Biotechnological development of a new bioinsecticide based on a *Helicoverpa armigera* nucleopolyhedrovirus from Spain

MAITE ARRIZUBIETA CELAYA

Pamplona - Iruña, 2015

Programa de Doctorado en Biotecnología
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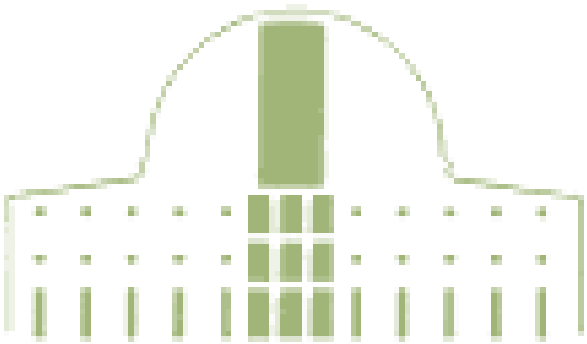

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MAITE ARRIZUBIETA CELAYA

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Biotechnological development of a new bioinsecticide based on a *Helicoverpa armigera* nucleopolyhedrovirus from Spain

Directores: Dr. PRIMITIVO CABALLERO MURILLO
Catedrático de Universidad
Departamento de Producción Agraria
Universidad Pública de Navarra

Dra. OIHANE SIMÓN DE GOÑI
Investigadora Ramón y Cajal
Instituto de Agrobiotecnología
CSIC-Universidad Pública de Navarra

Instituto de Agrobiotecnología
Universidad Pública de Navarra
Pamplona-Iruña, 2015

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Universidad de Córdoba

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Gesellschaft für Biotechnologie und Biologischen Pflanzenschutz mbH
e-nema

Revisores externos

Dra. Elisabete Figueiredo
Departamento de Protecção das Plantas e de Fitoecologia
Universidade Técnica de Lisboa

Dr. Benoit Graillot
Laboratoire de Génie de l´Environnement Industriel et des Risques Industriels et Naturels ((LGEI)
École des Mines d´Alès, Alès Cedex, Francia

Suplente

Dr. António Mexia
Departamento de Protecção das Plantas e de Fitoecologia
Universidade Técnica de Lisboa

Dr. PRIMITIVO CABALLERO MURILLO, Catedrático de Universidad del área de Producción Vegetal del Departamento de Producción Agraria y Director del Grupo de Investigación Bioinsecticidas Microbianos del Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC,

Dra. OIHANE SIMÓN DE GOÑI, Investigadora Ramón y Cajal del Instituto de Agrobiotecnología, Universidad Pública de Navarra-CSIC, y

INFORMAN:

que la presente memoria de Tesis Doctoral titulada **"Biotechnological development of a new bioinsecticide based on a *Helicoverpa armigera* nucleopolyhedrovirus from Spain"** elaborada por Dña. **MAITE ARRIZUBIETA CELAYA** ha sido realizada bajo nuestra dirección, y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona a 29 de abril de 2015,

Fdo. Dr. Primitivo Caballero Murillo

Fdo. Dra. Oihane Simón de Goñi

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RESUMEN

El taladro del tomate, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), una plaga mundial muy importante de cultivos de invernadero y de campo abierto, es una plaga clave de tomate en la Península Ibérica (España y Portugal). Los problemas asociados con el uso de insecticidas químicos, unidos a las resistencias desarrolladas por las poblaciones de *H. armigera*, han puesto de manifiesto la necesidad de desarrollar métodos de control alternativos, entre ellos los baculovirus. Así trabajos recientes han demostrado que el nucleopolyhedrovirus simple de *H. armigera* (HearSNPV) (*Alphabaculovirus*) es un agente eficaz para el control de *H. armigera* en otros cultivos en otras regiones. En la Península Ibérica, a pesar de que se han caracterizado varios aislados hasta la fecha ninguno de ellos se ha desarrollado como bioinsecticida. Por lo tanto en la presente tesis se han llevado a cabo los desarrollos biotecnológicos necesarios para obtener un producto eficiente basado en HearSNPV.

En primer lugar se evaluó la diversidad inter-poblacional del HearSNPV en la Península Ibérica. Para ello, se realizó la caracterización molecular y biológica de siete aislados procedentes de diferentes regiones, que se compararon con el genotipo de referencia, el genotipo chino HearSNPV-G4 (HearG4). Los valores de patogenicidad de los diferentes aislados no presentaron diferencias significativas frente a una población de *H. armigera* procedente de Oxford. Sin embargo, sí hubo diferencias en la virulencia. HearSNPV-SP1 (HearSP1) fue el aislado más rápido, y además fue uno de los más productivos. HearSP1 y HearG4 fueron igual de patogénicos frente a una población establecida recientemente procedente del sur de España, aunque HearSP1 fue más rápido matando a las larvas.

Tras ello, se evaluó la diversidad intra-poblacional de HearSP1 con el objetivo de identificar mezclas de genotipos con características insecticidas mejoradas. Únicamente se pudieron clonar dos variantes genotípicas, HearSP1A y HearSP1B, *in vitro*. También se evaluó la diversidad genotípica de varios aislados de HearSNPV obtenidos a partir de larvas recogidas en cultivos de algodón en el sur de España, y que murieron a causa del virus durante la cría en laboratorio. En este caso, se obtuvieron seis variantes genotípicas (HearLB1-HearLB6) mediante ensayo de dilución límite. El genotipo HearSP1B resulto ser ~3 veces más patogénico que el aislado silvestre HearSP1 y demás genotipos. En cuanto a las variantes HearLB, no se observaron diferencias de patogenicidad, pero HearLB1 y HearLB6 fueron de los genotipos más virulentos, siendo más rápidos incluso que aislado silvestre HearSP1. Además HearLB1 fue muy productivo, a pesar de ser una de los genotipos más rápidos, mientras HearLB3 y HearLB6 mostraron valores intermedios de

producción de OBs. Con el objetivo de seleccionar una mezcla de genotipos con mejores cualidades insecticidas, se construyeron ocho mezclas co-ocluidas tras infección *per os* utilizando cinco genotipos, que se seleccionaron en base a sus propiedades insecticidas (HearSP1A, HearSP1B, HearLB1, HearLB3 y HearLB6). La mezcla co-ocluida HearSP1B:LB6 en una proporción 1:1 fue entre 1.7-2.8 veces más patogénica que cualquiera de los genotipos individuales y mezclas ensayadas, y también mató a las larvas tan rápido como los genotipos más virulentos. La sucesión de pases *in vivo* modificó las proporciones de los genotipos que conforman la mezcla HearSP1B:LB6. Tras cinco pases, los OBs recuperados, compuestos por un 85% de HearSP1B y un 15% de HearLB6, fueron más patogénicos y virulentos que la mezcla original en proporción 1:1, lo que sugiere que la mezcla co-ocluida obtenida tras cinco pases *in vivo* es más adecuada como ingrediente activo para un producto biopesticida. Por lo tanto, este proceso de pases sucesivos podría mejorar aún más la transmisibilidad de la mezcla.

Con el objetivo de detectar los cambios genéticos responsables de estas diferencias en el fenotipo, se realizó la secuenciación completa del genoma de estos 5 genotipos. Estos genotipos mostraron un alto grado de identidad a nivel de nucleótido entre ellos (98-99%) y también con los aislados de HearSNPV y HearMNPV previamente secuenciados (95-99%). Las mayores diferencias entre todos estos genotipos se localizan en las *hrs* y en los genes *bro*. Además, se identificaron mutaciones puntuales en genes implicados en la replicación del ADN (*ie-1*, *lef-3*, *DNA polimerasa*), la transcripción viral (*lef-8*, *lef-1*), o genes estructurales (*p78/83*, *desmop*, *vp1054*, *calix/pep*, *odv-e66*), que podrían ser responsables de la reducida producción de OBs de los genotipos de HearSP1 o el aumento de la patogenicidad de HearSP1B. También, se identificaron diversas mutaciones localizadas en los genes *iap-2*, *iap-3* y *hoar* que podrían estar relacionadas con la estrategia de transmisión o con la capacidad para establecer infecciones encubiertas en el insecto huésped. El análisis de presión de selección mostró una fuerte selección en genes implicados en la transmisión y la dispersión del virus; como los implicados en la infección primaria (*p78/83*, *odv-e56 y p74*), la infección o replicación viral (*ie-0*), o la producción de OBs (*egt*). Esto indica claramente que estos procesos son importantes para la adaptación del virus a distintos huéspedes. Otros genes seleccionados positivamente de función desconocida (*bro-d*, *bv-ec31* y ORF130) están bajo investigación.

Aunque la mezcla HearSP1B:LB6 mostró unas características muy deseables para su desarrollo como bioinsecticida, su especificidad puede ser un factor limitante en tratamientos en campo, ya que será necesario el uso de ingredientes activos adicionales para el control de otras especies de plagas de lepidópteros que infecten el mismo cultivo. Desde esta perspectiva, el uso de baculovirus con un espectro de huéspedes más amplio, como HearMNPV, sería más deseable. Con el objetivo de ampliar el espectro de huéspedes de HearSNPV se aplicó la tecnología de co-oclusión de baculovirus para obtener muestras

de OBs en las que se encontrasen co-envueltos HearSNPV y HearMNPV, para obtener una mezcla con las características insecticidas deseables de HearSNPV y el amplio espectro de huéspedes de HearMNPV. Para la co-oclusión, se infectaron larvas de *H. armigera* primero con el virus menos patogénico, HearMNPV, para favorecer su replicación, y después a diferentes intervalos de tiempo con HearSNPV. Cuando el segundo virus se administró 12 o 24 h más tarde, los genomas de ambos se co-ocluyeron en los OBs en la misma proporción (1:1). Sin embargo, aunque HearSNPV constituyese el 50% de los OBs, sólo el 1% de sus nucleocápsidas se con-envolvieron en ODVs múltiples, sugiriendo que la mayor parte de las nucleocápsidas de HearSNPV se envolvieron de forma simple en los ODVs y que las mayoría de las nucleocápsidas múltiples la conformaron los genotipos HearMNPV. Sin embargo, la mezcla co-envuelta no presentó mejores características fenotípicas contra especies homólogas y heterólogas, pero aumentó el espectro de huéspedes de HearSNPV, ya que este virus fue capaz de entrar e infectar a especies no susceptibles como *S. frugiperda* y *M. brassicae*. Por otra parte, HearSNPV permaneció en la progenie de OBs durante al menos cinco pases sucesivos en estas dos especies no permisivas.

El sistema de producción masiva es una de las principales limitaciones en el desarrollo de baculovirus como bioinsecticidas. Para optimizar la producción de HearSP1B:LB6, se evaluaron los efectos de canibalismo, estadio larvario, la concentración del inóculo y la temperatura. El alto grado de canibalismo (>30%) indicó que los insectos deben ser criados individualmente después de la infección. Las larvas inoculadas un día después de mudar a L_4 y L_5 y las L_5 recién mudadas produjeron las mayores cantidades de OBs (6,7-9,1 x 10^9 OBs/larva). Sin embargo, la prevalencia de mortalidad (85,7%) fue notablemente mayor en larvas recién mudadas a L_5 que en las inoculados un día después de la muda (19,5%), lo que resultó en un incremento significativamente mayor de OBs global (6,0 x 10^{11} OBs/100 larvas inoculadas). La CL_{80} causó una mortalidad y una producción de OBs similar a la de concentraciones más altas en larvas L_5 recién mudadas. Por último, la temperatura de incubación no influyó en la producción de OBs, aunque las larvas incubadas a 30ºC murieron 13 y 34 horas antes que los incubadas 26ºC y 23ºC, respectivamente. La alta temperatura de incubación no afectó tampoco a las características insecticidas de los OBs. Por lo tanto, una producción eficiente de la mezcla co-ocluida HearSP1B:LB6 se lograría inoculando larvas L_5 recién mudadas de *H. armigera* con la CL_{80} (5,5 x 10^6 OBs/ml), e incubando las larvas individualizadas a 30ºC.

Por último, se determinó la eficacia y la persistencia de HearSP1B:LB6 en cultivos de tomate en invernadero y en campo abierto en Portugal y España. En cultivos de tomate protegidos, HearSP1B:LB6 fue tan eficaz como *Bacillus thuringiensis* (Bt) y spinosad reduciendo el porcentaje de frutos dañados, y además produjo una mortalidad larvaria mayor que Bt. En cultivos de tomate en campo abierto HearSP1B:LB6 fue igual de eficaz reduciendo el porcentaje de frutos dañados que spinosad, Bt y clorpirifos. La persistencia

de los insecticidas en plantas de tomate se correlacionó con la radiación solar en ambas condiciones. La persistencia de los OB en cultivos de tomate protegidos 6 días después de la aplicación fue un 55% y un 35% mayor que la de Bt y spinosad, respectivamente, lo que sugiere que las larvas pueden adquirir la infección viral varios días después del tratamiento. Sin embargo, en campo abierto la persistencia de los OBs fue significativamente menor que la de spinosad o clorpirifos. Estos resultados demuestran que HearSP1B:LB6 puede ser utilizado para controlar eficazmente las plagas ocasionadas por *H. armigera*.

Toda esta información ha sido objeto de una solicitud de patente (P201430956), y constituye la base para el desarrollo de un nuevo bioinsecticida. Este nuevo bioinsecticida es una herramienta muy útil para el establecimiento de una agricultura sostenible en los cultivos de tomate en la Península Ibérica, ya que puede ser incluido en programas de Manejo Integrado de Plagas.

SUMMARY

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), an important pest of field and greenhouse crops worldwide, is the key pest of tomato in the Iberian Peninsula (Spain and Portugal). Problems associated with chemical control of *H. armigera* populations, including resistance, have highlighted the need to develop alternative control methods, among which baculoviruses are top-ranking. The *H. armigera* single nucleopolyhedrovirus (HearSNPV) (*Alphabaculovirus*) has shown to be an effective agent for *H. armigera* control in other crops elsewhere. In the Iberian Peninsula, although several HearSNPV local isolates have been characterized, none has been developed yet as a bioinsecticide. The aim of this thesis was to conduct the biotechnological developments necessary to obtain an efficient HearSNPV-based product.

Firstly, the intraspecific diversity of Iberian HearSNPV isolates was evaluated at an interpopulation level. Seven isolates from different geographical regions were subjected to molecular and biological characterization and compared with the HearSNPV reference genotype, the Chinese HearSNPV-G4 (HearG4). Pathogenicity values did not differ significantly among Iberian isolates against a *H. armigera* colony from Oxford. However differences were found in virulence. HearSNPV-SP1 (HearSP1) was the fastest killing isolate while it was also among the most productive. HearSP1 and HearG4 were similarly pathogenic against a colony from southern Spain, but HearSP1 killed larvae faster.

Subsequently, the intrapopulation diversity of HearSP1 was evaluated with the aim of identifying mixtures of genotypes with improved insecticidal characteristics. Only two genotypic variants, HearSP1A and HearSP1B, were cloned *in vitro*. The diversity found within HearSNPV isolates obtained from larvae collected in cotton crops in southern Spain that died later on from virus disease during laboratory rearing was also evaluated. In this case, six genotypic variants (HearLB1 to HearLB6) were obtained by end point dilution. Phenotypic characterization revealed that HearSP1B was ~3 fold more pathogenic than HearSP1 and all the other variants. As for the HearLB variants, no differences were found in pathogenicity, but HearLB1 and HearLB6 were among the most virulent and killed larvae even faster than HearSP1-wt. Also, HearLB1 was highly productive, despite being one of the fastest-killing variants, whereas HearLB3 and HearLB6 showed intermediate OB yield values. With the aim of selecting a genotypic mixture with improved insecticidal activities, eight co-occluded mixtures were constructed following *per os* inoculation with five genotypes, which were selected on their insecticidal performance (HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6). The co-occluded mixture HearSP1B:LB6 at a 1:1 proportion was 1.7-2.8 fold more pathogenic than any single variant or any other mixture

tested, and also killed larvae as fast as the most virulent genotypes. Serial passage resulted in modified proportions of the component variants within the HearSP1B:LB6 co-occluded mixture. After five passages, the recovered OBs were composed of 85% HearSP1B and 15% HearLB6 and this mixture was more pathogenic and virulent than the original mixture at 1:1 proportion, suggesting that the co-occluded mixture obtained after five passages *in vivo* is the most suitable active ingredient for a biopesticide product. Therefore, serial passage could further improve the mixture transmissibility.

The complete genomic sequencing of these five genotypes was performed with the aim of detecting genetic changes responsible for the observed differences in phenotype. A high degree of identity was observed at the nucleotide level between them (98-99%), and also when compared to sequences from other HearSNPV isolates and from HzSNPV (95-99%). Major genomic differences between all the genotypes were located in the *hrs* and *bro* genes. Additionally, point mutations were found in genes involved in DNA replication (*ie-1*, *lef-3*, *DNA polymerase*), viral transcription (*lef-8*, *lef-1*) or structural genes (*p78/83*, *vp1054 desmop*, *calyx/pep*, *odv-e56*), which could be responsible for the reduced OB production of HearSP1 genotypes or the increased pathogenicity of HearSP1B. Also, several mutations were found in *iap-2*, *iap-3* and *hoar* genes, which could be related with transmission or with the ability to establish a covert infection in the insect host. Selection pressure analysis showed strong selection in genes involved in viral transmission and dispersion; such as those involved in the primary infection (*p78/83*, *odv-e66* and *p74*) virus infection and replication (*ie-0*) or OB production (*egt*). This clearly indicates that these processes are important features for virus adaptation to distinct hosts. Other positively selected genes of unknown function (*bro-d*, *bv-ec31* and ORF130) are under research.

Although the HearSP1B:LB6 mixture showed desirable characteristics for bioinsecticide development, its specificity constituted a limiting factor for field treatments, as additional active ingredients may be needed to control other lepidopteran pests species infesting the same crop. From this perspective, the use of a baculovirus with a broader host range, such as HearMNPV, is more desirable. With the aim of extending the host range of HearSNPV, a co-occlusion baculovirus technology was applied to HearSNPV and HearMNPV, to obtain a virus product comprising the desirable insecticidal characteristics of HearSNPV and the broader host range of HearMNPV. For co-occlusion, *H. armigera* larvae were infected first with the less pathogenic virus HearMNPV to favour its replication and afterwards, at different time intervals, with HearSNPV. When the second virus was administered 12-24 h later, the genomes of both viruses were co-occluded in the OBs at the same proportion (1:1). However, although HearSNPV constituted 50% of the OBs, only 1% of their nucleocapsids were co-enveloped in multiple ODVs, suggesting that most of the HearSNPV nucleocapsids were singly enveloped in ODVs and most of the multiple-nucleocapsids comprised HearMNPV genotypes. Nevertheless, the co-enveloped mixture

did not present improved phenotypic characteristics against homologous and heterologous species, but increased the HearSNPV host-range, as this virus was able to enter and infect non-susceptible species, such as *S. frugiperda* and *M. brassicae*. Moreover, HearSNPV remained in the OB progeny during at least five serial passages in these two non-permissive insect species.

The mass production system is one of the greatest limitations when developing baculoviruses as bioinsecticides. To optimize HearSP1B:LB6 production, the effects of cannibalism, larval instar, inoculum concentration and temperature in the final OB yield were evaluated. The high prevalence of cannibalism (>30%) indicated that insects should be reared individually after infection. Larvae inoculated one day after molting to L_4 and L_5, and right after molting to L_5 produced the greatest quantities of OBs (6.7-9.1 x 10^9 OBs/larva). However, the prevalence of mortality (85.7%) was markedly higher in recently molted L_5 larvae than in those inoculated one day after molting (19.5%), resulting in a significantly greater overall OB yield (6.0 x 10^{11} OBs/100 inoculated larvae). The LC_{80} caused similar mortality and OB production to that of higher concentrations in recently molted L_5 larvae. Finally, incubation temperature did not influence OB yield, although larvae maintained at 30ºC died 13 and 34 h earlier than those incubated at 26ºC and 23ºC, respectively. The high incubation temperature did not either affect the insecticidal characteristics of OBs. Therefore, an efficient production of HearSP1B:LB6 co-occluded mixture might be achieved by inoculating L_5 newly molted *H. armigera* with the LC_{80} (5.5 x 10^6 OBs/ml), followed by incubation of individualized larvae at 30ºC.

Finally, the insecticidal efficacy and persistence of HearSP1B:LB6 were determined in greenhouse and open-field tomato crops in Portugal and Spain. In protected tomato crops, treatment with HearSP1B:LB6 was as effective as *Bacillus thuringiensis* (Bt) and spinosad in reducing the percentage of damaged fruits, and produced higher larval mortality than Bt. In open-field tomato crops, HearSP1B:LB6 treatment was as effective in reducing the percentage of damaged fruits as spinosad, Bt and chlorpyrifos. The persistence of the insecticides on tomato plants was correlated with the solar radiation in both conditions. OB persistence on protected tomato crops at day 6 post-application was 55% and 35% higher than that of Bt and spinosad, respectively, indicating that larvae could acquire a virus infection several days after virus treatment. However, on field-grown tomatoes, OB persistence was significantly lower than that of spinosad or chlorpyrifos. These results demonstrate that HearSP1B:LB6 can be used to effectively control *H. armigera* pest outbreaks.

All this information has been subjected to a patent application (P201430956), and constitutes the basis for the development of a new bioinsecticide that will likely become a very useful tool for the establishment of a sustainable agriculture in tomato crops in the Iberian Peninsula, since it can be included in Integrated Pest Management programs.

CHAPTER I

Introduction

1. General introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is an important pest of field and greenhouse crops distributed worldwide (Czepak et al., 2013; Fitt, 1989). In Asia, Europe, Africa and Australasia, annual damage by *H. armigera* exceed US$2 billion in more than sixty cultivated plant species (Tay et al., 2013). Moreover *H. armigera* has recently invaded the American continent, where it causes greater economic losses than *H. zea* (Pogue, 2004). In the Iberian Peninsula (Spain and Portugal), *H. armigera* larvae originate serious injuries in peppers, cotton, maize, and a diversity of greenhouse vegetables and ornamental crops (Torres-Vila et al., 2002a). In particular, *H. armigera* is a key pest in tomatoes (Torres-Vila et al., 2003a), one of the most important crops in Spain, with an acreage of 59,300 hectares (ha) and a production over 4.3 mill. Tn/yr, making Spain the fourth largest tomato producing country, after the US (California), China and Italy (MAGRAMA, 2013). In Portugal, *H. armigera* also feeds on tomato, an important crop with a production over 1.1 mill. Tn/yr in 15,300 ha (INE, 2011). Although larvae may attack any phenological stage of this crop, flowering is the most susceptible one and, what is more, flowers and fruits are the preferred feeding organs, which lead to direct injuries in crop yields. This, coupled with other biological characteristics such as high mobility and high fecundity, make this insect a key pest (Fitt, 1989).

H. armigera pest populations have been traditionally controlled with chemical treatments, but they have turned ineffective as wild populations began to develop insecticide resistance (McCaffery, 1998; Torres-Vila et al., 2002a, b). The efficacy of natural insecticides, such as spinosad and neem-derived products, against *H. armigera* has been assayed in several crops, and showed similar protection than chemical insecticides (Gosh et al., 2010; Kranthi et al., 2000; Shankar and Parmar, 1999; Sharma et al., 1999a; Vojoudi et al., 2011; Vyas et al., 1999). In the Iberian Peninsula, *H. armigera* populations could be efficiently controlled by egg and larval parasitoids (Izquierdo et al., 1994; Meierrose and Araujo, 1986; Torres-Vila et al., 2000). Moreover, microbiological control based on entomopathogens such as *Metarhizium anisopliae* and *Bacillus thuringiensis* was also effective (Avila et al., 2005; Chakrabarti et al., 1998; Gajendra-Babu et al., 2002; Jalali et al., 2004; Kulkarni et al., 2008; Nahar et al., 2010). Nevertheless,

these natural products and entomopathogens affect non-target organisms and natural enemies (Ravi et al., 2008; Schmutterer, 1990; Zimmermann, 1993) reducing the efficiency of the latter in agroecosystems where biological control of other pests is necessary (CABI, 2014). Therefore, alternative control methods, more compatible with other insecticides and natural enemies, need to be developed (Moscardi, 1999).

