey test, $P < 0.05$) (Fig. 5). When comparing the OB production of HearSNPV-SP1 and HearSNPV-G4 between insect colonies, we found that the OB production per larva was significantly higher in fourth instars from the Almerian colony compared to the Oxford colony insects ($F_{3,8} = 17.42$, $P = 0.001$), which was correlated with a slower speed of kill against the Almerian colony.

4 DISCUSSION

The objective of this study was to select an HearSNPV isolate that could be used as the basis for a biological insecticide against *Helicoverpa armigera* in Spain. For this, the genomic and biological characteristics of seven isolates from the Iberian Peninsula[4,19] were compared with those of HearSNPV-G4[7,31] using a long- and short-term laboratory colonies of *H. armigera*. These strains were previously characterized using restriction profiles and LC_{50} and MTD values against second instars from a Portuguese colony.[4,19] The present study extends these findings by: (1) constructing the physical map of HearSNPV-SP1 and comparing it with the completely sequenced genotype from China, HearSNPV-G4,[7] (2) establishing the phylogenetic relationships among Iberian isolates and other *Helicoverpa* spp SNPV,[7–9] (3) determining the host stage susceptibility to the most virulent Iberian isolate, HearSNPV-SP1, and the widely used Chinese isolate, HearSNPV-G4,[7] against a Spanish colony of *H. armigera*, (4) determining the OB production for strains HearSNPV-SP1 and HearSNPV-G4[7] in *H. armigera* fourth and fifth instars from the Spanish colony.

The genome sizes reported here are similar to the size of the HearSNPV-G4 genome (131.4 kb)[7] and similar to the sizes of HzSNPV genomes estimated by physical maps (119 or 125 kb).[32,33] Chen *et al.*[34] estimated the HearSNPV-G4 genome size at 130.1 kb. In the present study, the HearSNPV-SP1 isolate had the largest genome (132.4 kb), whereas the other isolates lacked some restriction fragments present in HearSNPV-SP1, suggesting a shorter genome. Phylogenetic analysis grouped Iberian strains together, suggesting a recent common ancestor. Iberian strains were more closely related to the isolate from Kenya (HearSNPV-NNg1) than to isolates from China (HearSNPV-C1 and

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Table 4. LC_{50} values, relative potencies and mean time to death (MTD) values of the seven HearSNPV Iberian strains and HearSNPV-G4 genotype in *Helicoverpa armigera* second instars from the Oxford colony

Virus	LC_{50} (OBs mL^{-1})	Slope ± SE	Relative Potency	95% Fiducial limits		MTD (h)	95% Fiducial limits	
				Low	High		Low	High
HearSNPV-G4	1.5×10^4	1.525 ± 0.145	1	—	—	126.1 b	123.7	128.4
HearSNPV-SP1	3.2×10^4	0.798 ± 0.154	0.5	0.3	1.1	108.4 a	106.6	110.3
HearSNPV-SP2	3.5×10^4	1.015 ± 0.160	0.4	0.2	1.2	128.1 b	125.9	130.3
HearSNPV-SP4	1.6×10^4	0.867 ± 0.135	1.0	0.6	1.7	136.5 d	134.0	139.1
HearSNPV-SP7	3.3×10^4	0.943 ± 0.162	0.5	0.2	1.2	133.0 cd	130.7	135.4
HearSNPV-SP8	2.4×10^4	1.099 ± 0.157	0.6	0.3	1.2	137.7 d	135.3	140.2
HearSNPV-PT1	2.6×10^4	1.415 ± 0.175	0.6	0.4	1.3	128.5 bc	126.2	130.8
HearSNPV-PT2	2.0×10^4	1.732 ± 0.219	0.8	0.5	1.4	129.6 bc	127.2	132.0

Probit regressions were fitted in POLO Plus. A test for non-parallelism was significant ($\chi^2 = 29.2$, df = 7, $P < 0.001$). Relative potencies were calculated as the ratio of LC_{50} values relative to the HearSNPV-G4 strain. Mean times to death (MTD) values were estimated by Weibull analysis. Values followed by different letters differ significantly (*t*-test, $P < 0.05$).

HearSNPV-G4) or Australia (HearSNPV-Aus), possibly due to the geographical proximity of Africa compared to Asia or Australasia.

Iberian strains were as pathogenic to experimental insects as isolates from Kenya, South Africa and China.[6,14] However significant differences were observed in virulence – MTD values differed by 29 h with HearSNPV-SP1 being a notably fast-killing strain. This contrasts with HearSNPV isolates from China that differed by 14 h in speed of kill.[35] In this study, MTD values for HearSNPV-G4 were lower than that reported by Guo *et al.*,[35] probably due to the earlier instar insects used in our study.