Baculoviruses have very desirable characteristics as control agents, due to their high specificity, virulence, and compatibility with other beneficial organisms (Moscardi et al., 1999). In addition, baculovirus-based formulations, very similar to those of chemical insecticides, can be applied with conventional equipment, and are compatible with most insecticides (Harper, 1986; Cherry and Williams, 2001). Several isolates of the *H. armigera* single nucleopolyhedrovirus (Baculoviridae: *Alphabaculovirus*), HearSNPV, have been characterized all over the world (Chen et al., 2001; Gettig and McCarthy, 1982; Ogembo et al., 2007), including some from Spain (Figueiredo et al., 1999), and their efficiency for the control of *H. armigera* larvae has been widely demonstrated (Allen and Ignoffo, 1969; Figueiredo et al., 1999; Moore et al., 2004; Ogembo et al., 2005; Roome, 1975; Teakle et al., 1986). Interestingly, the genotypic characterization of single HearSNPV isolates has demonstrated a high intraspecific diversity, i.e. existence of distinct genotypes, which, in addition, differ in insecticidal activity (Ogembo et al., 2007; Sun et al., 1998; Wang et al., 2003). In other baculoviruses, infections with different mixtures of genotypes have revealed interactions among them (Barrera et al., 2013; Bernal et al., 2013; Simon et al., 2005) that make the mixture perform differently as an insecticide than single genotypes. Therefore, characterization and selection of genotype or mixtures of genotypes with the best possible insecticidal properties is a crucial factor in the development of baculovirus-based bioinsecticides (Bernal et al., 2013; Caballero et al., 2009).

The aim of this thesis was to develop a HearSNPV-based bioinsecticide to reduce the injuries generated by *H. armigera*. Firstly, the inter- and intra-population diversity of HearSNPV was evaluated, and the mixture of genotypes with the most suitable insecticidal characteristics against *H. armigera* larvae was selected. In addition, the complete genome sequence of five genotypes, including those present within the selected mixture, were obtained to determine the genome changes responsible for differences in biological activity. The narrow host-range of

HearSNPV, infective only for *H. armigera* and *H. zea,* limits the marketability of this virus, which led us to conduct a further research aiming at increasing its host-range. To this end, HearSNPV was co-enveloped with *H. armigera* multiple nucleopolyhedrovirus, HearMNPV, which has a broader host-spectrum. Finally, conditions for scaling up the production process were optimized for the selected mixture, and its efficacy as a biological control agent was evaluated both in greenhouse and in open-field tomato crops. The results obtained in this thesis have proved useful in the registration of a HearSNPV mixture as a biological insecticide for *H. armigera* pest control programs on tomato crops in Spain and elsewhere (Caballero et al., 2014).

2. *Helicoverpa armigera*

2.1 Taxonomy and synonymy

Helicoverpa armigera (Hübner, 1809) is a lepidopteran insect belonging to the Noctuidae family (CABI, 2014). Its taxonomic position has been controversial since its first rating to the present. Thus, there are several scientific names synonyms of *Helicoverpa armigera*: *Chloridea armigera* (Hübner), *Chloridea obsoleta* (Auctorum), *Helicoverpa obsoleta* (Auctorum), *Heliothis armigera* (Hübner), *Heliothis fusca* (Cockerell), *Heliothis obsoleta* (Auctorum), *Heliothis rama* (Bhattacherjee and Gupta) or *Noctua armigera* (Hübner) (CABI, 2014).

2.2 Morphology

H. armigera life cycle, as that of all holometabolous insect (complex metamorphosis), comprises four developmental stages: egg, larva, pupa and adult moth (Fig 1).

The adult moth morphology is typical of noctuids, 14-18 mm long and 3.5-4 cm wingspan; width across the thorax and then tapering. The male is greenish-grey and the female orange-brown. Forewings have a broad, irregular, transverse brown band and a line of seven to eight blackish spots on the margin. Hindwings have a pale-straw color with a broad dark-brown edge that contains a paler patch, yellowish margins, strongly marked veins and a dark spot in the middle. Antennas are covered with fine hairs (King, 1994) (Fig. 1A).

The eggs are shiny and yellowish-white at first, and gradually change to dark brown before hatching (Fig 1B). They are pomegranate-shaped with a 0.4-0.6 mm diameter. The apical area surrounding the micropyle is smooth, and around 24 longitudinal grooves extend from there to the base, with numerous finer transverse ridges between them (King, 1994) (Fig. 1B).

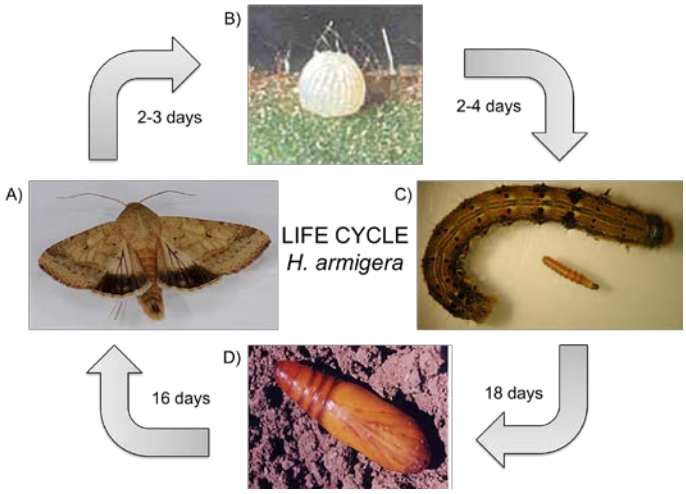

Figure 1: Stages of the life cycle of *H. armigera*: A) moth; B) egg; C) young and mature larva; D) pupa.

H. armigera larvae develop throughout 5-7 instars, depending on environmental conditions such as host plants or temperature (Hardwick, 1965; Shanower et al., 1999). *H. armigera* larvae bear five pairs of prolegs, emerging from the abdominal segments third to sixth and tenth. Neonate and second instar larvae are yellowish-white to reddish-brown, with head, prothoracic shield, supra-anal shield, prothoracic legs and spiracles brown to black (Fig. 1C). Mature larvae are 30-40 mm long; their color being extremely variable, from shades of green, straw-yellow, pinkish to red-brown or black, depending on the diet content. The head is yellow, with several spots; thoracic shield has dark pattern. Three dark lines run longitudinally along the dorsal region, while one yellow light line runs lengthwise under spiracles on the laterals. The ventral part of the larvae is pale (King, 1994) (Fig. 1C).

The pupae are 14-18 mm long, mahogany-brown, smooth surface and with two parallel spines at the posterior end (King, 1994) (Fig. 1D).

2.3 Geographical distribution and host plants

H. armigera is a polyphagous insect pest, originally from Africa, but widely distributed throughout Africa, Asia, Europe and Oceania today, where it causes damage in several crops (Fitt, 1989). It has recently been identified in Brazil (Czepak et al., 2013; Tay et al., 2013) and Argentina (Murúa et al., 2014) (Fig. 2). *H. armigera* probably arrived into America through an importation of cut flowers (Tay et al., 2013), although its natural spread cannot be ruled out as *H. armigera* moths can fly up to 2,000 km with favorable wind currents (Nibouche et al., 1998; Pedgley, 1985; Widmer and Schofield, 1983).

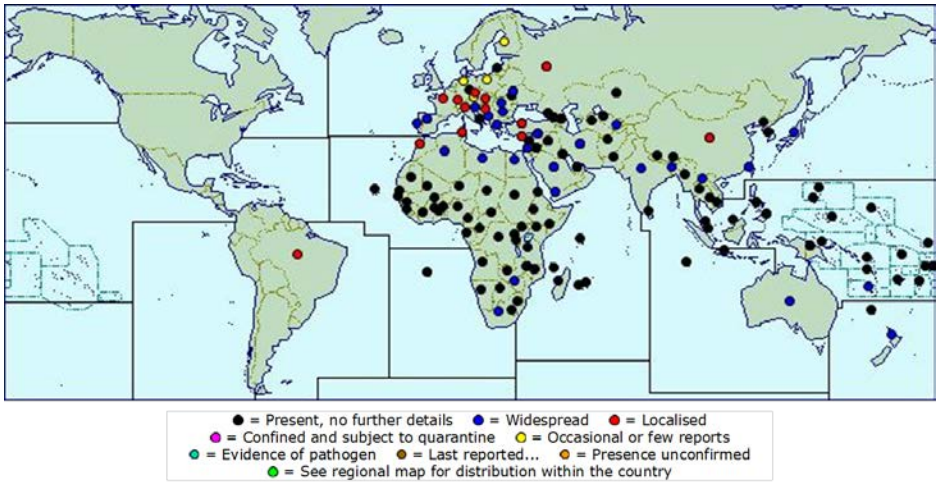

Figure 2: Geographical distribution of *H. armigera* (CABI, 2014).

The host range of *H. armigera* is very extensive (Manjunath et al., 1989; Matthews, 1991). The most important host crops of *H. armigera* are cotton, corn, chickpea, tomato and sorghum. However, it also may attack other crops such as pigeon pea, cowpea, groundnut, okra, peas, field beans, soybeans, lucerne, tobacco, potatoes, maize, flax, prunes, citrus, forest trees and wild plant species (CABI, 2014). Host preferences of *H. armigera* vary in different world regions.

In Europe, *H. armigera* is an important pest of several crops, being cotton, tomato, maize, and carnation most affected (Torres-Vila et al., 2002b). In Spain, *H. armigera* is the key pest of processing tomatoes, as occurs in other Mediterranean open-air cropping areas (Torres-Vila et al., 2003a).

In Africa, it is broadly distributed and may attack many crops such as cotton, citrus, pulses and tobacco (Cherry et al., 2003). In South Africa it is considered a key pest (Moran, 1983), where it causes damage primarily to cotton, citrus, sorghum and tomato crops (Hofs et al., 2006; Moore et al., 2004; Sivasupramaniam et al., 2007).

In Asia, *H. armigera* is one of the most important agricultural pests (Waterhouse, 1993), being a key pest on important crops in China such as wheat, cotton, corn, peanuts, soybeans, and vegetables (Wu and Guo, 2005). *H. armigera* is widely distributed in the major cotton-producing regions (Sun et al., 2002). In India it is also an important pest of cotton, tomato, okra, chili, cabbage, pigeon pea, gram, etc (Ghosh et al., 2010).

In Australia, *H. armigera* is one of the most devastating insect pests of irrigated soybean (Duffield and Jordan, 2000) and also causes important injuries in tomato, zucchini (Kay, 2007) and cotton crops (Mensah et al., 2005).

In America, *H. armigera* is considered a quarantine pest. However it has been recently identified in Brazil, specifically in soybean crops in the Goiás State, in volunteer soybeans in Bahia State, and in cotton crops in Mato Grosso State. Due to the invasiveness of this insect, it might already be widespread all over Brazil (Czepak et al., 2013). Moreover, this pest has also been described in Argentina, and is probably present all over Southamerica (Murúa et al., 2014).

2.4 Biology and ecology

H. armigera populations may give rise up to six generations per year, depending on the climatic conditions. In the Iberian Peninsula, *H. armigera* has two or three generations (Figueiredo et al., 2006). It is a migratory species, and adults can fly 1.000 km, and up to 2,000 km under favorable wind conditions (Nibouche et al., 1998; Pedgley, 1985; Widmer and Schofield, 1983). Adults take off in Autumn from cold to warmer regions, and return in Spring, as soon as the moths emerge from overwintering pupae (Feng et al., 2009).

In the Iberian Peninsula, adults appear in April-May, the first ones probably from migration, and are present until early November (Figueiredo et al., 2006). The life span of adults varies: 1-23 days for males and 5-28 days for female moths (Fig. 1), and depends on the availability of food, pupal weight and temperature (Pearson, 1958). Two to three days after adult emergence, oviposition begins.

Throughout this period (5 and 24 days), each female may lay between 200 and 1200 eggs, mainly at night and singly, presumably to prevent cannibalism (Figueiredo et al., 2006). Eggs hatch in 5.5 days at 20ºC, and in 2-4 days at 25ºC. Larval development is temperature-dependent, and takes 18 days at 25ºC (Fig. 1) (Figueiredo et al., 2006). Duration of the larval stage also depends on the quality of the host food (King, 1994). Fully grown larvae leave the plant to pupate in the soil at depths of 2-10 cm. *H. armigera* may also pupate inside the tomato fruits, although this behavior is unusual (Torres-Vila et al., 1996). After a pre-pupal stage (1-4 days) larvae molt into a pupa, which turns brown after about 24 hours. The pupal period at 25ºC lasts around 16 days after which adults emerge (Figueiredo et al., 2006).

H. armigera has a facultative pupal diapause which is induced in larvae exposed to shorter day lengths (11-14 h light/day) and lower temperatures (15-23ºC) (Kurban et al., 2007). Pupae may remain in diapause for several months (Hackett and Gatehouse, 1982; Roome, 1979). The end of the diapause is influenced mainly by the temperature (Mironidis and Savopoulou-Soultani, 2012).

2.5 Damage and injury

H. armigera is a very polyphagous pest, feeding on more than sixty cultivated plants (Fitt, 1989; Pawar et al., 1986; Pogue, 2004) and causing damage in economical important crops, such as cotton, corn, sorghum, tomato and chickpea (Fitt, 1989; Moral Garcia, 2006; Reed, 1965). *H. armigera* can feed on any phenological stage of the host plants, although flowering is the most susceptible one. Also, it may feed on different plant organs, but prefers harvestable parts such as flowers and fruits. Such feeding behavior is the cause of serious crop losses (Fitt, 1989). In Spain, *H. armigera* is the most important pest of tomato crops, feeding on leaves, buds, flowers and fruits (Torres-Vila et al., 2003a). The injury caused by this pest depends on the host plant. On cotton, larvae make holes at the base of flower buds, which can be hollowed. Larvae may also consume leaves and shoots. Larger larvae bore into green bolls, which fall after larval damage (Fig. 3A) (CABI, 2014). On tomatoes, young fruits are invaded and fall. Larger larvae may bore into older fruits, and secondary infections by other organisms, such as fungi and bacteria, lead to rotting (Fig. 3B) (CABI, 2014). On maize, larvae invade the cobs and feed on developing grain. In addition,

secondary bacterial infections are common following *H. armigera* injuries (Fig. 3C) (CABI, 2014). On sorghum, larvae feed on the developing grain, and hide inside the head during the daytime (Fig. 3D) (CABI, 2014). On chickpea, larger larvae bore into pods, consume developing seed and also foliage and entire small plants (Fig. 3E) (CABI, 2014). On pigeon pea, small larvae feed on flower buds and flowers, which may drop. Larger larvae bore into pods and consume developing seed (Fig. 3F) (CABI, 2014).

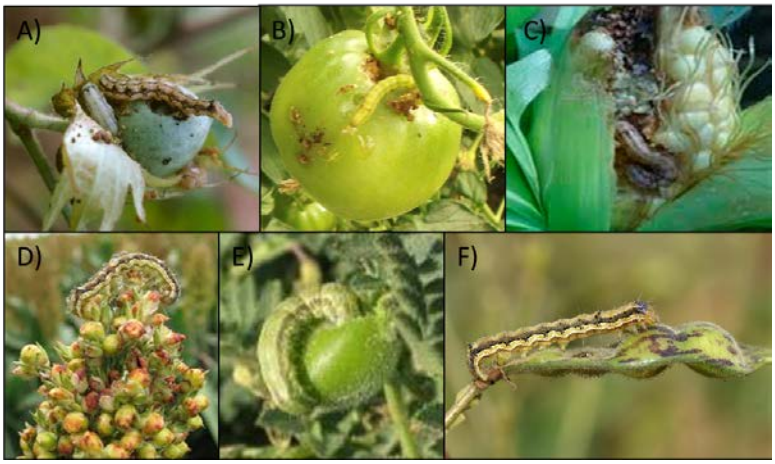

Figure 3: Injuries caused by *H. armigera* in crops: A) cotton, B) tomato, C) corn, D) sorghum, E) chickpea, F) pigeonpea.

2.6 Control methods

2.6.1. Cultural methods and monitoring

Cultural methods play an important role in pest management aimed to reducing pesticide use. Management options include manipulation of sowing dates, cropping season spacing and fertilizer application to minimize pest damage (Gowda, 2005). Cultural methods such as deep plowing and flooding reduce the survival of *H. armigera*, and intercropping with most susceptible crops can minimize damage to the main crops (Gowda, 2005).

Risk assessment, based on adult male catches in pheromone traps and egg counts is not reliable, since correlation between number of eggs on the plants and male caches in the traps and subsequent crop damage is frequently not found (Torres-Vila et al., 2003b). However, in processing tomatoes, percentage of seasonal damaged fruits is a reliable predictor in integrated pest management

(IPM) decision, being 3% of damaged fruits the economical threshold established in IPM programs (Torres-Vila et al., 2003b).

2.6.2. Chemical control

Control of *H. armigera* is typically performed by applying organophosphate insecticides, such as chlorpyrifos (Fig. 4A) (Torres-Vila et al., 2003a). In the Iberian Peninsula, other insecticides such as lufenuron and endosulfan are also commonly used to control this pest (Figueiredo et al., 2006). In China, the most used chemical insecticides are esfenvalerate, endosulfan, and pyrethroids (Sun et al., 2002). In South Africa, several organophosphates and carbamates are commonly used for the control of *H. armigera* (Nel et al., 2002). However, the use of chemical insecticides has led to insect resistance development,and, as a consequence low efficiency of chemical treatments (McCaffery, 1998; Torres-Vila et al., 2002a, b). Increasing environmental pollution generated by the indiscriminate use of chemical pesticides has also encouraged the use of pest control alternatives.

2.6.3. Natural products

The efficacy of spinosad (Fig. 4B), derived from fermentation of *Saccharapolyspora spinosa* (Mertz and Yao) (Actinomycetales), has been already demonstrated against *H. armigera*, and is commonly used in several parts of the world (Gosh et al., 2010; Kranthi et al., 2000; Vojoudi et al., 2011). Other plant products, such as custard apple seed extracts, karanj oil, neem oil, neem leaf extract, neem seed kernel extract, tobacco decoction and nicotine sulphate, have been also tested against *H. armigera* populations (Grzywacz et al., 2005); the most used ones being neem derivatives for their field-contrasted efficacy and simple commercial development (Shankar and Parmar, 1999; Sharma et al., 1999a; Vyas et al., 1999) (Fig. 4C). Neem tree (*Azadirachta indica* A. Juss) derivatives comprise several compounds with effects on insect behaviour and physiology, such as repellence, oviposition inhibition, growth disruption, etc (Shankar and Parmar, 1999; Sharma et al., 1999a). Therefore, the use of neem leaf and seed kernel extracts and also neem oil are recommended for pest management. In addition, neem derivatives have little or no effect against

beneficial insects such as beneficial spiders, parasitic wasps and predatory mites (Walter, 1999).

Figure 4: Commercial products commonly used against *H. armigera*: A) Chlorphyrifos (Dursban), B) spinosad (Spintor) and C) Neem oil.

2.6.4. Natural enemies

H. armigera has several natural enemies, which have shown efficacy in reducing pest populations. The species and importance of parasitoids and predators vary between crops and countries (CABI, 2014). For example, in Kenya, predators within the Anthocoridae and Formicidae families suppressed *H. armigera* population on sunflower, maize, sorghum and cotton, whereas parasitism was low (van den Berg et al., 1993). In contrast, in northern Tanzania, parasitism was the major cause of mortality on sorghum, cotton and weed, and the parasite varied with host plant (van den Berg et al., 1990).

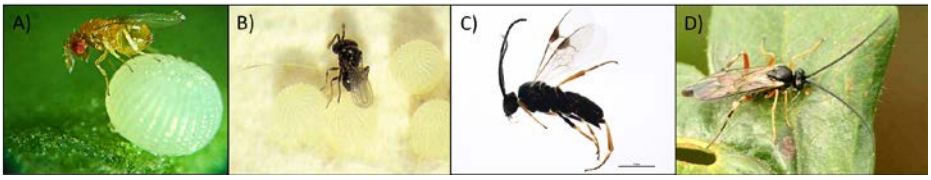

Figure 5: *H. armigera* control with natural enemies: A) *H. armigera* egg being parasitized by a *Trichogramma* spp. adult, B) *H. armigera* egg being parasitized by a *Telenomus* spp. adult, C) adult of the larval parasitoid *Cotesia kazak*, D) adult of the larval parasitoid *Hyposoter didymator*.

In the Iberian Peninsula, *H. armigera* populations could be efficiently controlled by egg and larval parasitoids (Izquierdo et al., 1994; Meierrose and Araujo, 1986; Torres-Vila et al., 2000). The most common *H. armigera* egg parasitoids belong to the genera *Trichogramma* (Hymenoptera: Trichogrammatidae) (Fig. 5A) and *Telenomus* (Fig. 5B) (Hymenoptera:

Scelionidae) (Meierrose and Araujo, 1986; Torres-Vila et al., 2000), which could reach a parasitism rate higher than 35% (Figueiredo et al., 2006). The number of parasitized eggs tends to increase as the growing period advances, and can reach up to 90% at the end of the crop season, reducing the hibernating population (Meierrose and Araujo, 1986). *H. armigera* populations could be also controlled by two larval parasitoids, *Cotesia kazak* (Telenga) (Hymenoptera: Braconidae) (Fig. 5C) and *Hyposoter didymator* (Thunberg) (Hymenoptera: Ichneumonidae) (Fig. 5D) (Izquierdo et al., 1994; Torres-Vila et al., 2000). In Spain, the parasitism rate caused by *C. kazak* and *H. didymator* can reach 70%, although generally the number of parasitized larvae varied between 25-35%, being the second and third larval instars the most susceptible stages for parasitization (Torres-Vila et al., 2000).

Many *H. armigera* predators have been described in the Iberian Peninsula, belonging to different insect orders such as Coleoptera (Coccinedillae and Staphylinidae families), Diptera (Syrphidae and Cecidomyiidae families) or Neuroptera (Chrysopidae family) (Figueiredo et al., 2006). However, these predators are non-specific, and measurement of their impact on the *H. armigera* population reduction is difficult (Figueiredo et al., 2006), and not commonly used for *H. armigera* control.

2.6.5. Microbiological control

Fungal entomopathogens have demonstrated to control *H. armigera* successfully. For instance, isolates of *Metarhizium anisopliae* (Fig. 6A) reduced *H. armigera* population in chickpea as efficiently as endosulfan (Kulkarni et al., 2008), and *M. anisopliae*, *Beauveria bassiana* and *Nonluraea rileyi* isolates were also as effective as endosulfan in controlling *H. armigera* in pigeon pea crops (Nahar et al., 2010).

Several studies have shown that *Bacillus thuringiensis* (Bt) toxins protect different crops against *H. armigera* efficiently in different countries (Fig. 6B) (Avila et al., 2005; Chakrabarti et al., 1998; Gajendra-Babu et al., 2002; Jalali et al., 2004). Also, transgenic crops expressing Bt toxins produced high mortality rates of *H. armigera* larvae in a wide variety of crops, such as cotton (Guo et al., 1999), chickpea (Kar et al., 1997), pigeon pea (Sharma et al., 1999b), tobacco (Selvapandian et al., 1998) and tomato (Mandaokar et al., 2000). However,

continuous applications of Bt based products may favour the development of resistance (Xu et al., 2005; Yang et al., 2007; Zhao et al., 2010).

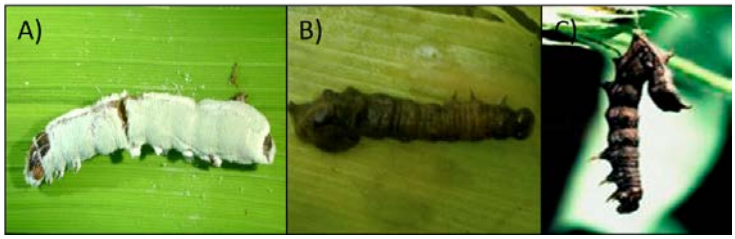

Figure 6: *H. armigera* larval cadavers after infection with different entomopathogens: A) *Metarhizium anisopliae*, B) *Bacillus thuringiensis*, C) nucleopolyhedrovirus.

Finally, field populations of *H. armigera* are naturally affected by several *Alphabaculovirus* (Fam. Baculoviridae), like the *H. armigera* single nucleopolyhedrovirus (HearSNPV) and the *H. armigera* multiple nucleopolyhedrovirus (HearMNPV) (Fig. 6C). HearSNPV showed better insecticidal characteristics than HearMNPV (Sun and Zhang, 1994), which has resulted in the characterization of several isolates all over the world (Chen et al., 2001; Figueiredo et al., 1999; Gettig and McCarthy, 1982; Ogembo et al., 2007), of which some have been commercialized as biological insecticides (Jones et al., 1998; Zhang, 1994). In China, *H. armigera* is successfully controlled with HearSNPV-based formulation, the most important commercial baculovirus to date (Yang et al., 2012). Baculoviruses are very promising control agents due to their high specificity, high pathogenicity and virulence, and compatibility with other beneficial organisms such as parasitoids, predators and pathogens, which facilitate their incorporation in integrated pest management (IPM) programs (Moscardi, 1999).

3. Baculoviruses

Baculoviruses are infectious agents that cause fatal disease in more than 700 insect species, the vast majority of them belonging to the orders Lepidoptera, Hymenoptera and Diptera (Caballero and Williams, 2008; Herniou et al., 2003). The presence of these viruses was first reported over 2,000 years ago in the silkworm culture in China (Benz, 1986). However, it was not until 1950-75 that baculoviruses were developed as biological insecticides (Ignoffo, 1981; Steinhaus,

1956, 1963). The first baculovirus-based biopesticide registered in the United States, Elcar®, based on the *Helicoverpa zea* single nucleopolyhedrovirus (HzSNPV), was a commercial failure for several reasons (Ignoffo and Couch, 1981). However, lately, the problems derived from the use of chemical control methods have intensified the interest in the development of baculoviruses as biological pest control agents and in the understanding of the biology and ecology of these viruses to develop biopesticide products with increased efficacy (Szewczyk et al., 2006).

3.1 Morphology and taxonomy

The advent of electron microscopy allowed the first observations of baculovirus morphology, which were described as refractile structures (occlusion bodies, OBs) containing rod-shaped virions packing the DNA (Bergold, 1953). This rod shape inspired the naming of these viruses; baculo meaning stick in Latin (Vago et al., 1974).

Baculoviruses have double-stranded, circular, supercoiled genomes, with lengths varying from 80 to 180 kb (Rorhmann, 2008). The DNA is bound to the P6.9, a protein present in all baculovirus genomes (Pearson et al., 1988; van Oers and Vlak, 2007), and encapsidated within a protein case composed mainly by VP39 protein (Pearson et al., 1988; Thiem and Miller, 1989), constituting the nucleocapsids (Fig. 7) (Funk et al., 1997). Nucleocapsids, the elementary genetic units of baculoviruses, are enveloped by a lipoprotein bilayer membrane forming the virions, which constitute the morphological units responsible for viral infection.

During the infection cycle of baculoviruses, two types of virions are produced, budded virions (BVs) and occlusion derived virions (ODVs). Although these two types of virions are similar in their nucleocapsid structure, they differ in both function and protein structure (Fig. 7). BVs are involved in cell-to-cell infection and are responsible for systemic infection. BVs contain a single nucleocapsid and are formed when the nucleocapsid buds out through the insect cell membrane at recognition sites, acquiring part of the membrane. This membrane contains glycoproteins such as GP64 or F protein, which allow budding and entry into new target cells (Blissard and Wenz, 1992; Garry and Garry, 2008; Ijkel et al., 2000; Monsma et al., 1996; Oomens and Blissard, 1999; Pearson et al., 2000).

ODVs are responsible for primary infection in the midgut epithelial cells of the host. ODVs are produced in the cell nucleus and may contain a variable number of nucleocapsids enveloped in a lipid bilayer synthesized *de novo* (Stolz et al., 1973). This envelope contains proteins that play a role in *per os* infectivity and the packaging of viral particles (Braunagel et al., 1996; Faulkner et al., 1997; Kikhno et al., 2002; Lapointe et al., 2004; Ohkawa et al., 2005; Pijlman et al., 2003; Zhang et al., 2005a). ODVs are produced in the very late stage of the infection and become surrounded by a protein matrix forming the occlusion bodies (OBs), which are responsible for the horizontal transmission of the virus and the virus survival in the environment (Rorhmann, 2008).

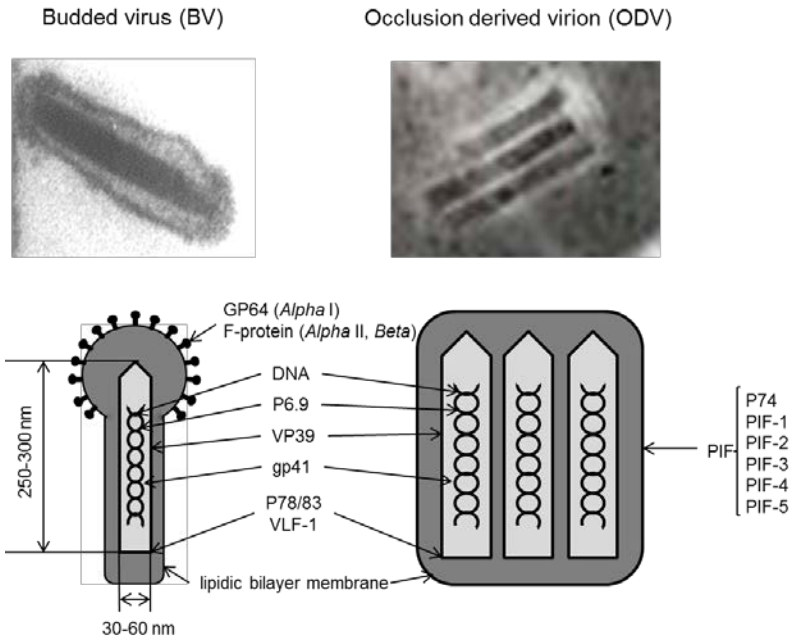

Figure 7: Scanning electron micrographs and schematic representation of virion forms produced during the baculoviruses infection cycle. Budded virions (BVs) and occlusion derived virions (ODVs) contain identical nucleocapsids but differ in the lipid and protein composition of their envelopes.

The International Committee on Taxonomy of Viruses (ICTV) has published nine reports since 1971 (King et al., 2012). In the 6th report, the *Baculoviridae* family was divided into two genera according to the OB morphology: *Granulovirus* (GV) and *Nucleopolyhedrovirus* (NPV) (Fig. 8) (Murphy et al., 1995). GVs are

granular shaped structures of 0.13 x 0.50 µm, composed by a protein matrix of granulin and each OB contains only one ODV with one nucleocapsid (Fig. 8) (Funk et al., 1997). In contrast, NPVs produce 0.15 to 15 µm polyhedral shaped structures composed by several ODVs embedded in a polyhedrin matrix (Fig. 8) and their virions may include one (single NPVs, SNPVs) or many nucleocapsids (multiple NPVs, MNPVs) (Fig. 8) (Funk et al., 1997). NPVs can be subdivided into two phylogenetic groups; group I and group II. GP64 is the major envelope glycoprotein in group I nucleopolyhedrovirus, in which *Autographa californica* multiple NPV (AcMNPV) or *Limantria dispar* NPV (LdMNPV) are included, while F protein constitutes the envelope of group II nucleopolyhedrovirus virions, in which *Spodoptera* NPVs (*S. exigua* NPV, SeMNPV, *S. littoralis* SpliNPV; S. frugiperda, SfMNPV) or HearSNPV are included (Fig. 7) (Westenberg et al., 2004). F protein is able to rescue infectivity in GP64-null group I nucleopolyhedrovirus, whereas GP64 cannot rescue infectivity in F-null group II (Long et al., 2006; Lung et al., 2002; Westenberg and Vlak, 2008). Group I NPVs are composed only by MNPVs, whereas group II is more diverse and comprises both MNPVs and SNPVs (Herniou et al., 2001, 2003). Moreover, all known MNPVs are only lepidopteran-infective, while SNPVs can also infect dipteran and hymenopetaran. As Lepidoptera is the most recently derived insect order, such host affinities suggest that MNPVs derived from SNPVs, and therefore, the MNPV phenotype may incur a selective advantage (Rohrmann, 1986; Washburn et al., 2003), which may favour the higher diversity of MNPVs compared to SNPVs (Vail et al., 1999; Volkman, 1997).

Formerly, another important characteristic for baculovirus taxonomy was their host range, which is defined as the spectrum of insect species susceptible to the virus. Initially, it was believed that baculoviruses could only infect a single host species, and therefore baculovirus isolates were classified according to its host range and its biological activity in each host. So, two different baculovirus species that infected the same insect host with similar insecticidal characteristics were considered as the same virus species. In fact, the different baculoviruses were named after the insect from which they were first isolated. Now we know that each baculovirus can infect a single or a few related host species, which has clear implications for baculovirus classification. Hence, a virus that infects several host species was initially given different names. That was the case of *Anagrapha*

falcifera NPV and *Rachiplusia ou* NPV, which were later shown to be variants of AcMNPV belonging to the same species (Harrison and Bonning, 1999). There were also cases in which different virus species were considered a single one because they were isolated from the same insect, for example *Spodoptera littoralis* NPV (Kislev and Edelman, 1982) and *Mamestra configurata* NPV (Li et al., 2002a,b). Moreover, MNPVs and SNPVs have also been isolated from a single host such as *Orgyia pseudotsugata* (Zanotto et al., 1993) or *Helicoverpa armigera* (Figueiredo et al., 1999; Gettig and McCarthy, 1982; Ogembo et al., 2005; Sun and Zhang, 1994; Williams and Payne, 1984; Zhang et al., 2005b). In general, most baculoviruses have a narrow host range, being infective to a few insect species, such as HearSNPV, which is infective only to *Helicoverpa* spp. (Gettig and McCarthy, 1982), but some baculoviruses have a broader host range, such as AcMNPV (Guo et al., 2005).

The development of molecular techniques, such as restriction endonuclease profiles or DNA sequencing, allowed a more accurate differentiation between different baculoviruses. Jehle et al. (2006) proposed a new classification based on phylogenetic analysis and on biological and morphological characteristics. According to this new classification, the 9th report of the ICTV divided the Baculoviridae family into four genera: *Alphabaculovirus* (lepidopteran-specific NPVs), *Betabaculovirus* (lepidopteran-specific GVs), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al., 2006; King et al., 2012). The 9th ICTV report proposed a method to classify two baculoviruses as different species according to the phylogenetic distance between them, based on the model proposed by Kimura et al. (1980). This method suggested that two or more baculovirus strains should be considered as the same baculovirus species when the distance measured by the Kimura 2-parameter (K2P) between single or concatenated *polh*, *lef-8* and *lef-9* nucleotide sequences is smaller than 0.015. However, when this distance is larger than 0.05 the baculovirus strains belong to different species. For baculovirus strains with K2P distances between 0.015 and 0.05, complementary information would be necessary for species demarcation (Jehle et al., 2006). So, morphology and biological characteristics such as insecticidal activity and host range of the baculovirus are also important for their classification (Cory and Myers, 2003).

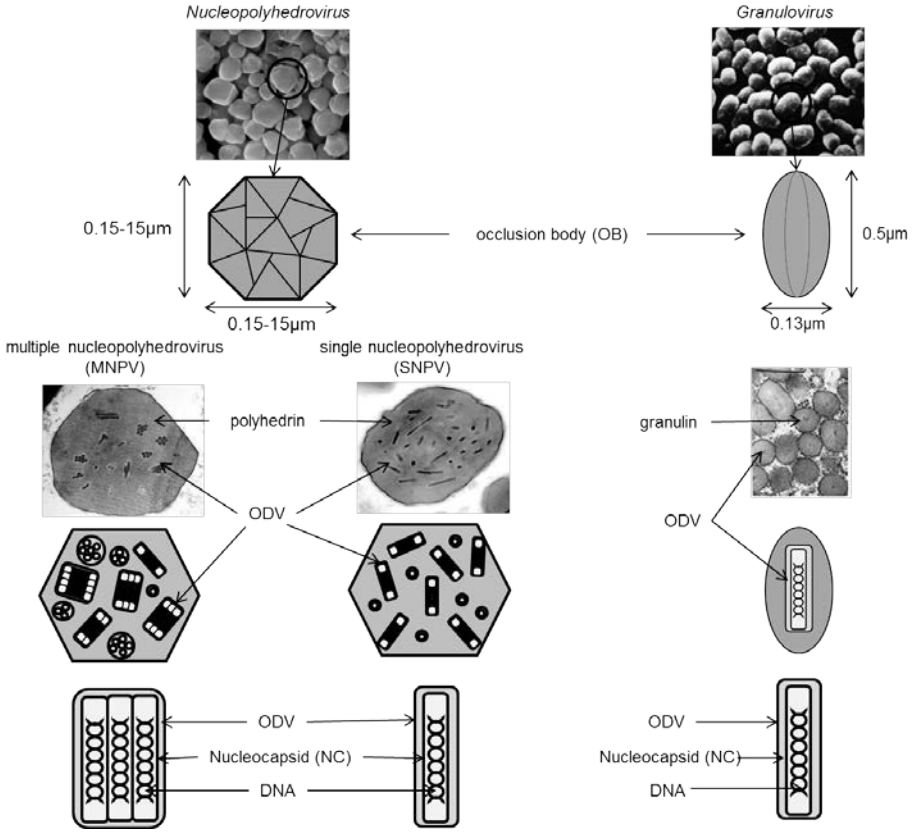

Figure 8: Scanning electron micrographs and schematic representation of occlusion body morphology of the former Baculoviridae genera, *Nucleopolyhedrovirus* and *Granulovirus (*Funk et al., 1997) and of their respective occlusion derived virions (ODVs).

The actual ICTV classification includes 50 different baculovirus species: 33 *Alphabaculovirus*, 14 *Betabaculovirus*, 1 *Deltabaculovirus* and 2 Gammabaculovirus (ICTV, 2013). The baculovirus classification is continuously being updated, as more baculoviruses are being studied and sequenced, and they have to be assigned to the different genera.

3.2 Infection cycle

3.2.1. *In vivo* replication and pathogenesis

The baculovirus infection cycle varies depending on the virus genus (Jehle et al., 2006). While *Alphabaculovirus* and *Betabaculovirus* show a secondary infection, and are able to infect all host tissues, the replication of *Deltabaculovirus* and *Gammabaculovirus* is restricted to the midgut (Jehle et al., 2006), which limits

their development as bioinsecticides. Since HearSNPV belongs to *Alphabaculovirus* the life cycle of this genus is described in detail (Fig. 10).

Alphabaculoviruses are present in the environment as OBs (Fuxa, 2004), a highly stable structure in natural conditions, being only dissolved at alkaline conditions (Jaques, 1985). The infection cycle starts when susceptible larvae ingest OBs present in plants or soils (Fig. 10A). In the insect midgut, due to high pH conditions (10 to 11), the polyhedrin matrix is solubilized and the ODVs and some infection-enhancing factors are released (Fig. 10B). The ODVs pass through the peritrophic membrane (PM) (Fig. 10C) helped by viral enhancins and host cell proteinases (Wang and Granados, 1997; Washburn et al., 1995), which degrade the mucin component of the PM. Then, the ODVs fuse with midgut epithelial cell membranes and the nucleocapsids are released into the cytoplasm, where they start the primary infection (Fig. 10C). The attachment and fusion requires the presence of some ODV envelope proteins, including *per os* infectivity factors (PIF) (Rohrmann, 2008), which are required only in the early stage of virus infection as they interact with receptors found in the midgut epithelial cells, favouring the entry into these cells. Deletion of any of these *pif* genes leads to a block in infection prior to viral gene expression in midgut epithelial cells (Faulkner et al., 1997; Haas-Stapleton et al., 2004; Ohkawa et al., 2005). Afterwards, the nucleocapsids head towards the cell nucleus, which they enter through nuclear pores (Whittaker et al., 2000). In the nucleus, viral DNA is replicated and new nucleocapsids are synthetized, assembled (Horton and Burand, 1993), and bud out of the nucleus. Some nucleocapsids bypass replication and are directly translocated to the basolateral side of the cell (Slack and Arif, 2007; Washburn et al., 1999). Viral proteins of the BVs envelope, GP64 or F protein, are transported to the cell surface and incorporated into BVs envelope when budding out takes place (Fig. 10C).

BVs pass into the hemocoel cavity through the tracheal system avoiding the basal lamina (Fig. 10C) (Engelhard et al., 1994; Lepore et al., 1996; Slavicek and Popham, 2005; Wang and Granados, 1997), and infect cells of other susceptible tissues such as fat body, muscles and haemocytes, initiating the secondary infection (Fig. 10D) (Flipsen et al., 1995). Some of the new assembled nucleocapsids remain in the nucleus and acquire an envelope synthetized *de novo* forming the ODVs. These new ODVs are embedded in the polyhedrin matrix to

form the OBs (Fig. 10D) (Hamblin et al., 1990; Wood et al., 1994). At the end of the infection cycle, the nuclear and the plasma membranes breakdown, releasing the OBs into the haemocoel. The *chitinase* and *cathepsin,* among other viral proteins, allow the liquefaction of the larval cuticles, and OBs are released to the environment (Hawtin et al., 1997), constituting the inoculum for new insects (Fig. 10A) (Federici, 1997).

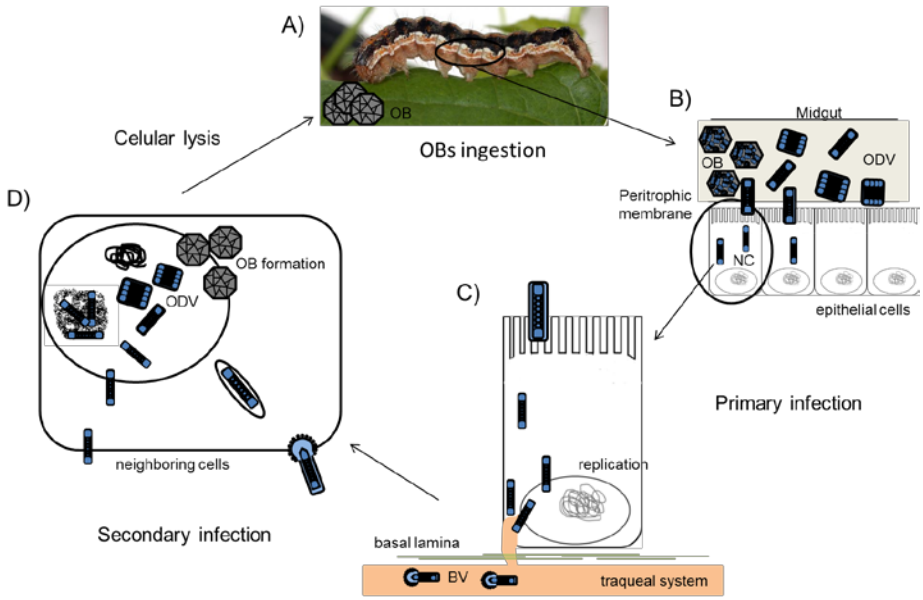

Figure 10: Squematic representation of *Alphabaculovirus* infection cycle. A) Larva ingests food contaminated with OBs. B) OBs are solubilised in the midgut releasing ODVs. C) ODVs, upon overcaming the peritrophic membrane, infect midgut epithelial cells. Newly formed nucleocapsids are released by budding through the plasma membrane of the infected cell forming the BVs. D) BVs are spread throughout the insect tissues where they initiate secondary infections.In the late stage of infection nucleocapsids acquire their envelope from the nucear membrane forming the ODVs, and are occluded within a polyhedrin matrix forming the OBs, which are released in the environment.

Alpahabaculovirus infections in Lepidopteran larvae are characterized by alterations in the physiology and metabolism of the host. Internally, host cell metabolism and RNA synthesis increase, and the hormonal titres in the larvae are also affected (Babu et al., 2009; Etebari et al., 2007; Granados and Williams, 1986). The external signs appear some days after infection, usually at the final stages of infection (Granados and Williams, 1986). These signs include changes in the colour and brightness of the tegument and in larval behavior (the larvae

become less active and lose the appetite) (Granados and Williams, 1986). Shortly before death, infected larvae climb to the top of the plants induced by the *egt* and *ptp* genes of the virus (Hoover et al., 2011; Katsuma et al., 2012), and hang from a branch by their last abdominal pseudopods (Federici, 1997), which favours its dispersion as larvae break due to mechanical actions such as wind or other animals, dispersing millions of OBs in the environment.

3.2.2. *In vitro* replication

The replication and morphogenesis of baculoviruses have been studied most intensely in cell culture systems (Blissard and Rohrmann, 1990; Erlandson, 2009; Granados and Lawler, 1981; Hitchman et al., 2007), which have provided fundamental knowledge of baculovirus DNA replication, gene function, gene expression, and gene regulation. Several other baculovirus characteristics, such as host specificity and baculovirus diversity present within natural populations can be investigated also using this system (Blissard and Rohrmann, 1990; Maruniak et al., 1984; Williams and Faulkner, 1997). Many insect cells are susceptible to baculovirus infection, so it is relatively easy to establish continuous cultures of insect cells *in vitro*. The most used system is that of *S. frugiperda* cell line (Sf21) (Vaughn et al., 1977) with AcMNPV. Several other baculoviruses have been grown in cell culture using different cell lines, including HearSNPV in HzAM1 cell line, established from ovarian *H. zea* cells (McIntosh and Ignoffo, 1981). However, few baculovirus-cell culture systems match the efficiency of the AcMNPV-Sf21 cell combination.

In cell culture, the first step of baculovirus infection, the primary infection in the midgut by ODVs is avoided. Instead, virus infection is established by harvesting haemolymph from infected larvae, containing BVs, and using it to inoculate susceptible cultured cells. Plaque purification techniques, based on cell culture infections with BVs, have allowed the cloning of genotypic variants present in wild-type isolates, which has demonstrated the heterogeneity present within baculovirus populations (Barrera et al., 2013; Bernal et al., 2013; Hitchman et al., 2007; Rowley et al., 2011; Simón et al., 2004).

However, continuous passage of viral progeny in cell culture favour the passage effect, i.e., the formation and accumulation of defective interfering particles (DIPs), which are deleted genotypes lacking genomic regions with genes

essential for primary infections (Heldens et al., 1996) or auxiliary genes (Dai et al., 2000: Pijlman et al., 2001, 2002), which are not necessary for secondary infection.

From a practical point of view, cell-virus systems have proven useful not only for generating engineered baculoviruses for biological control (Moscardi, 1999) or for the expression of novel genes (Jarvis, 1997), but also for the expression of heterologous genes and for the production of valuable proteins for therapeutic purposes (Kost et al., 2005).

3.3 Genome organization and evolution

The first baculovirus to be completely sequenced was the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Ayres et al., 1994). Since then, the number of complete sequences has grown rapidly. For instance, four *H. armigera* single nucleopolyhedrovirus (HearSNPV) genomes have been completely sequenced: HearSNPV-C1 (HearC1, GenBank accession number AF303045, Zhang et al., 2005b), HearSNPV-G4 (HearG4, AF271059, Chen et al., 2001) (Fig. 9), HearSNPV-NNg1 (HearNNg1, AP010907, Ogembo et al., 2009) and HearSNPV-Au (HearAu, JN584482, Zhang et al., 2014). To date, a total of 64 baculovirus genomes have been sequenced and deposited in the GenBank database, and include 44 *Alphabaculovirus*, 16 *Betabaculovirus*, 3 *Gammabaculovirus* and 1 *Deltabaculovirus*. The baculoviruses have double stranded circular genomes from 80 to 180 kb in size, containing between 79 and 181 open reading frames (ORFs). *Gammabaculovirus* and *Deltabaculovirus* have shorter genomes, probably because these baculoviruses are only infective to midgut host cells (Rohrmann, 2008). Moreover, *Alphabaculovirus* and *Betabaculovirus* show more variability in their genome sizes (Jehle et al., 2006). The representation of the whole genome of the baculovirus includes the adenine residue of the *polyhedrin*/*granulin* gene start codon as the zero point in the genome, and therefore, the *polyhedrin*/*granulin* as ORF number 1 (Fig. 9), and the following ORFs are numbered subsequently in a clockwise direction (Vlak and Smith, 1982).