The OB production in infected insects quantified in this study for HearSNPV-G4 was similar to that reported previously.[36] However, insects infected with HearSNPV-SP1, HearSNPV-SP4 and HearSNPV-SP7 produced a significantly greater number of OBs than those infected with HearSNPV-G4. Virulence (speed of kill) is often negatively correlated with OB production as fast killing viruses have less time to replicate and hosts have less time to develop and gain body weight.[12,15,37,38] However in our study, HearSNPV-SP1 was the fastest killing isolate but paradoxically produced as many OBs per larva as other slower-killing isolates. As transmission depends on the consumption of OBs on plant foliage, OB production per larva is likely to be advantageous to the transmissibility of the virus and may also improve the likelihood of OB persistence outside the host insect.[39] OB production per insect is also important for reducing the cost of virus mass production technology during the development of a biological insecticide. Differences in speed of kill and productivity values among the different Iberian strains against the laboratory colony are likely to be related to differences at the genomic level that can only be revealed by genomic sequencing.

The Iberian strains showed similar OB pathogenicities against the Oxford colony insects whereas a previous study on several of these isolates reported significant differences in OB pathogenicity against an insect colony from Portugal.[19] In general, colony-dependent differences in biological activity have been observed in NPVs and often conclude that local insect populations tend to be more susceptible to local virus isolates than to geographically distant strains.[15,40] As a result, prior to development of an isolate as the basis for a biological insecticide, the susceptibility of local insect colonies to native isolates has to be evaluated. For this, an insect colony from Almería (Spain) was used to determine the host stage response to the most virulent isolate, HearSNPV-SP1,

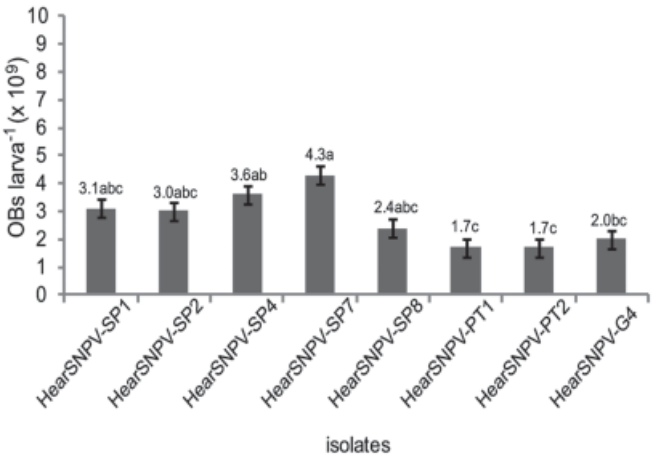

Figure 4. Median OB production ($\times 10^9$ OBs $larva^{-1}$) of *Helicoverpa armigera* fourth instars from the Oxford colony that died from polyhedrosis disease following infection with 10^6 OBs mL^{-1} of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4 strains. Values followed by identical letters did not differ significantly by ANOVA and Tukey test ($P < 0.05$).

and was compared to that of the Chinese genotype HearSNPV-G4. The host stage response of the colony from Almería that was originated from a field population was as susceptible to HearSNPV-SP1 as it was to the purified genotype HearSNPV-G4 across all instars tested. Similar patterns of instar-related susceptibility have been observed in *Helicoverpa armigera* larvae inoculated with HearMNPV.[41] The majority of studies have reported that susceptibility to infections tends to decrease with larval age both within and between instars.[42–45] This developmental resistance is related to the primary infection process in the midgut, probably involving a combination of variation in the porosity of the peritrophic matrix and an increased rate of sloughing of midgut cells in later instars.[46–48] However, in this case, the susceptibility of second, third and fourth instar to HearSNPV-SP1 was similar. This could be advantageous following applications of OBs in the field, as natural pest populations usually consist of a mixture of different larval stages present at the same time and place, and it is desirable to be able to control most of larval stages present following a single OB application. HearSNPV-SP1 killed the

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Table 5. LC_{50} values, relative potencies and MTD values of HearSNPV-SP1 and HearSNPV-G4 in *Helicoverpa armigera* second, third, fourth and fifth instars from the Almerian colony.