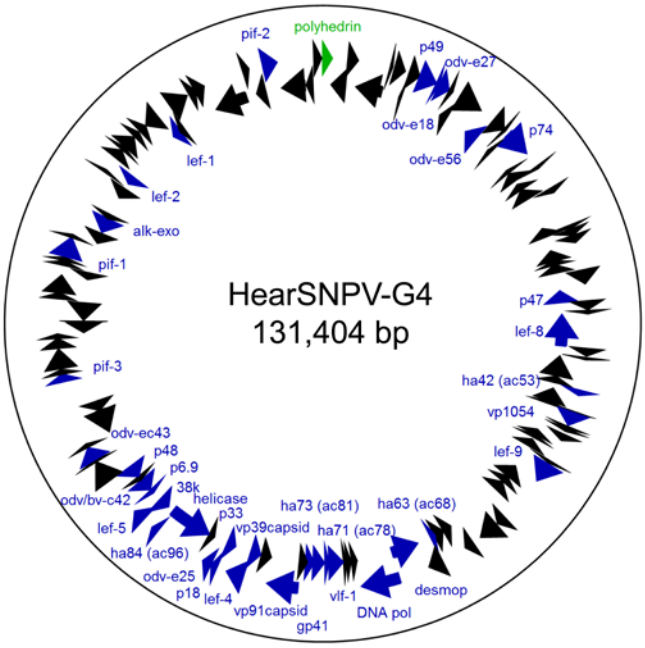

Figure 9: Genomic organization of *Helicoverpa armigera* single nucleopolyhedrovirus G4 genotype (HearSNPV-G4) (GenBank accession number AF271059). The 37 core genes present in all baculovirus genomes (Garavaglia et al., 2012; Miele et al., 2011) are indicated in blue. The zero point of the map, which corresponds with the first nucleotide of polyhedrin gene (Vlak and Smith, 1982), is indicated in green.

The ORFs within the genomes of baculoviruses are equally distributed over both strands with minimal intergenic regions (Fig. 9) (van Oers and Vlak, 2007). A set of 37 core genes have been identified in all baculovirus sequenced to date (Fig. 9) (Garavaglia et al., 2012; Miele et al., 2011), and are grouped into five functional categories: 1) genes involved in RNA transcription, 2) genes involved in DNA replication, 3) structural proteins, 4) auxiliary proteins or 5) genes with unknown function (Herniou et al., 2003). Moreover, a total of 62 common genes are described in all *Alphabaculovirus* and *Betabaculovirus* sequenced to date (Herniou et al., 2003; Jehle et al., 2006). The common and core genes are considered crucial for the life cycle of baculoviruses, and may develop important common functions such as infecting host midgut cells, encoding the viral RNA polymerase or play roles in the structure of virions (Miele et al., 2011; Rohrmann, 2008). In contrast, genes unique to each virus species, or characteristic of the different baculovirus genera, might influence individual phenotypic traits of

particular virus species (Herniou et al., 2003). Comparisons of complete genome sequence of several baculoviruses has also revealed differences in the gene content and order, which has led to establish phylogenetic relationships among the different baculovirus species (Herniou et al., 2003).

Although most ORFs are in single copies, the so-called baculovirus repeat ORFs (*bro* genes) have multiple copies. The number of *bro* copies varies in the different baculovirus species, with those having no *bro* genes like *Anagrapha falcifera* multiple nucleopolyhedrovirus (AfMNPV) (Federici and Hice, 1997), to those with just one unique *bro* gene, such as AcMNPV (Harrison et al., 2008), or those with up to sixteen *bros* identified, as in *Lymantria dispar* multiple nucleopolyhedrovirus (LdMNPV) (Kuzio et al., 1999). HearSNPVs encodes between two and four copies of *bro* genes (Chen et al., 2001; Ogembo et al., 2009; Zhang et al., 2005b, 2014). Although the function of these *bro* genes remain unclear, the location of bro proteins are viral cycle dependent (nucleus or cytoplasm), and its presence responds to frequent duplication events that would provide advantages regarding adaptive genomes with fewer *bro* copies (Kang et al., 2006). In addition, the absence of *bro* genes in several baculovirus species, in addition to their highly variability, suggest that *bro* genes resulted from virus-host interactions (Bideshi et al., 2003; Cohen et al., 2009), and are involved in the maintenance of the insecticidal activity against a specific host, avoiding the development of insect resistance to the viruses (Cohen et al., 2009).

In addition to genes, baculovirus genomes carry repeated sequences known as homologous repeat regions (*hrs*). *Hrs* are AT rich regions composed of repeated sequences which include both direct repeats and imperfect palindromes motifs, and are interspersed in the genome (Erlandson, 2009; Possee and Rohrmann, 1997). All HearSNPVs sequenced to date have five *hrs*, which are located at the same positions in their genomes (Chen et al., 2001; Ogembo et al., 2009; Zhang et al., 2005b, 2014). *Hrs* are enhancers of early promoters (Alves et al., 2002; Guarino et al., 1986; Guarino and Summers, 1986), and are implicated in DNA replication (Kool et al., 1993; Pearson et al., 1992), being hot spots for recombination and rearrangements (Chen et al., 2002; Harrison and Bonning, 2003; Hayakawa et al., 1999).

Transcription of baculovirus genes occurs in a temporally regulated cascade that includes four stages: immediate-early, delayed-early, late and very

late (Passarelli and Guarino, 2007; Rohrmann, 2008). Immediate early and delayed-early genes are transcribed between 0 and 6 hours post infection (hpi) by the host RNA polymerase (Passarelli and Guarino, 2007; Rohrmann, 2008). While the immediate early genes do not require the presence of any viral protein, delayed-early genes require the synthesis of some viral factors encoded by the immediate early genes (Passarelli and Guarino, 2007). Genes expressed in the early phase are involved in DNA replication, late gene expression and processes of host modification (Todd et al., 1996). The late and very late genes are transcribed from 6 to 24 hpi and 18 to 72 hpi, respectively, by the viral RNA polymerase (Passarelli, 2007; Passarelli and Guarino, 2007). These genes are transcribed at high levels and encode proteins responsible for viral assembly and occlusion (Lu and Miller, 1997).

3.4 Baculovirus diversity

Baculoviruses have been isolated from a great number of insect species, and also from different ecosystems, such as forests, fields, rivers and households (Martignoni and Iwai, 1986). The high prevalence in the environment is due to the inclusion in OBs and might explain the enormous baculovirus diversity, not only between isolates identified in different insect species, but also between different isolates or genotypes identified within the same viral species (Cory and Myers, 2003; Erlandson, 2009; Muñoz and Caballero, 2001).

3.4.1. Interspecific diversity

The variation between different baculovirus species is known as interspecific diversity. Baculoviruses have been isolated from more than 700 insect species (Caballero and Williams, 2008; Herniou et al., 2003), most of them from the orders Lepidoptera, Diptera, and Hymenoptera (Martignoni and Iwai, 1986). Baculovirus diversity is likely to be enormous, owing to the high diversity and ubiquity of arthropod species (Muñoz and Caballero, 2001). *Alphabaculovirus* is the most common and widely distributed genus of baculoviruses. It is estimated to be a great diversity of alphabaculoviruses, as more than 200,000 species of Lepidoptera have been described to date.

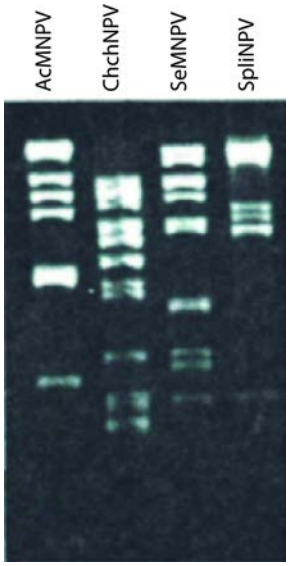

Figure 11: *Bgl*II restriction endonuclease profiles of the genomic DNA of different *Alphabaculovirus* species: *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV), *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV), *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) and *S. littoralis* nucleopolyhedrovirus (SpliNPV). Image adapted from Murillo et al. (2000).

The study of the natural diversity of baculoviruses will be beneficial for the development of baculovirus-based bioinsecticides with the best insecticidal characteristics (Muñoz and Caballero, 2001). This diversity stems from the unique genes of each baculovirus species, the genomic organization, the degree of homology between shared genes, small deletions or insertions and even by the structure of intergenic regions (Serrano et al., 2013). The development of DNA-based techniques, such as restriction endonuclease analysis (REN) (Fig. 11) and genome sequencing, allowed defining these differences at molecular level (Cory et al., 1997). Lately, phylogenetic analyses based on specific DNA sequences have favoured the classification and nomenclature of the different viral species, in continuously updating.

3.4.2. Intraspecific diversity

The intraspecific diversity refers to the variation existing within a single baculovirus species. Characterization of different geographical isolates of the same baculovirus revealed a great intraspecific variation, both at genotypic and phenotypic level (Fig. 12) (Erlandson, 2009; Simón et al., 2004). This

heterogeneity may be the consequence of host-pathogen adaptation processes and coevolution, as baculoviruses are usually more infective to local insect populations (Cory and Myers, 2003; Erlandson, 2009; Muñoz and Caballero, 2001). For instance, an isolate of *Spodoptera frugiperda* MNPV (SfMNPV) from Colombia (SfCOL) was more effective against local *S. frugiperda* insects than an isolate from Nicaragua (SfNIC) (Barrera et al., 2011), and local isolates of *Trichoplusia ni* SNPV (TnSNPV) or AcMNPV showed better insecticidal characteristics against local pest populations than those from geographically distant origins (Erlandson et al., 2007). Therefore, when developing a baculovirus as a bioinsecticide, it is very important to study the intraspecific variability existing in the geographical region where the control program is needed, in order to select the isolate with the most suitable insecticidal characteristics.

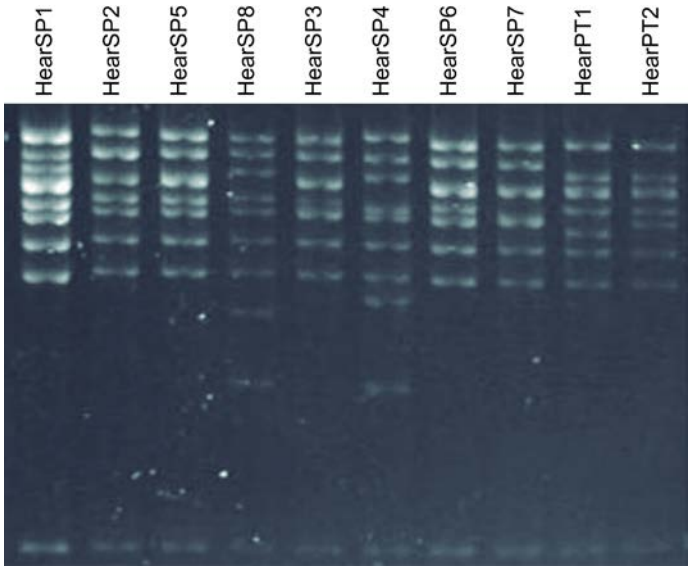

Figure 12: *Bgl*II restriction endonuclease profiles of the genomic DNA of different HearSNPV isolates: HearSP1, HearSP2, HearSP5, HearSP8, HearSP3, HearSP4, HearSP6 and HearSP7 from Spain, and HearPT1 and HearPT2 from Portugal. Image adapted from Figueiredo et al. (2009).

However, more interesting than differences found between geographical isolates within single baculovirus specie is the variation found within a single baculovirus isolate. Baculovirus isolates also present intrapopulation diversity, as isolates either from a pool of infected larvae or from a single infected insect, are usually composed of a mixture of distinct genotypes (Cory and Myers, 2003; Cory

et al., 2005) (Fig. 13). The presence of different genotypic variants within a single baculovirus isolate can be perceived by the appearance of submolar bands in the restriction endonuclease profiles of wild-type isolates or by detection nucleotide polymorphims after DNA sequencing (Chen et al., 2001; Erlandson, 2009). The natural conspecific genotypes can be separated by *in vivo* (Muñoz et al., 1999; Smith and Crook, 1988; Sun et al., 1998) or *in vitro* cloning (Barrera et al., 2013, Bernal et al., 2013; Ogembo et al., 2007; Simón et al., 2004) in a susceptible larvae or cell line, respectively, or also using a bacterial artificial chromosome (BAC) (Wang et al., 2003).

Although the reasons for this intraspecific variability are not clear, it can be the result of natural recombination between different genotypes after simultaneous infections (Hajós et al., 2000), the presence of transposon-like elements (Jehle et al., 1998), or changes such as mutations, gene duplication, insertion or deletions during the infection cycle (Erlandson, 2009; Muñoz and Caballero, 2001). This genetic variation in baculovirus genomes has been observed in hypervariable regions (Cory et al., 2005; Harrison et al., 2008; Muñoz et al., 1999; Stiles and Himmerich, 1998), such as homologous regions (*hr*) and baculovirus repeat ORFs (*bro* genes), suggesting that these regions may act as recombination hot spots (Erlandson, 2009). Additionally such variations have also been described in regions rich in auxiliary genes, which are not necessary for viral replication but provide selective advantage depending on the host or environmental conditions (Barrera et al., 2013; Hodgson et al., 2002; Simón et al., 2004, 2005).

Moreover, there is a selective pressure for natural variation within virus genomes that allow the virus to persist, as competition between baculoviruses represents a natural selection process in which the better suited for survival has an advantage over the other competitors (Hodgson et al., 2002). Therefore, the identification of the individual genes subjected to an important positive selection may be a way to select those genes that likely are key factors in the baculovirus infection cycle or in host range determination (Harrison and Bonning, 2004).

Closely related genotypes do not usually show large phenotypic differences, but sometimes minimal differences in the genome of these genotypes may affect important morphological and biological functions such as pathogenicity, virulence, OB yield or even host range (Muñoz et al., 1999; Simón et al., 2004). For example, the genotypes found within SfMNPV and SeMNPV wild type isolates showed

important differences at biological level in terms of pathogenicity, virulence or productivity (Muñoz et al., 1999; Simón et al., 2004). The phenotypic differences between these genotypes may alter baculovirus-host population dynamics. However, many of these minimal genomic changes are unnoticeable and do not confer any advantage at biological level, as showed with the genotypes isolated from AcMNPV or TnSNPV (Erlandson et al., 2007).

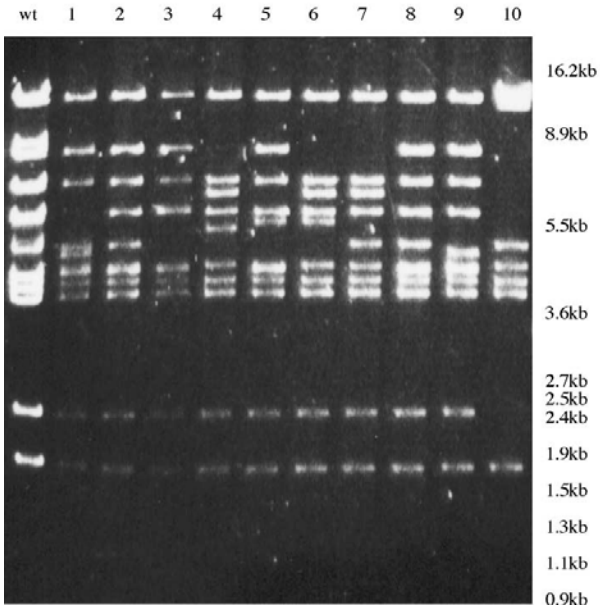

Figure 13: *Hin*dIII restriction endonuclease profiles of the genomic DNA of HearSNPV wild type isolate (wt) and the bacmid genotypes cloned from the wild type (1-10). Image adapted from Wang et al. (2003).

Additionally, the presence of naturally occurring deletion mutants in baculovirus populations is very common. For example, within SfMNPV and SeMNPV isolates, defective genotypes that were not able to infect larvae *per os,* but retained their infectivity in cell culture or by injection of the ODVs, were identified (Muñoz et al., 1999; Simón et al., 2004, 2005). The deletion found within these genotypes affected genes encoding for the so call *per os* infectivity factors (*pifs*), essential for ODV attachment to midgut cells and therefore for initiating primary infection (Dai et al., 2000; Kikhno et al., 2002; Simón et al., 2004). In these cases, the maintenance of these deletion mutants within natural populations is possible due to the co-occlusion and/or co-envelopment of multiple genotypes

within the same OB and/or ODV (Clavijo et al., 2010). This strategy favors the arrival of defective genotypes to the same cell (Bull et al., 2001) and therefore the co-infection of a single cell by the defective genotype into the virus population and complete genotypes, allowing the maintenance of the defective genotypes throughout successive passages (López-Ferber et al., 2003).

Additionally, infections with mixtures of genotypes could result in neutral (Milks et al., 2001), antagonistic (Arends et al., 2005; Barrera et al., 2013; Muñoz et al., 1998; Muñoz and Caballero, 2000) or synergistic interactions (Bernal et al., 2013; Espinel-Correal et al., 2012; Hodgson et al., 2004; López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simon et al., 2005), modifying the insecticidal traits (pathogenicity, virulence or OB production) of single genotypes. For instance, the wild type isolate of SfMNPV was more pathogenic than any of the genotypes, indicating a positive interaction (López-Ferber et al., 2003; Simón et al., 2005). In contrast, some genotypes of the SeMNPV reduced the insecticidal activity of the wild type, and were considered parasitic genotypes (Muñoz et al., 1998). This highlights the importance of the genotypic and phenotypic characterization of genotypes or mixtures of genotypes when developing a baculovirus-based bioinsecticide (Erlandson, 2009; Ogembo et al., 2007). The genotypic interactions that modulate the insecticidal characteristics have been studied in detail to develop bioinsecticides, as certain genotypic combinations in specific proportions result in improved insecticidal properties of the final mixture (Caballero et al., 2009, 2013). Therefore, when developing a baculovirus-based insecticide, the genotypic structure of the wild type isolates has to be analysed and their individual genotypes characterized at the molecular and biological level and then assembled into novel mixtures of genotypes to select the mixture with the best insecticidal characteristics against the target pest (Erlandson, 2009; Ogembo et al., 2007).

Recently the co-occlusion of different genotypes of a given isolate within the same OB was clearly demonstrated, in both SNPV (Bernal et al., 2013) and MNPV (Clavijo et al., 2010). Bernal et al. (2013) identified three different ChchSNPV genotypes within a single OB by end point dilution of the OBs. Clavijo et al. (2010) was able to detect several SfMNPV genotypes in a single OB. and also demonstrated the co-envelopment of different SfMNPV genotypes within the same ODV by plaque assay with the ODVs released from the OB mixtures. More

interestingly, Beperet (2014) was able to co-envelop two different baculovirus species within the same ODV, regardless of the phylogenetic distance between the baculvoirus species. These findings have clear implications when developing baculovirus-based bioinsecticides, as the co-occlusion or co-envelopment of different baculovirus species might facilitate the control of different target pests that infect a given crop at the same time. Mixed virus production technology offers a new possibility to control specific complexes of pests on agricultural crops or forests.

Additionally, it was demonstrated that the co-occlusion or co-envelopment of different genotypes has more advantages at the biological level than the simple mixtures of OBs (Bernal et al., 2013; López-Ferber et al., 2003; Serrano et al., 2013), as it seems to favor the infection of later instars and less permissive host species (Zwart et al., 2009). Bernal et al. (2013) also found that co-occluded mixtures of ChchSNPV genotypes were more virulent than OB mixtures, and suggested that this improvement in the speed of kill might be due to the physical proximity of the ODVs, that may favor the entry of the different genotypes in midgut cells in frequencies similar those present in the inoculum. This frequency likely provides the maximum transmissibility, and this is less probable to occur when genotypes are separated in different OBs.

3.4.3. Maintenance of diversity

Several mechanisms have been described as potentially responsible for the maintenance of baculovirus diversity, including trade-offs between virus fitness components, differential selection for genotypes, multiple infections and interactions between genotypes, interspecific competition, frequency dependent selection and host immune specificity (Cory and Myers, 2003; Hitchman et al., 2007; Hodgson et al., 2001, 2003).

The different genotypes present in a nucleopolyhedrovirus isolate are naturally occluded within the same OB and, in the multiple nucleopolyhedroviruses, co-enveloped within the same ODV (Clavijo et al., 2010). This fact contributes to the maintenance of diversity, as defective particles can replicate and be maintained in the virus population through trans-complementation by complete genotypes (López-Ferber et al., 2003; Simón et al., 2005).

In the environment, baculoviruses are usually found as a mixture of different genotypes. The insect host plays a key role in the transmission and maintenance of these genotypic variants, promoting the persistence of selected genotypes (Cory, 2010), as the genotypic structure and biological activity of a genotypic mixture may be different after several passages in different host species (Cory and Myers, 2003; Hitchman et al., 2007). For instance, several passages of *Panolis flammea* nucleopolyhedrovirus (PaflNPV) in five host species resulted in different proportion of the genotypes depending on the host in which the virus was replicated (Hitchman et al., 2007). The differential selection of genotypic variants also may be influenced by the tritrophic interaction between virus, insect and plant (Hodgson et al., 2002). For example, when *P. flammea* insects infected with PaflNPV were fed on two different pine species, the OB progeny differed in their biological characteristics (Hodgson et al., 2002).

Mixtures of genotypes that are subjected to sequential passage through a given host population tend to reach the most suitable genotypic frequencies, namely, those that improve the efficacy of the OBs. For instance, experimental genotypic mixtures of SfMNPV that were subjected to successive passages *in vivo* rapidly converged to the frequencies present in the wild type isolate, as the wild type presented the best insecticidal properties (Clavijo et al., 2009; Simón et al., 2006). In fact, when different genotypes of ChchSNPV were mixed in proportions similar to those in which they were isolated, their frequencies were maintained over successive passages (Bernal et al., 2013).

The transmission strategy of the different genotypes also contributes to the maintenance of diversity. Two mains routes of transmission have been associated with these viruses; horizontal from one host to another one in the same generation, and vertical from parents to offspring (Fuxa and Tanada, 1987). Horizontal transmission, that occurs when a susceptible insect feeds on contaminated foliage, is the main route of transmission. However the existence of vertical transmission has also been observed for decades for different species of NPVs in their natural hosts (Young and Yearian, 1982; Shapiro and Robertson, 1987; Smits and Vlak, 1988; Young, 1990; Fuxa and Richter, 1994; Goulson and Cory, 1995; Cabodevilla et al., 2011a, b). However, only until lately it has been recognized as an important mechanism for the survival of baculovirus populations, especially during periods of low host densities when horizontal opportunities are

limited (Burden et al., 2006). A number of hypotheses have been proposed to explain how baculovirus persist when hosts are rare. One hypothesis is that baculoviruses may become persistent or latent, in a way similar to some human viruses. Additionally, certain genotypes are involved in specific routes of transmission, and in addition, their pathogenicity and virulence traits vary according to their principal transmission strategy, horizontal or vertical (Cabodevilla et al., 2011a). Vertically transmitted genotypes are usually less pathogenic than horizontally transmitted ones, probably because horizontal transmission depends entirely on achieving per oral infection.

The genetic variability present in an insect population may also favour the maintenance of some genotypes, as individuals from the same host population may show different susceptibility to a given genotype. For instance, European populations of *Cydia pomonella* exhibited resistance to the *C. pomonella* granulovirus (CpGV) strain M, while being susceptible to other genotypes (Schmitt et al., 2013). Moreover, parasitism may influence the efficacy and transmission of the genotypic variants (Cossentine, 2009; Escribano et al., 2000, 2001).

3.5 Baculoviruses as biological control agents

In the last few decades, pest control has relied on synthetic chemical pesticides (Nicholson, 2007). However, their indiscriminate use has caused several problems, including contamination of the environment, reduction of the ozone layer, development of resistance in target pests, reduction of non-target populations, and impact on human health (Al-Zaidi et al., 2011; Casida and Quistad, 1998; Fitches et al., 2010). Therefore, the development of alternative safer methods, such as baculoviruses, is needed (O´Callaghan and Brownbridge, 2009).

Baculoviruses have been used for biological control of forestry and agriculture pests since 1900´s (Inceoglu et al., 2006), as they have many advantages as bioinsecticides. Baculoviruses are highly specific, being sometimes infective to only one or two insect species (Caballero et al., 2009; Moscardi, 1999; Rodgers, 1993). Consequently, they are safe for vertebrates, humans and non-target organisms, and contribute to maintain biodiversity in agroecosystems (Ahmad et al., 2011; Ashour et al., 2007; Gröner, 1986). Moreover, as baculoviruses are highly persistent in the environment they are available to infect

new susceptible insects (Carinhas et al., 2010). Furthermore, the fact that they can be applied by conventional methods, favours their commercial development (Moscardi, 1999).

Baculoviruses have showed high efficacy in controlling field pests, and around 60 baculovirus-based pesticides have been developed all over the world. The most prominent example is the Brazilian program against *Anticarsia gemmatalis,* which has successfully control outbreaks of this pest since 1982 in more than 2 million soybean hectares with a local *A. gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) (Moscardi, 1999; Sosa-Gómez et al., 2008; Szewczyk et al., 2006; Yang et al., 2012). In Europe, *Cydia pomonella* granulovirus (CpGV) has been used to protect pome fruit crops from pest caused by *C. pomonella* (Kutinkova et al., 2012; Lacey et al., 2008; Moscardi, 1999; Vincent et al., 2007). However, *C. pomonella* populations have developed resistance to CpGV, reducing its efficacy (Asser-Kaiser et al., 2007; Schulze-Bopp and Jehle, 2012; Undorf-Spahn et al., 2012). Novel effective isolates or genotypes are being sought to combat this resistance, which has enlightened the importance of studying NPV diversity, in particular, intraspecific diversity (Berling et al., 2009). A more familiar example is that occurring in greenhouses in southern Spain, where *Spodoptera exigua* populations that developed resistance to chemical insecticides have been controlled efficiently with SeMNPV (Caballero et al., 2009). Furthermore, the gypsy moth, *Lymantria dispar*, an important defoliator in forestry, is controlled worldwide with its homologue nucleopolyhedrovirus, LdMNPV (Moscardi et al., 2011). The insect pest studied in this thesis, *H. armigera*, considered one of the most serious pest around the world in a wide variety of crops (Sun et al., 2002), has developed resistance to most chemical insecticides as well as to Bt crops (Jouben et al., 2012; Luttrell and Jackson, 2012; Miriondis et al., 2013; Yang et al., 2013). However, it is successfully controlled with applications of HearSNPV in Australia, India and China (Moscardi et al., 2011), where it is considered the most important commercial baculovirus (Yang et al., 2012).

Baculovirus-based bioinsecticides present several limitations; their most important one being a slow speed of kill, as compared to chemical insecticides. Chemical insecticides kill the larvae in a matter of hours, whereas baculovirus infected larvae die between three and twenty days after OB ingestion (Caballero et

al., 1992; Shapiro, 1986). During this period, larvae continue feeding and causing damage to the crop. Improvement of the speed of kill has been one of the major research objectives on baculoviruses exploited as pest control agents. Different approaches have been employed to develop recombinant baculoviruses with increased virulence, as expression of insecticidal toxins, enzymes or hormones (Hammock et al., 1990; Maeda, 1989; Stewart et al., 1991; Tomalski and Miller, 1991), deletion of virus genes involved in the host metabolism, such as *egt* (O'Reilly and Miller, 1991), or a combination of both (Bonning et al., 1995). Only a few genetically modified baculoviruses have been tested in the field (Eberle et al., 2012) and none has been commercialized (Summers, 2006), owing to public aversion to release of genetically modified organisms (GMOs) (Szewczyk et al., 2006). This has highlighted the importance of the search and selection of new isolates, genotypes or mixtures of genotypes with improved phenotypic characteristics. For instance, a co-occluded mixture of three genotypes of the ChchSNPV reduced the median time to death down to 33 h, which improves the results obtained with recombinant baculoviruses (Bernal et al., 2013). The limitation of the slow speed of kill may also be overcome by formulation, ie. by incorporating compounds such as optical brighteners (Morales et al., 2001; Zou and Young, 1994), free radical inhibitors, or certain enzymes (Zhou et al., 2004).

The narrow host range of baculoviruses, an advantage from an environmental standpoint, is at the same time undesirable at the commercial level for crops in which two or more insect pests are present. In these instances, baculovirus-based bioinsecticides with wider host ranges would be much more attractive. However extending the host range of baculoviruses has been an ardours task. Several attempts have been carried out in various ways with little success; one of the most important ones being the construction of recombinant baculoviruses bearing genes from other baculoviruses with a distinct host range. For instance, AcMNPV, which is not infective to *Bombix mori* larvae, is able to infect this insect species following recombination with a 0.6 kb fragment from *B. mori* MNPV (BmMNPV) (Maeda et al., 1993). Moreover, a recombinant AcMNPV bearing the *hrf-1* gene from LdMNPV could infect *L. dispar* larvae, whereas the wild type of AcMNPV is not infective to this species (Chen et al., 1998). However, most of these advances have been obtained by chance, since it has been very difficult to identify genes involved in host range. Another approach has been the

incorporation of chemical compounds, such as optical brighteners, to virus formulations. The addition of Phorwite AR to AcMNPV suspensions increased its host range to the non-permissible species, *L. dispar* (Shapiro and Dougherty, 1995). However the public aversion to release of genetically modified organisms or to the use of chemical products in bioinsecticide products makes the search for alternative methods necessary. It is in this context that research on the natural diversity of NPVs to select genotypes or mixtures with improved characteristics becomes remarkable.

Another important drawback of baculoviruses as pest control agents is found at their scaling-up production. Baculoviruses are obligate pathogens, and host larvae (*in vivo*) or cell cultures (*in vitro*) are needed to replicate the virus, implying high costs. Therefore, massive production conditions have to be optimized in order to cut costs and make the product commercially competitive. In *in vitro* production (Inceoglu et al., 2001), the formation of defective genotypic variants that accumulate by serial passage in cell culture (Moscardi et al., 2011) and result in reduced occlusion and loss of virulence (Krell, 1996), challenge researchers even further. The superior selective advantage of deletion mutants is often attributed to their elevated BV production (Pedrini et al., 2005; Slavicek et al., 2001), and to a higher budding rate (Pedrini et al., 2011), which results in a dramatic decline in both BV and polyhedra production (Pedrini et al. 2005; Pijlman et al., 2001). In view of this, *in vivo* production is the most suitable option for massive virus production so far. To make the production systems economically efficient, it is necessary to obtain greatest amounts of active virus per larva with the lowest virus dose and in the shortest possible time, while keeping contamination at minimally acceptable levels (Ravensberg, 2011). Cannibalism is an important factor to take into account in the *in vivo* mass production, as it reduces the number of larvae, which, in turn, reduces OB production (Chapman et al., 1999; Shapiro, 1986). So, massive production of cannibalistic insect species, such as *H. armigera*, may be performed in individualized larvae, increasing production costs. Several factors that directly affect larval growth rate may also influence final OB production (Ignoffo and Couch, 1981). For instance, several studies have found a direct relationship between larval age and viral productivity (Bernal, 2013; Gupta et al., 2007; Kalia et al., 2001). Moreover, increased viral inoculum may have a negative effect on the production of OBs/larva since the

death of the larva occur in a shorter time, larvae attain a lower weight and, consequently, produces less OBs (Grzywacz et al., 1998; Kalia et al., 2001). Viral productivity may also be influenced by incubation temperature, as it directly affects larval development, and viral replication (Subramanian et al., 2006), as larvae incubated at higher temperature die faster than larvae inoculated at lower ones (Ribeiro and Pavan, 1994; Subramanian et al., 2006). Therefore, biotic and abiotic factors should be determined in detail in each baculovirus-host system in order to optimize mass production. Several studies have been conducted in order to optimize the mass production of HearSNPV *in vivo* (Gupta et al., 2007; Kalia et al., 2001; Mehrvar et al., 2007). In general, the maximum yield per larva (6 to 12 x 10^9 OBs/larva) was obtained when fifth instar larvae were inoculated with a virus dose that produced approximately 80% mortality (Gupta et al., 2007; Kalia et al., 2001; Mehrvar et al., 2007), by incubating the infected larvae at 25ºC (Mehrvar et al., 2007) and harvesting the larvae as cadavers (Gupta et al., 2007; Mehrvar et al., 2007).

Finally, once the efficacy of NPV has been demonstrated under laboratory conditions and its mass-production can be attained at profitable costs, it is still necessary to determine the efficiency of the baculovirus under natural field conditions (Grzywacz et al., 2008; Gupta et al., 2010; Lasa et al., 2007a). Baculovirus efficacy might vary between laboratory and field conditions, as laboratory insect populations are often more susceptible than those in the field (Bianchi et al., 2000; Dwyer et al., 2005; Grant and Bouwer, 2009). Moreover, insects from different geographical regions can present differences in susceptibility (Barrera et al., 2011; Briese and Podgwaite, 1985; Fuxa, 1993). Other factors, such as temperature, pest density or crop phenology may also influence the virus activity in the field (Moscardi, 1999). Additionally, the efficacy of a baculovirus-based biopesticide is strongly influenced by its persistence in the field (Jones and McKinley, 1987). As baculovirus are inactivated by UV radiation (Ignoffo, 1992), their persistence is longer in a greenhouse than in the open air, as plastic films filter a part of the UV spectrum (Lasa et al., 2007b). Finally, plant exudates (Elleman and Entwistle, 1985; Hoover et al., 1998), high temperatures and alkaline pH conditions (Young et al., 1977) may reduce the field persistence of baculoviruses. HearSNPV has been validated as a protecting agent for different crops in the field in different countries (Cherry et al., 2000; Gupta et al., 2010; Kay,

2007; Moore et al., 2004) and has proved as effective as chemical insecticides and *Bacillus thuringiensis* in crops such as chickpea (Cherry et al., 2000; Gupta et al., 2010), citrus (Moore et al., 2004), tomato (Gupta et al., 2010; Moore et al., 2004), capsicum or zucchini (Kay, 2007). Moreover, HearSNPV is compatible with other pesticides (Harper, 1986) and may be used in combination with biological and chemical insecticides in order to manage insecticide resistance (Duraimurugan and Regupathy, 2005).

3.6 *Helicoverpa armigera* nucleopolyhedroviruses

H. armigera larvae are naturally infected by different *Alphabaculovirus* (*Baculoviridae* family). Single and multiple nucleopolyhedroviruses have been isolated from this insect species (Figueiredo et al., 1999, 2009; Gettig and McCarthy, 1982; Ogembo et al., 2005; Sun and Zhang, 1994; Williams and Payne, 1984; Zhang et al., 2005b), each having its pros and its cons. *H. armigera* single nucleopolyhedrovirus (HearSNPV) shows better insecticidal characteristics than *H. armigera* multiple nucleopolyhedrovirus (HearMNPV) (Sun and Zhang, 1994) but also a too specific host range, as it is infective only to *Helicoverpa* spp. (Gettig and McCarthy, 1982), which limits its commercial development. HearMNPV has a broader host range (Rovesti et al., 2000; Tompkins et al., 1988), but lower pathogenicity values. Therefore, a mixture of both viruses that presents the pathogenicity of HearSNPV and the wide spectrum of HearMNPV might meet commercial requirements.

Several HearSNPVs have been isolated from *H. armigera* larvae all over the geographical distribution area of this pest (Chen et al., 2001; Figueiredo et al., 1999, 2009; Gettig and McCarthy, 1982; Ogembo et al., 2005; Zhang et al., 2014), and several HearSNPVs have been used as biological pest control agents in many countries, such as China and Thailand (Jones et al., 1998; Zhang, 1994). This favors the commercial development of a local HearSNPV to control *H. armigera* in the Iberian Peninsula, as local insect populations are usually more susceptible to native virus isolates than to foreign ones (Barrera et al., 2011; Erlandson et al., 2007), and constitutes the main goal of the present thesis.

Apart from the interpopulational diversity of HearSNPV, this virus species likely presents intrapopulation diversity within an individual field strain, as occurs in other HearNPV isolates in which several genotypic variants were purified *in vivo*

(Sun et al., 1998), *in vitro* (Ogembo et al., 2007) or using the bacterial artificial chromosome (BAC) system (Wang et al., 2003). Different genotypes may differ in biological activity (Muñoz et al., 1999; Simón et al., 2004), and interact with one another (Barrera et al., 2013; Bernal et al., 2013; Espinel-Correal et al., 2012; Simon et al., 2005). Thus, characterization of different genotypes and genotypic mixtures needs to be accomplished before selecting the active ingredient with the most appropriate insecticidal properties (Erlandson, 2009; Ogembo et al., 2007).

To date five *Helicoverpa* spp. SNPVs have been completely sequenced: HearSNPV-G4 (HearG4) (Chen et al., 2001) and HearSNPV-C1 (HearC1), from China (Zhang et al., 2005b), HearSNPV-NNg1 (HearNNg1), from Kenya (Ogembo et al., 2009), HearSNPV-Au from Australia (HearAu) (Zhang et al., 2014), and one genotype of the *H. zea* SNPV (HzSNPV) from USA (Chen et al., 2002). These genotypes showed high nucleotide sequence identity except for the homologous regions (*hrs*) and the baculovirus repeat ORFs (*bro*) (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005b, 2014). Moreover, some genes are subjected to an important positive selection, indicating that they are probably key factors in the baculovirus infection cycle or in host range determination (Harrison and Bonning, 2004). Therefore, comparative analysis of different *Helicoverpa* spp. SNPV genomes could allow focusing on the key genes involved in desirable characteristics for the development of bioinsecticides.

As mentioned previously, the exclusive specificity of HearSNPV for *Helicoverpa* spp. larvae (Gettig and McCarthy, 1982) limits its use as a control agent in agroecosystems where different lepidopteran species inflict economic losses. In these instances, HearMNPV could be more amenable in terms of host range coverage (Rovesti et al., 2000; Tompkins et al., 1988) but its insecticidal activity compares unfavorably to that of HearSNPV (Sun and Zhang, 1994). Based on previous studies that demonstrated co-envelopment of two different baculovirus species in the same ODV (Bepertet, 2014), co-occlusion and co-envelopment of HearSNPV and HearMNPV could result in a new baculovirus species with the suitable insecticidal characteristics of HearSNPV and the host range of HearMNPV.

4. Scope of research

The aim of this study was the biotechnological development of a new bioinsecticide based on a local HearSNPV to control pest outbreaks caused by *H. armigera* larvae in the Iberian Peninsula.

In **Chapter II**, seven isolates of HearSNPV from the Iberian Peninsula were subjected to molecular and biological characterization in order to select the isolate with higher insecticidal properties. Although the Iberian isolates showed similar pathogenicity values, HearSNPV-SP1, despite being the fastest killing isolate, was one of the most productive strains. Although HearSNPV-SP1 was as pathogenic as the reference Chinese genotype HearSNPV-G4 against an insect colony from southern Spain, HearSNPV-SP1 was faster at killing the larvae of a range of instars, demonstrating the importance of the use of native isolates for control local pest. Therefore, HearSNPV-SP1 merited further evaluation as the basis for a biological insecticide for control of *H. armigera* in the Iberian Peninsula.

With the aim of selecting a mixture of genotypes with improved biological characteristics, the genotypic diversity of two Spanish isolates of HearSNPV was evaluated in **Chapter III**. Two genotypes present within the previously selected isolate HearSNPV-SP1 were cloned by *in vitro* plaque assay. Moreover, six genotypes were isolated by end point dilution from larvae died in an epizooty occurred in a *H. armigera* laboratory colony established with larvae collected in cotton crops in Spain. Comparison of the biological activity of these genotypes and those of several genotypic mixtures allowed selection of the HearSP1B:LB6 mixture at 1:1 proportion as a bioinsecticide product to control *H. armigera* larvae in fields, as this mixture was more pathogenic than pure genotypes and other mixtures, and also as fast in killing the larvae as the most virulent genotypes.

In **Chapter IV**, the complete genome sequence of five pure genotypes, including those of the mixture, were described. Moreover, in **Chapter V**, the sequences of the complete genomes of these five genotypes and those of previously sequenced *H. armigera* spp. SNPV isolates were compared, with the aim of determine the genetic changes responsible for the phenotypic diversity of this virus. This sequence comparison has also been valid to design the primers necessary for the quantification by qPCR of the relative proportion of each genotype in the mixture selected in **Chapter III**. Complete genome sequences

analysis showed overall minimal differences, except in the homologous regions and *bro* genes. Selection pressure analyses were performed in order to identify genes potentially involved in biological characteristics or in determining population adaptations.

Subsequently, we aimed to expand the host range of HearSNPV, as this virus is only infective to *Helicoverpa* spp. In **Chapter VI**, HearSNPV was co-enveloped within HearMNPV, a virus with a wider host range, in order to obtain an OB sample that presented the insecticidal characteristics of HearSNPV and conserved the wider host range of HearMNPV. The co-envelopment of HearSNPV and HearMNPV had no effect on the biological activity against *H. armigera*, *S. frugiperda* and *M. brassicae* larvae, but HearSNPV remained in the OB progeny during at least five serial passages in these three insect species, which meant an expansion of the host range of this virus, as it was able to persist in non-permissisve hosts such as *S. frugiperda* and *M. brassicae.*

After the selection of the active material and prior to its commercialization as a bioinsecticide, it was essential to optimize its mass-production in order to reduce costs and make the product commercially competitive. **In Chapter VII**, the influence of different parameters such as larval density, larval stage, inoculation time, viral concentration and incubation temperature on HearSNPV production was determined, and the optimum conditions for HearSNPV production were selected.

Finally, field trials were performed to test the efficacy of this bioinsecticide to control *H. armigera* in the Iberian Peninsula. In **Chapter VIII**, the efficacy and persistence of this virus was determined in protected and open-field tomato crops, in Lisbon and Badajoz, respectively. Application of HearSNPV provided control comparable to that of commercial insecticides. In addition, the high persistence of HearSNPV increased the efficacy of the viral application, as larvae could effectively acquire the virus on contaminated leaves for a long time.

These results showed that HearLB6:SP1B merits registration as a biological insecticide to control *H. armigera* pest on tomato crops in Spain, which has prompted a patent application based on this virus.

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CHAPTER II

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

Abstract

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). 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. 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. 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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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 (Fitt, 1989). 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 (Torres-Vila et al., 2002). 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 (Moscardi, 1999).

Several NPVs have been isolated from *Helicoverpa armigera* larvae in different parts of the world (Chen et al., 2001; Figueiredo et al., 1999; Gettig and McCarthy, 1982; Ogembo et al., 2005). The genomes of four of these isolates, HearSNPV-G4 (Chen et al., 2001) (GenBank accession number AF271059) and HearSNPV-C1 (Zhang et al., 2005) (GenBank accession number AF303045) from Hubei province, China, HearSNPV-NNg1 (Ogembo et al., 2009) (GenBank accession number AP010907) from Kenya, and HearSNPV-Au (GenBank 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 (Zhang, 1994), whereas in Thailand a locally-produced HearSNPV-based product is available for control of this pest (Jones et al., 1998). 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 (Chen et al., 2000a; Sun et al., 2002, 2004).

However, the use of recombinant baculoviruses is not presently authorized in most countries, so that the selection of wild-type 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 (Barrera et al., 2011; Erlandson et al., 2007; Milks, 1997) and second, under certain circumstances the use of exotic NPV isolates can have a negative impact on the biological activity of native strains (Muñoz and Caballero, 2000). 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 (Figueiredo et al., 1999). However, subsequent studies indicated that three different isolates, HearSNPV-SP7, HearSNPV-PT1 and HearSNPV-PT2 had more favorable insecticidal characteristics against *Helicoverpa armigera* (Figueiredo et al., 2009), 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. Material and 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± 1ºC, 70±5% relative humidity and 16h:8h day/night photoperiod on a semi-synthetic diet (Greene et al., 1976). 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) (Figueiredo et al., 1999, 2009). 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) (Figueiredo et al., 2009). 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 x *g* for 5 min. Pellets were resuspended in 1 mg/ml sodium dodecyl sulphate (SDS) and centrifuged for 5 min at 2500 x *g*. The resulting pellets were washed twice in distilled water and finally resuspended in 300 µl distilled water and stored at 4ºC until required.

The Chinese genotype HearSNPV-G4 (Sun et al., 1998), isolated by *in vivo* cloning, was kindly provided by J.M. 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 at 10^9 OBs/ml were incubated with one volume of 0.5 M Na_2CO_3, 0.5 volumes of 100 mg/ml SDS and 2.5 volumes of distilled water at 60ºC during 10 min to dissolve the polyhedrin matrix. Undissolved OBs were pelleted at 6000 x *g* for 5 min. The virion-containing supernatant was transferred to sterile 1.5 ml vials and incubated at 50ºC with 500 µg proteinase K 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 x *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 4ºC until use.

For restriction endonuclease digestion, 2 µg of viral DNA, quantified in a spectrophotometer (BioPhotometer Plus, Eppendorf, Freiberg, Germany), were incubated with 10 U of *Bgl*II or *Eco*RI (Takara, Japan) at 37ºC for 4 to 12 h. These enzymes were selected as they previously had proved useful in the differentiation of HearSNPV strains (Figueiredo et al., 1999, 2009). Each reaction was stopped by the addition of 1/6 volume of 6 x loading buffer (2.5 µg/ml bromophenol blue, 40 mg/ml sucrose). Fragments were separated by electrophoresis in gels containing 10 mg/ml agarose in TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) buffer with 0.25 µg/ml 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 (Figueiredo et al., 2009) 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. 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 (Altschul et al., 1990; Pearson, 1990). Baculovirus sequences used in the comparative analysis were GeneBank (accession numbers included): HearSNPV-G4 (AF271059), HearSNPV-C1 (AF303045), HearSNPV-NNg1 (AP010907) and HearSNPV-Au (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. (2009) 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 (Tamura et al., 2011) 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 (Hughes and Wood, 1981). 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 sucrose and 0.05 mg/mL 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) 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±1ºC 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 (Le Ora Software, 1987). 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 x 10^5, 2.1 x 10^5, 1.1 x 10^5 and 1.0 x 10^5 OBs/ml, 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±1ºC 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 (Crawley, 1993). 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 x 10^6 OBs/ml, 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 28ºC until death and subsequently stored at -20ºC. 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 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 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 for fifth instar) which resulted in ~95% to 5% mortality in preliminary assays. Statistical 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 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, 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.