					95% Fiducial limits			95% Fiducial limits	
Instar	Virus	LC_{50} (OBs mL^{-1})	Slope ± SE	Relative potency	Low	High	MTD (h)	Low	High
L_2	HearSNPV-G4	3.3×10^4	0.513 ± 0.127	1	—	—	145.1 c	141.9	148.3
	HearSNPV-SP1	1.2×10^4	1.244 ± 0.156	2.8	1.0	8.3	134.1 a	131.1	137.1
L_3	HearSNPV-G4	1.4×10^4	0.901 ± 0.127	2.5	0.8	7.2	156.0 d	153.4	158.6
	HearSNPV-SP1	1.1×10^4	0.740 ± 0.126	3.0	1.0	9.1	136.5 ab	134.5	138.6
L_4	HearSNPV-G4	5.1×10^4	0.567 ± 0.135	0.7	0.2	2.3	156.8 de	153.7	159.9
	HearSNPV-SP1	2.2×10^4	0.631 ± 0.125	1.5	0.4	5.5	142.1 bc	137.3	147.0
L_5	HearSNPV-G4	3.4×10^5	0.792 ± 0.126	0.1	0.03	0.3	163.0 e	159.5	166.7
	HearSNPV-SP1	2.9×10^5	0.661 ± 0.120	0.1	0.04	0.4	144.9 c	141.6	148.3

Probit regressions were fitted in POLO Plus. A test for non-parallelism was significant ($\chi^2 = 19.0$, df = 7, $P < 0.001$). Relative potencies were calculated as the ratio of LC_{50} values relative to the HearSNPV-G4 strain in second instars. Mean time to death (MTD) values were estimated by Weibull analysis. Values followed by different letters differ significantly (*t*-test, $P < 0.05$).

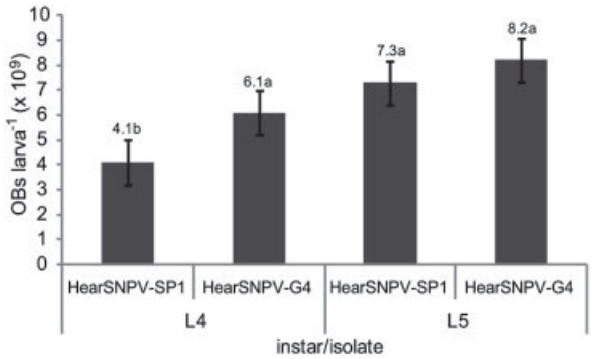

Figure 5. Median OB production (× 10^9 OBs $larva^{-1}$) of *Helicoverpa armigera* fourth and fifth instars from the Almerian colony that died from polyhedrosis disease after infection with 2.4×10^6 and 2.5×10^7 OBs mL^{-1} of HearSNPV-SP1, respectively, and with 1.2×10^6 and 1.4×10^7 OBs mL^{-1} of HearSNPV-G4. Values followed by identical letters did not differ significantly by ANOVA and Tukey test ($P < 0.05$).

Almerian colony insects faster across all instars than HearSNPV-G4, whereas OB production in insects from this colony was similar in both virus strains. Finally, when comparing at the population level, the present study showed that the virulence of HearSNPV-SP1 and HearSNPV-G4 was lower and resulted in higher OB productivity against the Almerian insects than the Oxford colony insects. However, this difference in virulence and OB production per larva could be due to differences in the size of insects from different colonies or their susceptibility to virus replication, resulting in the observed differences in these variable between the Oxford colony and Almerian colony insects. This difference in MTD between *H. armigera* populations was also reported in previous studies.[49] In the field, virus survival depends on a diversity of biotic and abiotic factors including climatologic conditions, so the probability of virus survival under field conditions would be expected to increase with increasing OB productivity and pathogenicity.

In summary, when tested against a long-standing colony from Oxford, HearSNPV-SP1 was identified as the fastest killing isolate compared to the other Iberian strains tested. HearSNPV-SP1 also presented a rapid speed of kill against different instars from a recently established Spanish colony. Baculovirus based insecticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest. In this respect, the similar pathogenicity but the higher virulence of HearSNPV-SP1 suggest that this isolate is likely to prove useful as the active material for development of a virus-based biological insecticide for control of *Helicoverpa armigera* populations in Spain.

ACKNOWLEDGEMENTS

The authors thank Noelia Gorría and Itxaso Ibáñez for insect rearing, and Jesús Murillo for technical assistance. This study was funded by MEC project numbers AGL2008-05456-CO3-01 and AGL2011-30352-CO2-01 as well as the Gobierno de Navarra project IIQ14065.RI1. M.A. received a pre-doctoral fellowship from CSIC.

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