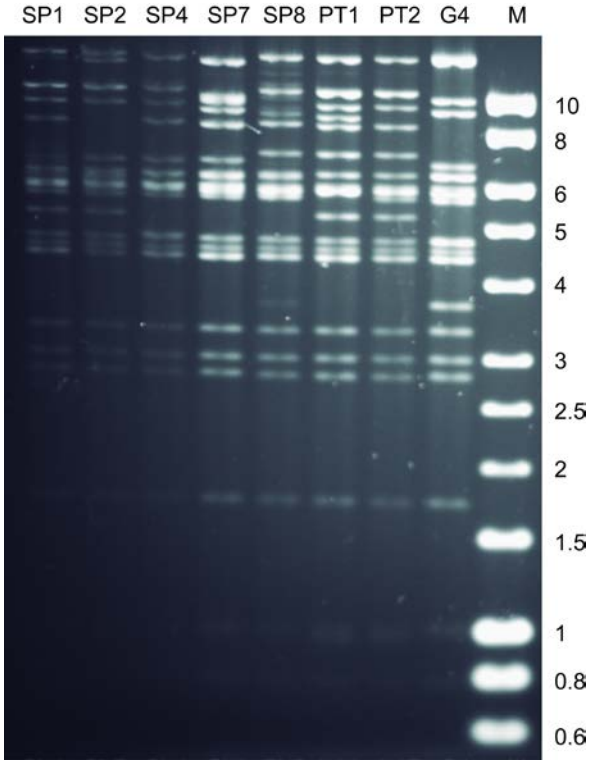

Figure 1: *Eco*RI profiles of the genomic DNAs of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4 isolates. The DNA HyperLadder I (Bioline) was used as a molecular size marker (kb) (M), and fragment sizes are indicated to the right.

The 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 (Chen et al., 2001), 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.

Table 1: Molecular size (kb) of *Eco*RI restriction endonuclease fragments of HearSNPV genomic DNAs from Iberian strains and HearSNPV-G4. DNA fragments are named alphabetically, starting with A for the largest fragment and their sizes are given in kb.

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

* Fragments cloned into pUC19 vector and showed in REN profiles

^ Fragments generated by PCR amplification using primers on each side of the gaps (Table 2) and not visible by REN.

α HearSNPV-G4 *Eco*RI fragments generated *in silico* using Clone Manager 9 (Scientific & Educational Software)

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 (Figueiredo et al., 1999, 2009). 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 large sequence similarity (99.5%) with the African HearSNPV-NNg1 isolate, whereas HearSNPV-G4, HearSNPV-C1 and HearSNPV-Au displayed sequence similarities of 99.4, 99.4 and 99.3% with the HearSNPV-SP1 genome, respectively.

The EcoRI physical maps of HearSNPV-SP1 and HearSNPV-G4 were closely related with few differences between them (Fig. 2). Both viruses showed differences in EcoRI 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.

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.

Primer	Direction	*Eco*RI fragment	Amplified fragment	Nucleotides HearSNPV-G4	Purpose
5´-CAGTTTTGACAGTG GCAA-3´	Forward	R	a	8,443-8,460	Physical map
5´-GTGTTGGCAAATGT GTAC-3´	Reverse	B		9,085-9,102	
5´-GTTACACGACACAG AACA-3´	Forward	L	Z	37,578-37,595	
5´-CCGCTTTAAACATTC AGCA-3´	Reverse	S		38,320-38,338	
5´-CCTCCTGGTCATATC ATGTG-3´	Forward	S	d	40,796-40,815	
5´-ACTTTGACCGCTG ATCA-3´	Reverse	N		41,544-41,560	
5´-GTCAAGATGGTTGCG TAAAG-3´	Forward	C	b / e	55,244-55,263	
5´-GCGTTTCATTCC TTTCTCTG-3´	Reverse	J		56,006-56,025	
5´-GCCAACAGTATTTA CGGA-3´	Forward	J	c	61,455-61,472	
5´-CAATGAGCACATAC GGAA-3´	Reverse	T		62,188-62,205	
5´-TACTCGTATGCGGT GAGCAG-3´	Forward		*lef-8*	33,255-33,274	Phylogenetic analysis
5´-CACCATGCGTCAAG ATATGC-3´	Reverse			33,739-33,758	
5´-CATCGTTCTATGCC AACGTG-3´	Forward		*lef-9*	45,122-45,141	
5´-GAGAGCACAATCGC GTAACA-3´	Reverse			45,620-45,639	
5´-CAAATACTTGGTGGC GGAAG-3´	Forward		*polh*	156-175	
5´-TCCTCTTCTTCGGCA GAATC-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. The percentage of amino acid sequence identity and similarity to homologous ORFs of other HearSNPV isolates is shown.

Fragment	Gene family	Dir.*	Size (aa) estimated in this study	HearSNPV isolates **ORF**/% Identity (Similarity)			
				NNg1	G4	C1	Au
*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)

* Direction of transcription in the same (>) or opposite (<) sense of *polyhedrin* gene.

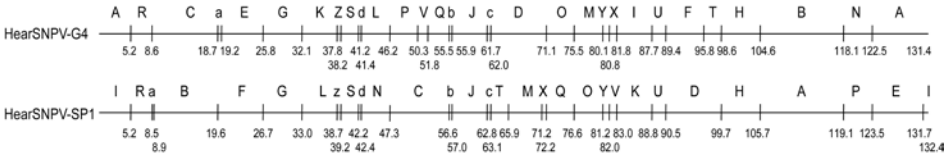

Figure 2: Physical maps based on *Eco*RI for HearSNPV-SP1 and the Chinese genotype HearSNPV-G4. The first nucleotide of the map is the first nucleotide of the *polyhedrin* gene. The circular HearSNPV DNA is represented in linear form. Nucleotide positions representing restriction sites are indicating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

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 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-Au), or HzSNPV (Chen et al., 2002).

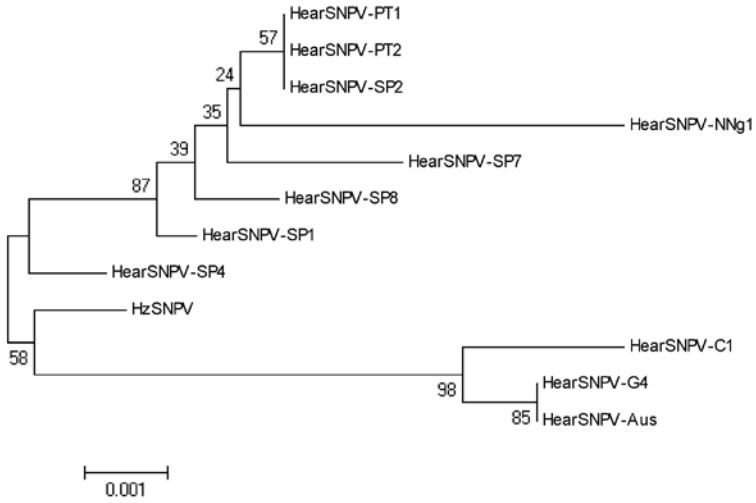

Figure 3: Phylogenetic analysis of *lef-8*, *lef-9* and *polh* partial sequences using MEGA5 software for alignment. Baculovirus sequences used in this phylogenetic analysis were GeneBank (accession numbers indicated in parentheses): *Helicoverpa armigera* (Hear) SNPV-C1 (AF303045), HearSNPV-G4 (AF271059),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

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 x 10^4 OBs/ml for HearSNPV-G4 to 3.4 x 10^4 OBs/ml for HearSNPV-SP2. The 95% fiducial limits of the relative potency values, representing the ratio of LC_{50} values (Robertson and Preisler, 1992), 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.

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 *H. armigera* second instars from the Oxford colony.

Virus	LC_{50} (OBs/ml)	Slope ± SE	Relative Potency	95% Fiducial limits Low	95% Fiducial limits High	MTD (h)	95% Fiducial limits Low	95% Fiducial limits 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-PC. A test for non-parallelism was significant (χ^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).

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 (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, while HearSNPV-PT1 and HearSNPV-PT2 were the least productive isolates with 1.7×10^9 OBs/larva.

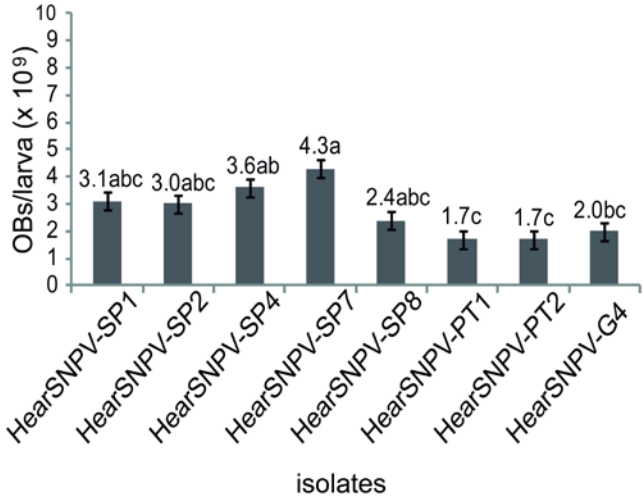

Figure 4: Median OB production (x 10^9 OBs/larva) of *H. armigera* fourth instars from the Oxford colony that died from polyhedrosis disease following infection with 10^6 OBs/ml of HearSNPV-SP1, HearSNPV-SP2, HearSNPV-SP4, HearSNPV-SP7, HearSNPV-SP8, HearSNPV-PT1, HearSNPV-PT2 and HearSNPV-G4 strains.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

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 x 10^5 OBs/ml) than the other younger stages tested. In contrast, HearSNPV-G4 showed similar LC_{50} values in second and third instars (3.3 x 10^4 and 1.4 x 10^4 OBs/ml, respectively) or in fourth and fifth instars (5.1 x 10^4 and 3.4 x 10^5 OBs/ml, 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.

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

Instar	Virus	LC_{50} (OBs/ml)	Slope ± SE	Relative Potency	95% Fiducial limits		MTD (h)	95% Fiducial limits	
					Low	High		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-PC. A test for non-parallelism was significant (χ^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).

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 x 10^9 OBs/larva) than the production for HearSNPV-G4 (6.1 x 10^9 OBs/larva) (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.

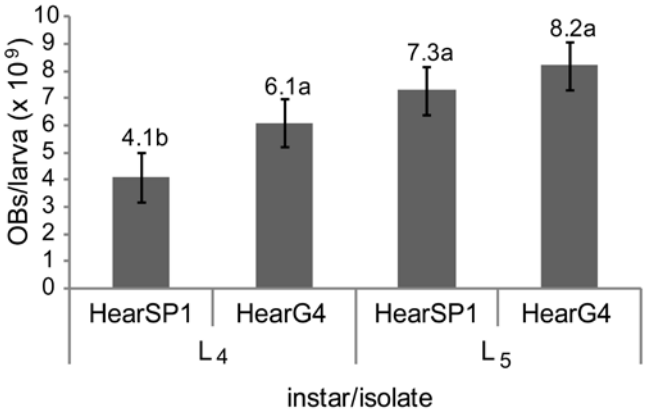

Figure 5: Median OB production (x 10^9 OBs/larva) of *H. armigera* fourth and fifth instars from the Almerian colony that died from polyhedrosis disease after infection with 2.4 x 10^6 and 2.5 x 10^7 OBs/ml of HearSNPV-SP1, respectively, and with 1.2 x 10^6 and 1.4 x 10^7 OBs/ml of HearSNPV-G4. Values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

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 (Figueiredo et al., 1999, 2009) were compared with those of HearSNPV-G4 (Chen et al., 2001; Zhang et al., 1981) 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 (Figueiredo et al., 1999, 2009). 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 (Chen et al., 2001), (2) establishing the phylogenetic relationships among Iberian isolates and other *Helicoverpa* spp SNPV (Chen et al., 2001; Ogembo et al., 2009; Zhang et al., 2005), (3) determining the host stage susceptibility to the most virulent Iberian isolate, HearSNPV-SP1, and the widely used Chinese isolate, HearSNPV-G4 (Chen et al., 2001), against a Spanish colony of *H. armigera*, (4) determining the OB production for strains HearSNPV-SP1 and HearSNPV-G4 (Chen et al., 2001) 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) (Chen et al., 2001) and similar to the sizes of HzSNPV genomes estimated by physical maps (119 or 125 kb) (Corsaro and Fraser, 1987; Knell and Summers, 1984). Chen et al. (2000b) 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 HearSNPV-G4) or Australia (HearSNPV-Au), 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 (Ogembo et al., 2005; Sun et al., 2004). 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 (Guo et al., 2006). In this study, MTD values for HearSNPV-G4 were lower than that reported by Guo et al. (2006), 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 (Sun et al., 2005). 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 (Barrera et al., 2011; Chen et al., 2000a; Hodgson et al., 2004; Simón et al., 2004). 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 (Cory and Myers, 2003). 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 (Figueiredo et al., 2009). 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 (Barrera et al., 2011; Erlandson, 2009). 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, 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 (Rovesti et al., 2000). The majority of studies have reported that susceptibility to infections tends to decrease with larval age both within and between instars (Ali and Young, 1991; Briese, 1986; Escribano et al., 1999; Martinez et al., 2003). 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 (Hegedus et al., 2009; Kirkpatrick

et al., 1998; Washburn et al., 1998). 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 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 (Grant and Bower, 2009). 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.

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CHAPTER III

A novel binary mixture of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) genotypic variants provides improved insecticidal characteristics for control of the cotton bollworm

Abstract

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. From the most pathogenic wild type isolate of the Iberian Peninsula, HearSNPV-SP1 (HearSP1-wt), two genotypic variants, HearSP1A and HearSP1B, were cloned *in vitro*. Similarly six genotypic variants (HearLB1-6) were obtained by end point 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 production (OBs/larva). HearSP1B was ~3 fold more pathogenic than HearSP1-wt and the other variants. HearLB1, HearLB2, HeaLB5 and HearLB6 were the fastest killing variants. Moreover, although highly virulent, HearLB1, HearLB4 and HearLB5 produced more OBs/larva than other variants. The co-occluded mixture HearSP1B:LB6 at a 1:1 proportion was 1.7-2.8 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, suggested 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.

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1. Introduction

Alphabaculoviruses (Baculoviridae) are lepidopteran specific nucleopolyhedroviruses that have been used successfully as biological control agents against several agricultural and forest pests (Moscardi, 1999). These viruses are characterized by high intraspecific heterogeneity, not only between isolates from different geographic regions (Escribano et al., 1999; Rowley et al., 2011), but also within single isolates that often comprise mixtures of several genotypes present in different proportions (Cory et al., 2005; Hodgson et al., 2001; Muñoz et al., 1999; Redman et al., 2010; Simón et al., 2004). The molecular heterogeneity of genotypes is often associated with phenotypic differences in pathogenicity, speed of kill and virus production (Simón et al., 2004; Erlandson, 2004), that are traits of practical importance in the use of these viruses as biological insecticides (Eberle et al., 2012). Infections involving mixtures of genotypes can result in positive (Hodgson et al., 2004; López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simón et al., 2005) or negative (Arends et al., 2005; Barrera et al., 2013; Muñoz and Caballero, 2000; Muñoz et al., 1998) 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 (Bernal et al., 2013; Clavijo et al., 2009; Espinel-Correal et al., 2012; Simón et al., 2004), 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 (Caballero et al., 2009, 2013). 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, Oceania and is in the process of invading South America (Fitt, 1989; Tay et al., 2013). The *H. armigera* single NPV (HearSNPV) (genus *Alphabaculovirus*) has been shown to

be an effective agent for control of *H. armigera* (Allen and Ignoffo, 1969; Figueiredo et al., 1999; Moore et al., 2004; Ogembo et al., 2005, Roome, 1975; Teakle et al., 1986). Several isolates of HearSNPV have been characterized at the molecular and phenotypic levels (Chen et al., 2001; Gettig and McCarthy, 1982; Ogembo et al., 2007, 2009), including some isolates from the Iberian Peninsula (Figueiredo et al., 1999; Arrizubieta et al., 2014, 2015). Genotypic diversity within field isolates of HearSNPV has been demonstrated by cloning the genotypic variants *in vivo* (Sun et al., 1998), *in vitro* (Ogembo et al., 2007), or using BAC technology (Wang et al., 2003). Moreover, various isolates of this virus have been commercialized as the basis for biological insecticides in different parts of the world (Jones et al., 1998).

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, when compared with isolates from other parts of the Iberian Peninsula (Arrizubieta et al., 2014). Some of the HearSP1 component genotypes were identified as the active ingredient for a potential biological insecticide (Caballero et al., 2014). Additional HearSNPV isolates 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*.

2. Material and methods

2.1 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±2ºC, 70±5% relative humidity and 16:8 h light:dark photoperiod on a semisynthetic diet (Greene et al., 1976). The HzAM1 cell line (McInthos and Ignoffo, 1981) was maintained at 28±2ºC, using EX-CELL 420 medium (Sigma, St. Louis, Missouri, USA) supplemented with 10% fetal bovine serum (FBS) (Lonza, Washington, 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 (Arrizubieta et al., 2014; Figueiredo et al., 1999). From a total of seventeen 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 *Eco*RI restriction endonuclease profiles were identified. These variants were named HearLB1 to HearLB6.

2.2 Isolation of HearSNPV genotypic variants

2.2.1. From HearSP1 isolate

The isolation of individual genotypes of HearSP1 was performed once by plaque assay. For this, the hemolymph from 25 *H. armigera* fourth instars previously infected with 10^9 OBs/ml was taken by bleeding at 48 h post-inoculation and filtered through a 0.45 µm pore filter. Six-well tissue culture plates were seeded with 1.5 x 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% (w/v) SeaPlaque agarose (Lonza), and antibiotics were 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 to a vial containing 0.1 ml of PBS (phosphate buffered saline). Finally, ten *H. armigera* fourth instars were intrahemocelically injected with 8 µl of each plaque suspension and then reared individually on semisynthetic diet. Mortality was recorded daily, and virus-killed larvae were individually transferred to a microcentrifuge tube and stored at -20°C until required.

2.2.2. From HearLB isolates

Due to difficulties with cell adhesion and the need to check plaques by microscopy, the purification of genotypes from the seventeen diseased cadavers from Lebrija was performed by end point dilution (EPD). Hemolymph of 25 fourth instar insects previously infected with 10^9 OBs/ml of each isolate was taken at 48 h post-inoculation, filtered through a 0.45 µm pore filter and serially diluted using 5-fold dilutions (1 to 2.56 x 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 x 10^5 HzAM1 cells/ml, and a 100 µl volume of the resulting suspension was placed in 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 28ºC. 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 ≤10% of infected wells, which meant that almost all wells were infected by a single budded virion, i.e. a single genotype (King and Possee, 1992), were removed individually using a sterile Pasteur pipette, and volumes of 8 µl were injected in groups of 10 *H. armigera* fourth instars that were reared individually on semisynthetic diet until death. The resulting OBs were collected and stored at -20 ºC.

2.2.3. OB purification

OBs were extracted from virus-killed larvae as described previously (Arrizubieta et al., 2014). 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 -20ºC.

2.3 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 OB/ml with 100 µl of 0.5 M Na_2CO_3, 50 µl of 10% SDS and 250 µl of distilled water at 60ºC during 10 min. Debris was pelleted at 6,000 x *g* for 5 min and the supernatant was incubated with 25 µl proteinase K (20 mg/ml) at 50ºC during 1 h. Viral DNA was extracted twice with phenol (pH 7.8), once with chloroform, precipitated with 10% (v/v) 3 M sodium acetate (pH 5.2) and ice-cold ethanol at 12,000 x *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 4ºC until use.

Viral DNA (2 µg) was incubated with 10 U *Eco*RI (Takara Bio Inc., Shiga, Japan) at 37ºC 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 µg/ml ethidium bromide and visualized on a transilluminator, GeneSnap (Syngene, San Diego, California, USA). DNA fragment sizes were estimated by comparison to standard molecular weight markers (HyperLadder I, Bioline, or 1kb, NIPPON).

2.4 Biological activity of genotypic variant OBs

The biological activity of genotypic variant OBs was compared in terms of pathogenicity (expressed as OB concentration-mortality metrics, LC_{50}), mean time to death (MTD) and OB productivity (OBs/larva) in *H. armigera* second instars inoculated using the droplet feeding method (Hughes and Wood, 1981). The wild type HearSP1 isolate was included as a reference isolate.

Concentration-mortality responses were determined using five OB concentrations of each variant that were estimated to result in a range of mortalities between ~5 and ~95% (Arrizubieta et al., 2014). Groups of 24-second instars 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 to 25 ml cups with diet and reared at 25±1ºC 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 (Le Ora Software, 1987).

Speed of kill and OB production were determined in groups of 24 second instars (<12 h post-molting) inoculated with the corresponding LC_{90} concentration of OBs, previously determined by 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 to microtubes, incubated at 28ºC for up to 8 h until death, and were subsequently stored at -20ºC. Time-mortality results of individuals that died due to lethal polyhedrosis disease were subjected to Weibull survival analysis in GLIM (Crawley, 1993). For OB counting, infected cadavers were thawed, individually homogenized in 1 ml of distilled water and triplicate samples were counted using a Neubauer improved hemocytometer. OB production values were normalized by log transformation and subjected to analysis of variance (ANOVA) and Tukey test using the SPSS 21.0 program.

2.5 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 following the methods developed by us (Bernal et al., 2013). Briefly, OBs of each genotype were mixed in the desire proportion and 50 *H. armigera* fourth instars were orally inoculated with a LC_{90} concentration (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 semisynthetic diet at 25±1ºC until death. The resulting OBs were purified from virus-killed insects and these were considered co-occluded genotype mixtures. Their pathogenicity (LC_{50}), 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

non-specific mortality. OBs of each of the single genotypes HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 were also included as reference isolates.

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

Mixture	Genotypes	Proportions
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

2.6 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, 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 (P_0) OBs. Groups of 24 fourth instars were allowed to drink a suspension of 1 x 10^7 OBs/ml of P_0 OBs, which resulted in 96-100% mortality. Following incubation and death, virus-killed insects were homogenized and OBs were extracted and purified. These OBs, pooled among the 23-24 larvae that died following inoculation with P_0 OBs, were considered passage one (P_1) OBs, and were used as inoculum to infect the subsequent group of larvae (24 for each passage). The virus population was followed for four additional passages (P_2, P_3, P_4 and P_5). 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 HearSP1B and HearLB6 complete genomes (GenBank accession numbers KJ701033 and KJ701031, respectively) (Arrizubieta et al., 2015) was used to design a set of primers and a probe specific to HearLB6 in the homologous region 1 (*hr1*); LB6-f (5´-GCACACGGCACTATTCCAAC-3´; nucleotides 22,326 to 22,345 in the HearLB6 genome), LB6-r (5´-ACGAACACATCCAAGACCAG-3´; nucleotides 22,414 to

22,433) and LB6-probe (5´-/56-FAM/TAACAAATC/ZEN/ACGCCACGCCCAAAG/3IABkFQ/-3´; nucleotides 22,374 to 22,397). Due to the unavailability of specific primers for HearSP1B, a set of primers was designed using conserved sequences in HearSNPV in the *ha29* gene (Guo et al., 2005); ha29.1 (5´-CTCGTATCATGCAAAACGCC-3´; nucleotides 25,140 to 25,159 in the HearLB6 genome and 25,212 to 25,231 in the HearSP1B genome) and ha29.2 (5´-GAATCTGGCTTCGACTGGC-3´; nucleotides 25,201 to 25,219 in the HearLB6 genome and 25,273 to 25,291 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 using 5 µl of Premix (Takara Bio Inc.) and probe at 0.2 µM final concentration for LB6-f and LB6-r primers and 5 µl of SsoAdvanced SYBR Green Supermix (Bio-Rad, Berkeley, California, USA) for ha29.1 and ha29.2 forward and reverse primers at 0.2 µM final concentration, and 1 µl of template DNA in a total reaction volume of 10 µl. Non-template controls (NTCs), standard curves (30 to 1.9 x 10^{-3} ng/ µl of serial 5-fold dilutions of template DNA from HearLB6) and samples were analyzed twice. The PCR reactions were performed in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). The program used was: 2 min 30 sec at 95ºC; 40 cycles of 98ºC for 15 sec and 60ºC for 30 sec. For reactions performed with SYBR green, this program was followed by a melting curve (60-95ºC). Data were analyzed using Bio-Rad CFX Manager software (Bio-Rad). Results were subjected to repeated-measures analysis of variance (ANOVA) and Tukey test ($P<0.05$) for homogeneous groups using SPSS 21.0 program.

In addition, the concentration-mortality response, speed of kill and OB production of virus OBs sampled at P_0, P_1 and P_5 were determined 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 x 10^3 OB/ml of the selected co-occluded mixture (P_1). These larvae drank less than 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 in larvae that drank a volume of 1 µl of suspension. As larvae drank less than 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 prevalence greater than 26% of virus-killed insects. OBs from each dead larva were purified individually, DNA was extracted and subjected to qPCR analysis as described above to determine the relative prevalence of each genotypic variant in each infected insect.

3. Results

3.1 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 *Eco*RI restriction profiles of their genomic DNA (Fig. 1A) and were named HearSNPV-SP1A (abbreviated as 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, end-point dilution was used for the isolation of HearLB genotypes. End point dilution of HearLB isolates indicated that each of the 17 larvae analyzed was infected by a single genotypic variant, as every clone obtained had an identical *Eco*RI profile to that of the original isolate. In total, six distinct *Eco*RI profiles were identified from the 17 larvae (Fig. 1B); these were designated HearSNPV-LB1, HearSNPV-LB2, HearSNPV-LB3, HearSNPV-LB4, HearSNPV-LB5 and HearSNPV-LB6 (or abbreviated, 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 genotypes HearLB4, HearLB5 and HearLB6 were each isolated from a single larva (5.9% each).

*Eco*RI digestion of variant DNAs revealed the presence of polymorphic fragments that could be used as markers (Fig. 1). The *Eco*RI-B fragment of HearLB4 (11.0 kb) was larger than in 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 (*Eco*RI-D), HearSP1A (*Eco*RI-D) and HearSP1B

(*Eco*RI-D) showed a common fragment of 9.2 kb, whereas in HearLB1 (*Eco*RI-D), HearLB2 (*Eco*RI-D), HearLB3 (*Eco*RI-D), HearLB4 (*Eco*RI-D), HearLB5 (*Eco*RI-C) and HearLB6 (*Eco*RI-D) this fragment was slightly larger (9.38 kb). The *Eco*RI-E (8.7 kb) fragment of HearLB4 is only present in this and in the HearLB5 (*Eco*RI-D) profile. HearSP1A and HearLB2 showed a common fragment of 7.16 kb (*Eco*RI-F), whereas the *Eco*RI-M fragment of HearSP1A (5.26 kb) was absent in HearLB2 and HearLB3. Finally, HearLB5 showed a unique fragment of 3.10 kb (*Eco*RI-S), whereas this variant lacked a 2.83 kb fragment which was present in all the other variant profiles. In addition, the *Eco*RI profile of HearSP1A and HearSP1B showed a fragment of 9.73 kb that was absent in the HearSP1 profile.

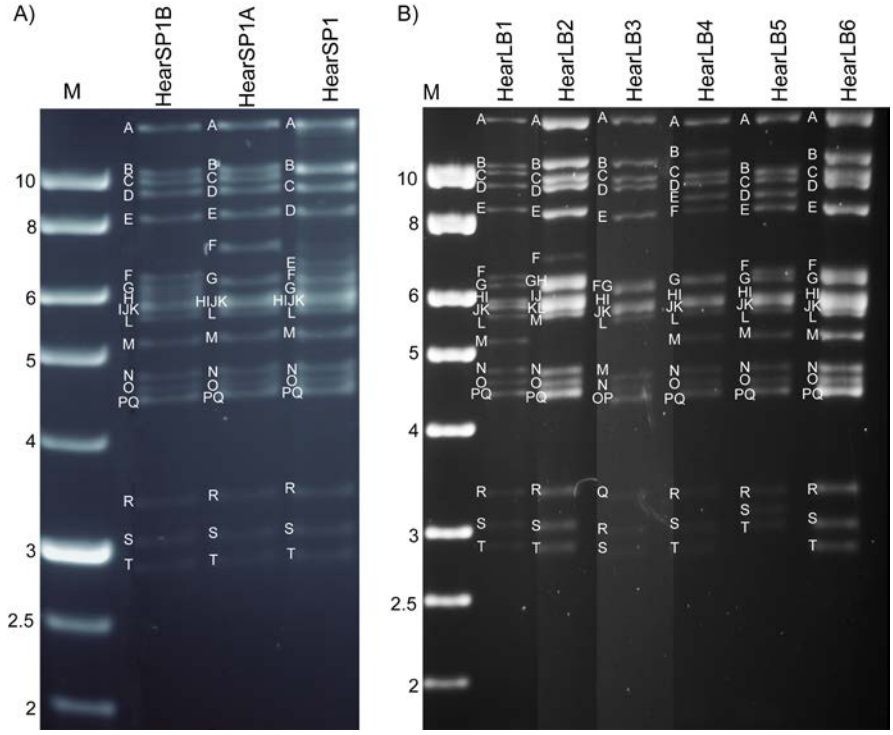

Figure 1: A) *Eco*RI profiles of the genomic DNAs of HearSP1 wild type and the genotypes HearSP1A and HearSP1B. The DNA ladder 1kb (NIPPON) was used as a molecular size marker (M), and fragment sizes are indicated at left (kb). B) *Eco*RI restriction endonuclease profiles of the genomic DNAs of HearLB1, HearLB2, HearLB3, HearLB4, HearLB5 and HearLB6 genotypes. The DNA HyperLadder I (Bioline) was used as a molecular size marker (M), and fragments sizes are indicated at left (kb). Fragments were named alphabetically giving the letter A to the largest *Eco*RI fragment, respectively.

Some of the bands found in the 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 in band L of HearLB1 or HearLB3, which, being a single band, appeared at lower intensity, in comparison with those formed by JK fragments in the same genotypes (Fig. 1B).

3.2 Biological activity of individual variants and mixtures

3.2.1. 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 were observed in mean time to death, since HearSP1A (99.6 hours post inoculation, h.p.i.) and HearSP1B (98.3 h.p.i.) variants killed second instars *H. armigera* at a similar mean time as the wild type HearSP1 (102.8 h.p.i) (Table 2). OB production values were similar for the two variants and slightly lower (~25% reduction) than observed in the wild type isolate, possibly reflecting the faster speed of kill of the variants, although this difference was not significant ($F_{2,6}$=0.46; P>0.05) (Fig. 2A).

3.2.2. Biological activity of HearLB variants

The LC_{50} values of the six HearLB variants were all extremely similar to that of the reference isolate HearSP1 with an LC_{50} value of 1.6×10^4 OBs/ml in second instars (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 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 - 118.4 h.p.i.), whereas HearSP1 wild-type was intermediate in 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 the wild-type HearSP1 was the least productive, producing approximately half the number of OBs per larva as insects infected by HearLB1 (Tukey, P>0.05). HearLB2, HearLB3, HearLB5 and HearLB6 produced intermediate numbers of OBs per larva (Fig. 2B).

Table 2: LC_{50} values, relative potencies and mean time to death (MTD) values of HearSP1 wild-type and the single genotypes HearSP1A and HearSP1B; HearSP1 isolate and HearLB1, HearLB2, HearLB3, HearLB4, HearLB5 and HearLB6 genotypes; and mixtures of genotypes and individual genotypes from two HearSNPV isolates in second instar insects.

		LC_{50}	95% Fiducial limits		Slope	Relative	MTD	95% Fiducial limits	
	Virus	(OBs/ml)	Low	High	± S.E.	potency	(h.p.i.)	Low	High
HearSP1 genotypic 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 genotypic 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
Genotypic mixtures									
Genotypic mixtures	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
Genotypes	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

Probit regressions were fitted in POLO-PC. A test for parallelism was not significant (χ^2=5.57, df=2, P=0.06 for HearSP1 genotypes; χ^2=6.93, df=6, P=0.33 for HearLB genotypes; and χ^2=16.84, df=12, P=0.16 for mixtures of genotypes and individual genotypes). Relative potencies were calculated as the ratio of LC_{50} values relative to HearSP1 wild-type, and to HearLB3:LB6 (1:1). MTD values were estimated by Weibull analysis (Crawley, 1993). Values followed by different letters are significantly different for comparisons among values within each of the four sections of the table (t-test, P<0.05).

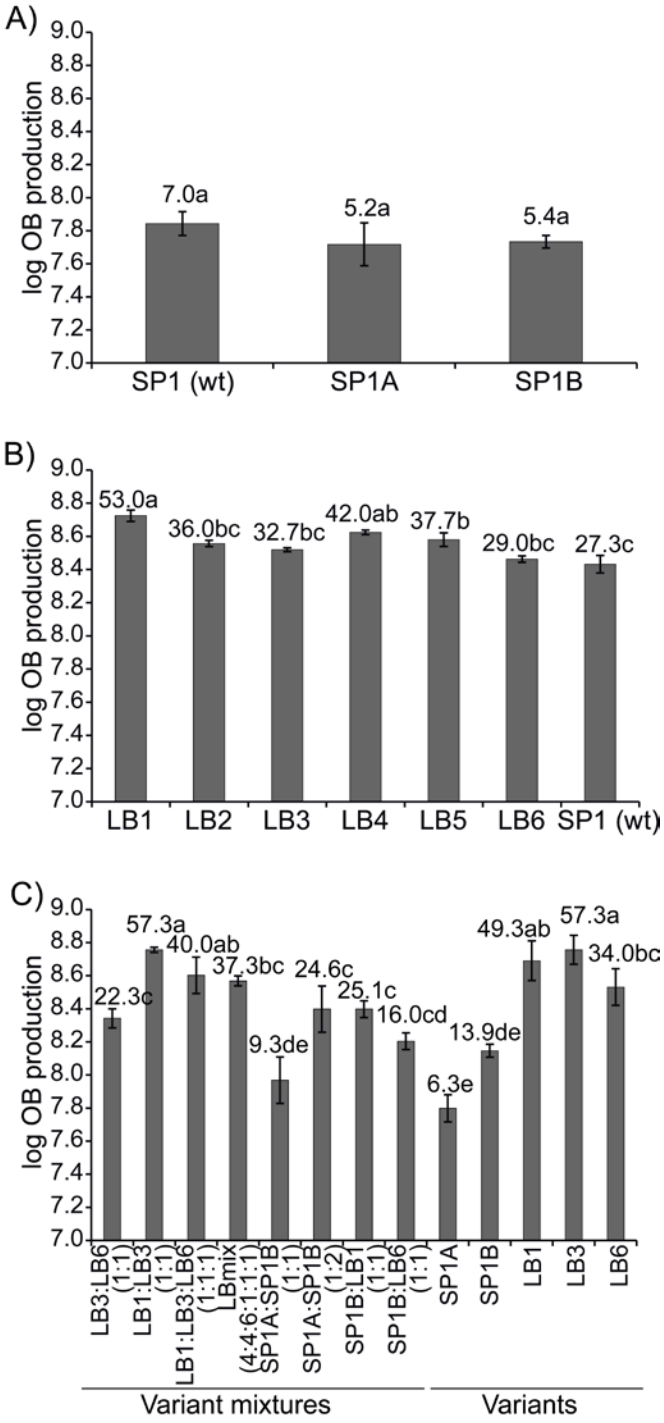

Figure 2: Mean OB production of *H. armigera* second instars that died from virus disease following infection with the LC_{90} of A) HearSP1 wild type (from a total of 68 larvae) and the genotypes HearSP1A (65 larvae) and HearSP1B (65 larvae), B) HearLB1 (66 larvae), HearLB2 (64 larvae), HearLB3 (63 larvae), HearLB4 (68 larvae), HearLB5 (66 larvae), HearLB6 (65 larvae) genotypes and HearSP1 strain (64 larvae), and C) HearSP1A (68 larvae), HearSP1B (64 larvae), HearLB1 (62 larvae), HearLB3 (67 larvae) and HearLB6 (65 larvae) genotypes, and the genotypic mixtures HearSP1A:SP1B (1:1) (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). Vertical lines indicate the standard error. Values above bars represent mean OB production ($x10^7$ OBs/larva) and those followed by identical letters did not differ significantly by ANOVA and Tukey test ($P<0.05$).

3.3 Biological activity of the genotypic mixtures

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

Individual variants and variant mixtures differed significantly in 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 that produced 9-fold fewer OBs per insect (Fig. 2C). The remaining variants had intermediate production of OBs per larva although they differed significantly from one another (Tukey, P<0.05). 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 compared to the HearLB1:LB3 mixture (Tukey, P<0.05). OB production by the HearLBmix mixture, that 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 (Tukey, P<0.05).

3.4 Stability of a co-occluded variant mixture during passage *in vivo*

Following examination of the results on the biological characteristics described above, the HearSP1B:LB6 co-occluded mixture was selected as the mixture with improved insecticidal characteristics compared with single variants, co-occluded variant mixtures or the wild-type isolates.

The relative frequency of HearSP1B and HearLB6 varied significantly throughout the five successive passages ($F_{1,2}$=51.85, P<0.02 for HearLB6 and $F_{1,2}$=171.49, P<0.006 for HearSP1B) (Fig. 3). The frequency of HearSP1B in the mixed variant population increased from 47.7% at P_1 to 84.6% at P_5. Correspondingly, the relative frequencies of HearLB6 decreased from 52.3% at P_1 to 15.4% at P_5.

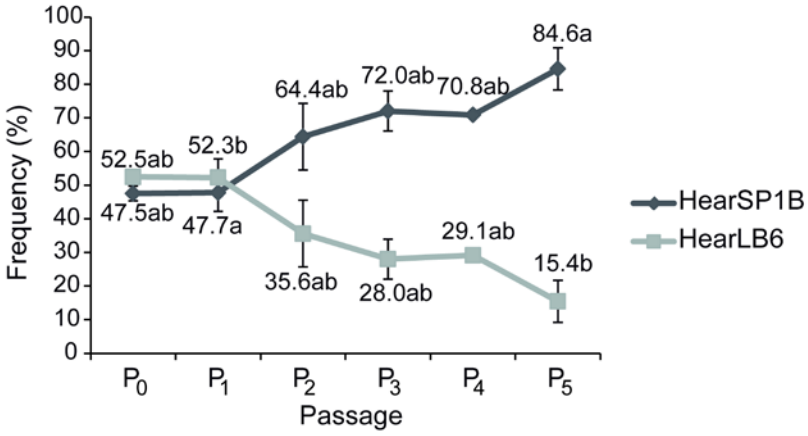

Figure 3: Relative frequencies of HearSP1B and HearLB6 in the genotypic mixture HearSP1B:LB6 throughout five serial passages *in vivo* (P_0-P_5). Vertical lines indicate the standard error. Values followed by identical letters did not differ significantly by ANOVA and Tukey test (P<0.05).

The pathogenicity of the co-occluded mixture HearSP1B:LB6 at P_0 did not change significantly at P_1, but subsequently increased slightly and was 1.7-fold more pathogenic at P_5 (Table 3). The mean time to death decreased significantly between P_0 and P_1 and again between P_1 and P_5 (Table 3). The decrease in MTD value was reflected in significantly decreased values of OB production per larva

between P_0 and P_5, whereas the P_1 value was intermediate (Tukey, P<0.05) (Fig. 4).

Table 3: LC_{50} values, relative potencies and mean time to death (MTD) values of at different passages (P_0, P_1 and P_5) of HearSP1B:LB6 (1:1 mixture) in second instar *H. armigera*.

Passage	LC_{50} (OBs/ml)	Slope ± S.E.	Relative Potency	95% Fiducial limits Low	95% Fiducial limits High	MTD (h.p.i.)	95% Fiducial limits Low	95% Fiducial limits 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

Probit regressions were fitted in POLO-PC. A test for parallelism was not significant (χ^2=4.80, df=2, P=0.09). Relative potencies were calculated as the ratio of LC_{50} values relative to the passage zero (P_0) value. MTD values were estimated by Weibull analysis (Crawley, 1993). Values followed by different letters are significantly different (t-test, P<0.05).

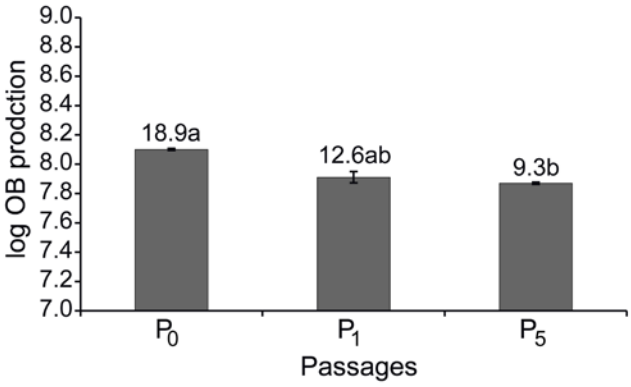

Figure 4: Mean OB production of *H. armigera* second instars that died from virus disease following infection with the LC_{90} of P_0 (from a total of 66 larvae), P_1 (62 larvae) and P_5 (64 larvae) of HearSP1B:LB6 (1:1). Vertical lines indicate the standard error. Values above bars represent mean OB production ($x10^7$ OBs/larva) and those followed by identical letters did not differ significantly by ANOVA and Tukey test (P<0.05).

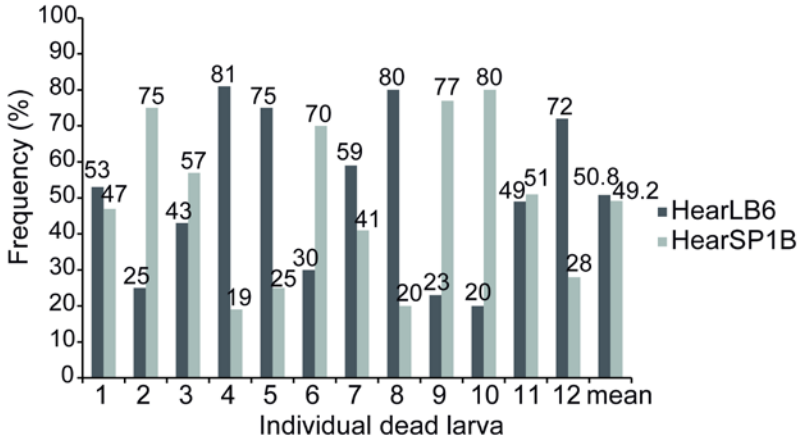

Figure 5: Relative frequencies of HearSP1B and HearLB6 in the OBs obtained from larvae that died after consumption of a single OB. The presence of both genotypes in larvae infected by a single OB corroborated the co-occlusion of both within the same OB.

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

4. 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, or 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 (Simón et al., 2004; Erlandson, 2009; Hodgson et al., 2002).

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 may have been present that could not be detected using this technique. 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 (Bernal et al., 2013). Therefore, other genotypes may have also been present that were not amenable to replication in cell culture. The presence of helper genotypes has also been reported in alphabaculoviruses (Clavijo et al., 2009; López-Ferber et al., 2003). For instance, using an *in vivo* cloning technique, Muñoz et al. (1998) were unable to isolate two of the genotypes from a SeMNPV wild type isolate, as these genotypes required the presence of complementary genotypes to replicate in the insect host. Sun et al. (1998) isolated seven HearSNPV genotypes from a HearSNPV wild type strain using *in vivo* techniques. Similarly, Wang et al. (2003) isolated ten HearSNPV genotypes from a single wild type strain using BAC cloning. In contrast, Ogembo et al. (2007) identified between one and five genotypes from different HearSNPV strains by plaque assay, supporting the idea that some HearSNPV genotypes replicate poorly in cell culture. Unexpectedly, given the previous results in this field, the seventeen HearLB killed larvae that we analyzed were each infected by a single genotypic variant. These could be classified into six different variants by their *Eco*RI restriction profiles, that had not been observed in previously characterized HearSNPV variants (Figuiredo et al., 1999, 2009; Ogembo et al., 2005, 2007; Sun et al., 1998).

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. However, as occurs among other genotypes of many alphabaculoviruses, small changes in the genome can result in important differences in biological activity (Barrera et al., 2013; Bernal et al., 2013; Ogembo et al., 2007). OBs of HearSP1B were ~3 fold more pathogenic than HearSP1-wt, although its prevalence in the population was estimated at 31%. Alternatively, interactions between genotypes might decrease the pathogenicity of the mixture

as previously observed in a SeMNPV population (Muñoz et al., 1998). 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 (Muñoz et al., 1998) and SfMNPV (Barrera et al., 2013). In contrast, among the different variants isolated from larvae that died during laboratory rearing, no marked differences were detected in pathogenicity, but differences were observed in speed of kill, 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 (Arrizubieta et al., 2014). These results are in line with previous studies performed with different variants of HearSNPV, in which variants of similar pathogenicity presented marked differences in speed of kill (Arrizubieta et al., 2014; Guo et al., 2006). Generally, the fastest variants tend to produce lower numbers of OBs in each infected insect, because insects die faster, feed and grow less during the incubation period of the infection, and the virus has less time to replicate (Barrera et al., 2011; Chen et al., 2000; Guo et al., 2006; Sun et al., 2005). However, we observed that HearLB1 was highly productive, despite being one of the fastest-killing variants. This fact has also been observed in other alphabaculovirus species, as SeMNPV, in which the most productive genotype was also among the fastest killing genotypes present in the population (Cabodevilla et al., 2011). Curiously, MTD values of 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 be faster killing than HearLB3, although the difference was significant in one experiment, that comparing individual genotypes, but not in the other, which compared genotypic mixtures. This is why statistical comparisons were performed among treatments within an experiment and not between different experiments (Table 2).

The genetic structure of natural populations of alphabaculoviruses are adapted to improve the survival of the virus which depends on a combination of the 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 where 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 proved of great value for the development of several novel virus insecticides (Caballero et al., 2009, 2013, 2014). 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 virus phenotype. Consequently 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 similar speed of kill to HearLB6, but with low OB productivity. These characteristics might favor its use in field, as the increased pathogenicity and speed of kill could favor the rapid suppression of the pest using the 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 in ChchSNPV, in which co-occluded mixtures of variants were faster killing than mixtures of OBs of each variant (Bernal et al., 2013). Bernal et al. (2013) 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 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 higher pathogenicity of HearSP1B was likely responsible for its increased transmission capacity compared with 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 85%HearSP1B:15%HearLB6 mixture obtained after five passages *in vivo* is the most suitable active ingredient for a biopesticide product. This process represents a unique mechanism for 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 *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) that were subjected to successive passages *in vivo* rapidly converged to the frequencies present in the wild type isolate (Clavijo et al., 2009; Simón et al., 2006). However, the genotypes HearSP1B and HearLB6 were isolated from different wild type isolates and this genotypic mixture reached genotypic frequencies that apparently favored improved transmission.

In conclusion, the genotypic mixture HearSP1B:LB6 was selected to be developed as a bioinsecticide for control *H. armigera* larvae, as showed the highly suitable insecticidal characteristics. Moreover, before marketing such a bioinsecticide, it would be necessary to determine the most suitable conditions for the mass 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 simple binary mixture of HearSNPV genotypic variants selected from the 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.

5. Acknowledgements

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CHAPTER IV

Genomic sequence of five closely related *Helicoverpa armigera* single nucleopolyhedrovirus genotypes from Spain showing significant differences in their insecticidal properties

Abstract

Helicoverpa armigera single nucleopolyhedrovirus (HearSNPV) has been proved effective as the basis for a biological insecticide. Complete genome sequences of five HearSNPV genotypes differed principally in the homologous regions (*hrs*) and the baculovirus repeat ORFs (*bro*) genes suggesting that they may be involved in the phenotypic differences observed between genotypes.

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Helicoverpa armigera (Lepidoptera: Noctuidae) is a widely distributed, polyphagous insect pest that results in economic losses in many crops (cotton, maize, tomato, etc.) (Fitt, 1989). This insect has been recently introduced in Latin America where it causes extensive damage to important crops, particularly soybean (Czepak et al., 2013). The *H. armigera* single nucleopolyhedrovirus, HearSNPV (*Baculoviridae*: *Alphabaculovirus*), has proved to be an effective alternative to chemical control methods (Jones et al., 1998; Zhang, 1994). Several naturally-occurring HearSNPV isolates have been characterized in terms of genome sequence and phenotypical traits such as pathogenicity, speed of kill and occlusion body production in infected insects. These isolates originate from different geographical regions, including some from Spain (Figueiredo et al., 1999, 2009), reflecting the broad geographical distribution of the pest. In a previous study, two genotypes (named HearSNPV-SP1A and HearSNPV-SP1B), purified in vitro from a highly insecticidal Iberian isolate HearSNPV-SP1 (Arrizubieta et al., 2014), and three other genotypes (HearSNPV-LB1, HearSNPV-LB3 and HearSNPV-LB6), isolated from single larvae that died from patent polyhedrosis disease during laboratory rearing, were selected as the most suitable genotypes to form the basis for the active ingredient of a new baculovirus insecticide (Caballero et al., 2014). In the present study, the sequencing of the genomes of these five genotypes was determined using the PacBio technique (Pacific Bioscience, Merlo Park, USA) and assembled using the HGAP v2.0.2 program.

The genomes of HearSNPV-SP1A, HearSNPV-SP1B, HearSNPV-LB1, HearSNPV-LB3 and HearSNPV-LB6 were 132,481, 132,265, 131,966, 130,949 and 130,992 bp in size, respectively. The size of these genomes is very similar to other genotypes of *H. armigera* SNPV that have been sequenced previously such as HearSNPV-C1 (AF303045) of 130,759 bp, HearSNPV-G4 (AF271059) of 131,405 bp, HearSNPV-NNg1 (AP010907) of 132,425 bp, HearSNPV-Au (JN584482) of 130,992 bp and HzSNPV (AF334030) of 130,869 bp (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). The G+C content of the four novel Iberian virus genomes is 39% which is also very similar to other *H. armigera* SNPV isolates (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014) and lower than commonly observed in other species of alphabaculoviruses (Ayres et al., 1994; Simón et al., 2011; Thézé et al., 2014).

A total of 136 ORFs were identified in the genomes of HearSNPV-SP1A and HearSNPV-SP1B, whereas the genomes of HearSNPV-LB1, HearSNPV-LB3 and HearSNPV-LB6 lacked one ORF, as only two instead of three *bro* genes were found between the homologous regions (*hrs*) 2 and 3. The 136 ORFs are present at least in one of the HearSNPVs and HzSNPV genomes that have been sequenced previously (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). The Iberian genotypes also conserved the 62 core genes (Herniou et al., 2003), whereas only 10 of the 17 ORFs that are unique to *Helicoverpa* spp. SNPVs (Ogembo et al., 2009) were present in the five Spanish genotypes. ORF5 of unknown function was present in all the sequenced *Helicoverpa* spp. SNPVs (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). ORF58 (*bro-a*), unique to the Chinese genotypes, HearSNPV-C1 (Chen et al., 2001) and HearSNPV-G4 (Zhang et al., 2005), was not identified in the genomes of the Spanish genotypes. Additionally, five *hrs* were identified in these genotypes at the same genomic positions (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). Overall, 43 ORFs were 100% homologous among the Spanish genotypes, but only 13 ORFs presented 100% homology to the previously sequenced genotypes.

Although the insecticidal properties of the five Spanish genotypes showed significant differences between them and with respect to other genotypes; however, they showed 98-99% homology at the nucleotide level, and 95-99% homology with the previously sequenced HearSNPVs and HzSNPV. Alignments of complete sequences revealed that the principal differences among the isolates were located in the homologous regions (*hrs*) and baculovirus repeat ORFs (*bro*) genes, as previously reported for other isolates (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014), suggesting that these genes could be involved in genotype-specific differences in a number of insecticidal properties. Finally, phylogenetic analysis derived from this alignment revealed that the genotype from Kenya, HearSNPV-NNg1, is the most similar to Spanish genotypes. The Spanish genotypes grouped in the same cluster with HearSNPV-NNg1, whereas the HearSNPV isolates from China (HearSNPV-C1 and HearSNPV-G4) and Australia (HearSNPV-Au), and HzSNPV, from USA, were grouped in another cluster, possibly reflecting geographical dispersal of the pest, that has its origin in Africa.

The complete genome sequences presented in this study provide a useful resource for further exploring the genetic diversity of this virus and the factors that are involved in differences in the insecticidal properties observed among the different genotypes.

Nucleotide sequence accession numbers

The complete genome sequences of HearSNPV-LB1, HearSNPV-LB3, HearSNPV-LB6, HearSNPV-SP1A and HearSNPV-SP1B were submitted to GenBank under the accession numbers KJ701029, KJ701030, KJ701031, KJ701032 and KJ701033, respectively.

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CHAPTER V

Phenotypically diverse *Helicoverpa armigera* single nucleopolyhedrovirus genotypes differ minimally in *bro* genes and homologous regions

Abstract

A binary co-occluded mixture of *Helicoverpa armigera* nucleopolyhedrovirus (HearSNPV) variants has proved effective as the basis for a biological insecticide in the Iberian Peninsula. Genomic analysis revealed that the five Spanish genotypes, HearSNPV-SP1A (HearSP1A), HearSNPV-SP1B (HearSP1B), HearSNPV-LB1 (HearLB1), HearSNPV-LB3 (HearLB3) and HearSNPV-LB6 (HearLB6), were 130,949-132,481 bp in size, very close to the genome sizes of other *Helicoverpa* spp. SNPVs (130,759-132,425 bp). A total of 136 ORFs were identified in the HearSP1A and HearSP1B genomes, compared to 135 ORFs in HearLB1, HearLB3 and HearLB6 as they lacked one *bro* gene between *hrs* 2 and 3. The Spanish genotypes showed high degree of shared identity at the nucleotide level (98-99%) and to the other HearSNPVs and HzSNPV (95-99%), and were more closely related to the Kenyan genotype. Major differences were located in the *hrs* and *bro* genes, influencing viral phenotype. Point mutations in genes involved in DNA replication (*ie-1*, *lef-3*, *DNA polymerase*), viral transcription (*lef-8*, *lef-1*) or structural genes (*p78/83*, *vp1054*, *desmop*, *calyx/pep*, *odv-e66*) could also be involved in the reduced OB production of HearSP1 genotypes or the increased pathogenicity of HearSP1B. Additionally, mutations in the *iap-2*, *iap-3* and *hoar* genes could be related to the transmission strategy or the ability to establish a covert infection in the insect host. Selection pressure analysis showed strong selection in genes involved in transmission and dispersion of the virus, such as those involved in primary infection (*p78/83*, *odv-e56* and *p74*), virus infection and replication (*ie-0*) or insect development (*egt*). This clearly indicates that these processes are important features where adaptation acts during the transmission of these viruses in distinct hosts. In contrast, other positively selected genes of unknown function (*bro-d*, *bv-ec31* and ORF130) require additional study.

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1. Introduction

Helicoverpa armigera (Lepidoptera: Noctuidae) is an insect pest distributed worldwide that causes important damage in several crops (Fitt, 1989; Czepak et al., 2013). Several strains of *H. armigera* single nucleopolyhedrovirus (HearSNPV) have been proved effective as biological control agents against *H. armigera* (Arrizubieta et al., 2015; Jones et al., 1998; Zhang, 1994). A previous study showed that a binary co-occluded mixture, composed of HearSNPV-SP1B (or abbreviated HearSP1B) and HearSNPV-LB6 (HearLB6), named HearSP1B:HearLB6, had potential as a bioinsecticide for the control of *H. armigera* pest populations in the Iberian Peninsula (Arrizubieta et al., 2015). These genotypic variants were selected for the mixture due to the high pathogenicity of HearSP1B and the rapid speed of kill of HearLB6 in comparison with wild-type isolates or other pure genotypes. The HearSP1B is a plaque-purified genotype obtained from the most prevalent and pathogenic isolate in the Iberian Peninsula, HearSNPV-SP1 isolate (Arrizubieta et al., 2014; Figueiredo et al., 1999), along with HearSP1A (Arrizubieta et al., 2015). In contrast, HearLB6 was isolated from a single larva that died in the laboratory, as were HearLB1, HearLB2, HearLB3, HearLB4 and HearLB5 (Arrizubieta et al., 2015).

In the present study, the sequencing of the complete genome of HearSP1B and HearLB6 was performed. For comparison, the genomes of HearSP1A, HearLB1 and HearLB3 were also included in the analysis in an attempt to identify the genetic factors responsible for the observed differences in the biological activity of these genotypes. Additionally, these sequences were compared with the previously sequenced *Helicoverpa* spp. nucleopolyhedrovirus genomes, comprising four HearSNPVs and one *H. zea* SNPV (HzSNPV). Two of HearSNPV were isolated in China, HearSNPV-G4 (HearG4) (Chen et al., 2001) and HearSNPV-C1 (HearC1) (Zhang et al., 2005), one in Kenya, Africa, HearSNPV-NNg1 (HearNNg1) (Ogembo et al., 2009) and the last one in Australia, HearSNPV-Au (HearAu) (Zhang et al., 2014), while the HzSNPV isolate was obtained in USA (Chen et al., 2002). These genotypes although showing important differences in their insecticidal activities against *H. armigera* larvae (Ogembo et al., 2007; Zhang et al., 2005), presented high nucleotide sequence identity, except for the homologous regions (*hr*) and the baculovirus repeat ORFs (*bro*), which

might be involved in the observed differences at the phenotypic level (Ogembo et al., 2009). In addition, minimal changes at genome level, such as individual amino acid substitutions, might also influence the insecticidal activity of the virus (Argaud et al., 1998; Kamita and Maeda, 1997; Simón et al., 2012; Yang, 1998). Moreover, single nucleotide substitutions could affect the activity of the encoded protein, thus facilitating or hampering viral adaptation to a given host species or population (Harrison and Bonning, 2004). Therefore, in addition to genome sequence comparisons, maximum-likelihood models of codon substitutions, in terms of the non-synonymous to synonymous substitution rate ratio ($\omega = d_N/d_S$), were also examined to determine positively selected ORFs. The ω ratio provides a sensitive measure of selection on amino acid sequences, and allows the identification of genes undergoing positive or diversifying selection. Nucleotide substitutions may lead to alterations in the activity of the encoded protein that facilitate adaptation to a new host or effectively overcome the defenses of a current host. Such mutations may confer a selective advantage and could be fixed in the population at a higher rate than silent or neutral substitutions (Harrison and Bonning, 2004). Comparative analysis of the different *Helicoverpa* spp. SNPV genomes from geographically distinct sources could be an effective method for highlighting the genetic basis for differences in their insecticidal activity, thereby focusing attention on the key genes involved in desirable characteristics for the development of bioinsecticides, particularly with respect to host range, occlusion body (OB) pathogenicity or speed of kill, for which several genes have been implicated in studies on other nucleopolyhedrovirus-insect pathosystems (Chen and Thiem, 1997; Chen et al., 1998; Popham et al., 1998).

2. Experimental methods

2.1 Viruses

A binary co-occluded mixture HearSP1B:HearLB6, previously found to be highly pathogenic against *H. armigera* (Arrizubieta et al., 2015), was composed by HearSNPV-SP1B (HearSP1B), purified from the most prevalent and active Iberian isolate HearSNPV-SP1 (Arrizubieta et al., 2014), and by HearSNPV-LB6 (HearLB6), obtained from a larva that died during laboratory rearing (Arrizubieta et al., 2015). Additionally HearSP1A, isolated from HearSP1, and HearLB1 and

HearLB3, also isolated during laboratory rearing, were included in the analysis in an attempt to identify the genomic regions involved in the OB pathogenicity and speed of kill characteristics of HearSP1B and HearLB6, respectively.

To produce large quantities of OBs, groups of 50 *H. armigera* fifth instars from a laboratory colony were inoculated with 2.5 x 10^7 OBs/ml using the droplet feeding method, previously estimated to kill 90% of inoculated insects (Arrizubieta et al., 2014), and incubated with semisythetic diet until death or pupation. OBs were extracted from virus-killed insects, mixed with an equal volume of distilled water, filtered through muslin, and centrifuged at 2,500 x *g* for 5 min. Pellets were washed once in 0.1% (wt/vol) sodium dodecyl sulfate (SDS) and twice in distilled water, and finally resuspended in distilled water (Arrizubieta et al., 2014).

2.2 Viral DNA isolation and sequencing

DNA was obtained from purified OBs by CsCl gradient purification (King and Possee, 1992). Briefly, 2 ml of 10^{10} OBs/ml of each genotype were mixed with an equal volume of 0.1 M Na_2CO_3 and incubated at 28ºC for 30 min. Debris was removed by low-speed centrifugation (6,000 x *g*, 5 min). The supernatant containing occlusion derived virions (ODVs) was placed on a 70-30% continuous sucrose gradient and centrifuged at 4ºC at 98,000 x *g* during 1 h in a Beckman centrifuge using a SW 32 ti rotor. Thereafter a single band, corresponding to single nucleocapsid ODVs, was extracted for each genotype by puncturing the centrifugation tube with the needle of a 1 ml syringe at the height of the band. The extracted volume was finally diluted in 2 vol. of TE buffer (10 mM Tris, 1 mM EDTA). The ODVs were pelleted by centrifugation at 4ºC at 98,000 x *g* during 1 h and resuspended in 400 µl of TE. DNA was extracted from ODVs by mixing with 100 µl of 20% sarkosyl (N-Laurylsarcosine sodium salt, Sigma) and incubating during 30 min at 60ºC. This lysate was transferred to a 5 ml tube of cesium chloride (50% w/w TE) containing ethidium bromide, and centrifuged at 126,000 x *g* during 18-24 h at room temperature in a Beckman centrifuge using a 70 Ti rotor. After centrifugation DNA was collected from the centrifuge tube by puncturing the tube with a 1 ml syringe needle just below the DNA bands. Ethidium bromide was removed from the DNA by mixing 6 times with an equal volume of butanol. The DNA samples were then dialyzed during 48 h in TE buffer at 4ºC, changing the TE every 8 hours. Finally DNA was kept at 4ºC until use.

Between 5-10 µg of DNA of each genotype were used for DNA sequencing using the PacBio technique (Pacific Bioscience, Merlo Park, USA) by Lifesequencing S.L. (Paterna, Spain). For this, a genomic library was constructed for each genotype in a sequencing vector and sequence information was generated from 1,962-24,627 reads. The genome sequences were assembled using the HGAP v2.0.2 program (Pacific Bioscience).

2.3 Sequence analysis

Open reading frames (ORFs) consisting of more than 50 amino acids (150 nucleotides) with minimum overlaps were considered to be protein encoding and were designated putative genes. The DNA and amino acid deduced sequences of each genotype were compared with one another and with the previously sequenced *Helicoverpa* spp. SNPVs obtained from the GenBank database: HearSNPV-G4 (HearG4) (AF271059) (Chen et al., 2001), HearSNPV-C1 (HearC1) (AF303045) (Zhang et al., 2005), HearSNPV-NNg1 (HearNNg1) (AP010907) (Ogembo et al., 2009), HearSNPV-Au (HearAu) (JN584482) (Zhang et al., 2014) and HzSNPV (AF334030) (Chen et al., 2002) using BLASTn (Altschul et al., 1990; Pearson, 1990). Sequence alignments were performed using Blosum62 substitution matrix (Henikoff and Henikoff, 1992) present in the Clone Manager 9 program (Scientific and Educational Software, Morrisville, USA). Muscle program was used for multiple sequence alignments, whereas MEGA5 software (Tamura et al., 2011) was used to determine optimal nucleotide substitution models and to construct the phylogenetic trees. Maximum-likelihood phylogenetic tree was generated using MEGA5 with the optimal model parameters and the complete deletion option was performed to eliminate positions containing gaps. Confidence levels for the branching points were determined using 1,000 bootstrap replicates.

2.4 Positive selection analysis

Selective pressures acting on each ORF were initially screened using a Bayesian inference approach on Selecton Server with default parameters (Doron-Faigenboim et al., 2005; Stern et al., 2007), in order to calculate the ratio of non-synonymous/synonymous ($\omega=d_N/d_S$) nucleotide substitution acting on every ORF and to infer single amino acid sites subjected to these processes. The d_N to d_S ratio, ω, is a measure of the magnitude of selection acting on a gene. Genes with

a value of ω=1 are considered to be subjected to neutral selection, and non-synonymous mutations have no effect on fitness. Genes with ω<1 are undergoing negative or purifying selection, in which non-synonymous mutations are eliminated at a faster rate than synonymous mutations because of their deleterious influence on fitness. Finally, genes with ω>1 are undergoing positive or diversifying selection in which non-synonymous mutations are fixed at a faster rate than synonymous mutations as they positively influence fitness. To achieve this, sequences were examined for the presence of indels causing a frameshift and the affected codons were removed from the alignments; indels that did not result in a frameshift were not eliminated. For those ORFs identified as undergoing significant selection ($P<0.05$), additional analyses were performed using the multiple sequence alignments and phylogenetic trees constructed by Selecton Server with two maximum likelihood methods; Paml4 (Yang, 2007) and the HyPhy package (Pond et al., 2005). Only those ORFs and sites undergoing significant selection with the three methods were scored and the rest were considered as potential false positives for selection.

3. Results and discussion

3.1 General characteristics of the Spanish HearSNPVs genomes

The complete nucleotide sequences of the HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 genomes were 132,481, 132,265, 131,966, 130,949 and 130,992 bp in size, respectively, very closed to the genomes size of other *Helicoverpa* spp. SNPV genotypes previously sequenced, such as those of HearG4 (130,759 bp), HearC1 (131,405 bp), HearNNg1 (132,425 bp), HearAu (130,992 bp) or HzSNPV (130,869 bp) (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014) (Table 1). These differences in the genome size of the different *Helicoverpa* spp. SNPVs were attributed to short deletions and insertions along the whole genome, and predominantly those found in the homologous regions (*hr*) and *bro* genes. When comparing the Spanish genomes, the HearSP1A genotype had the largest genome sequence, and was used as the reference genome to perform the sequence comparisons. In contrast, the HearSP1B, HearLB1, HearLB3 and HearLB6 genomes were 216 bp, 515 bp, 1,532 bp and 1,489 bp shorter than the HearSP1A genome, respectively. Those

deletions were mostly located in the *hrs*, and specifically within *hr1* (22,062 to 24,129 nt in HearSP1A genome). Compared to HearSP1A, the HearSP1B genome contained a deletion of 150 bp (nt 22,514-22,663 in the HearSP1A genome) and HearLB1 a deletion of 202 bp (nt 22,262-22,463 in the HearSP1A genome). Similarly, the HearLB3 and HearLB6 genomes showed also a 202 bp deletion, but in this case located between nt 22,117-22,318 in the HearSP1A genome. The HearLB3 genome contained an additional deletion of 111 bp (nt 23,363-23,474 of HearSP1A). Within the other *hrs*, HearLB3 and HearLB6 had a deletion in *hr2* of 414 bp (nt 49,185 to 49,598 in HearSP1A genome) and another of 421 bp in *hr4* (nt 93,237-93,657 in HearSP1A genome). Finally the HearLB1, HearLB3 and HearLB6 genomes contained a 391 bp deletion (nt 51,814-52,204 in HearSP1A genome) that overlapped and eliminated the *bro-b* gene (nt 51,851 to 52,156 in HearSP1A genome). The remaining differences in the genome sizes among the Spanish HearSNPV genomes were attributed to short deletions and insertions along the whole genome.

Table 1: General characteristics of *Helicoverpa* spp. SNPV genomes.

	SP1A	SP1B	LB1	LB3	LB6	G4	C1	NNg1	Au	Hz
Size (bp)	132,481	132,265	131,966	130,949	130,992	131,405	130,759	132,425	130,992	130,869
% G+C	39.2	39.1	39.2	39.2	39.2	39	38.9	39.2	39.0	39.1
nº ORFs	136	136	135	135	135	136	135	143	134	139
nº *hr* (bp)	5 (7,367)	5 (7,177)	5 (7,151)	5 (6,230)	5 (6,278)	5 (9,301)	5 (6,325)	5 (6,399)	5 (9,430)	5 (8,348)

The computationally-derived *Eco*RI restriction profiles of HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 were in agreement with the empirical restriction endonuclease analysis performed previously (Arrizubieta et al., 2015). However, minimal differences were observed between genotypes in the number and position of restriction sites due to these short deletions and insertions, which resulted in slightly different *Eco*RI profiles for each isolate.

The G+C content was very similar among the Spanish genomes, being 39.2% for HearSP1A, HearLB1, HearLB3 and HearLB6 and 39.1% for HearSP1B, compared to the previously sequenced HearSNPVs and HzSNPV, with a G+C content between 38.9% (HearC1) and 39.2% (HearNNg1), while the others

presented an intermediate G+C content (Table 1) (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014).

Table 2: Percentage of nucleotide sequence identity among *Helicoverpa* spp. SNPV complete genomes.

% Identity	SP1B	LB1	LB3	LB6	G4	C1	NNg1	Au	Hz
SP1A	99.4	98.6	97.9	97.9	96.1	95.5	98.7	96.0	96.5
SP1B	-	98.7	97.9	97.9	96.1	95.5	98.8	95.9	96.6
LB1	-	-	98.4	98.5	96.2	95.8	97.9	96.1	96.9
LB3	-	-	-	99.7	96.1	95.7	97.9	96.1	97.1
LB6	-	-	-	-	96.2	95.8	97.7	96.1	97.0

The nucleotide sequence alignment showed an overall identity of 97.9%-99.7% among the Spanish genotypes, with the HearLB3 and HearLB6 genomes being the most identical (99.7%), while HearSP1A and HearLB3 genomes showed slightly more divergence (97.7%). A higher identity was observed among the HearSP1 genomes, with HearSP1A and HearSP1B showing 99.4% identity at the nucleotide level, while genotypes also isolated during laboratory rearing showed a little more divergence; HearLB1 had 98.4% identity with HearLB3 and 98.5% identity with HearLB6, while HearLB3 and HearLB6 genomes were more proximal with 99.7% identity (Table 2). These identity relationships are consistent with the differing origins of the HearSP1A and HearSP1B genotypes that were isolated from a distinct source compared to the HearLB1, HearLB3 and HearLB6 genotypes (Table 2), as shown in the phylogenetic tree where they form two distinct branches (Fig. 1). The high identity was also maintained with the previously sequenced HearSNPVs and HzSNPV genomes (95.5% to 98.8%). In fact, the high percentage of identity observed among these genomes confirmed that the 10 genotypes clearly represent strains of the same virus species. The Kenyan genotype genome, HearNNg1, was the most identical to the five Spanish genomes (Table 2), as the Spanish genotypes clustered with HearNNg1. This might be due to their geographical proximity, whereas the Chinese HearC1 genome was the most distantly related. Moreover, the Spanish genotypes were more closely related to HzSNPV from the USA, than to the HearSNPVs isolates from China (HearG4 and HearC1) and Australia (HearAu), which were grouped in another cluster (Fig. 1). These phylogenetic relationships among the different

HearSNPV might be explained by the seasonal migration of *H. armigera*, adults flying from cold regions to warmer ones and vice versa depending on the time of year (Spain-Africa or China-Australia), facilitating virus dispersion between these regions (Feng et al., 2009; Pedgley, 1985). The close relationship between the Spanish HearSNPVs and the American HzSNPV suggest that the latter could have evolved from a European isolate, although this has not been demonstrated to date.

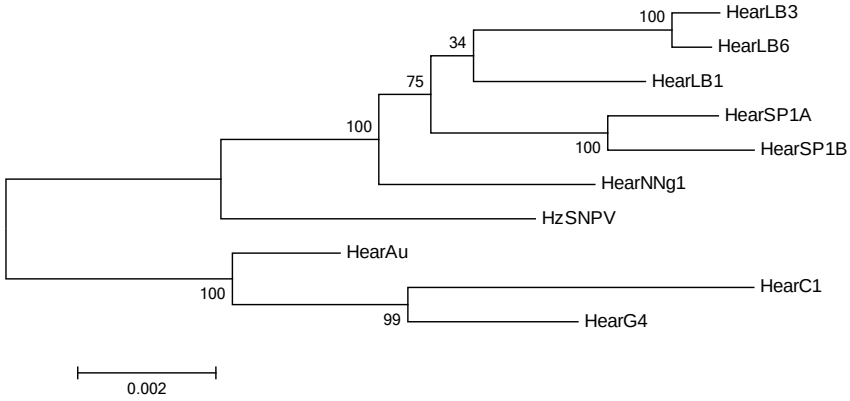
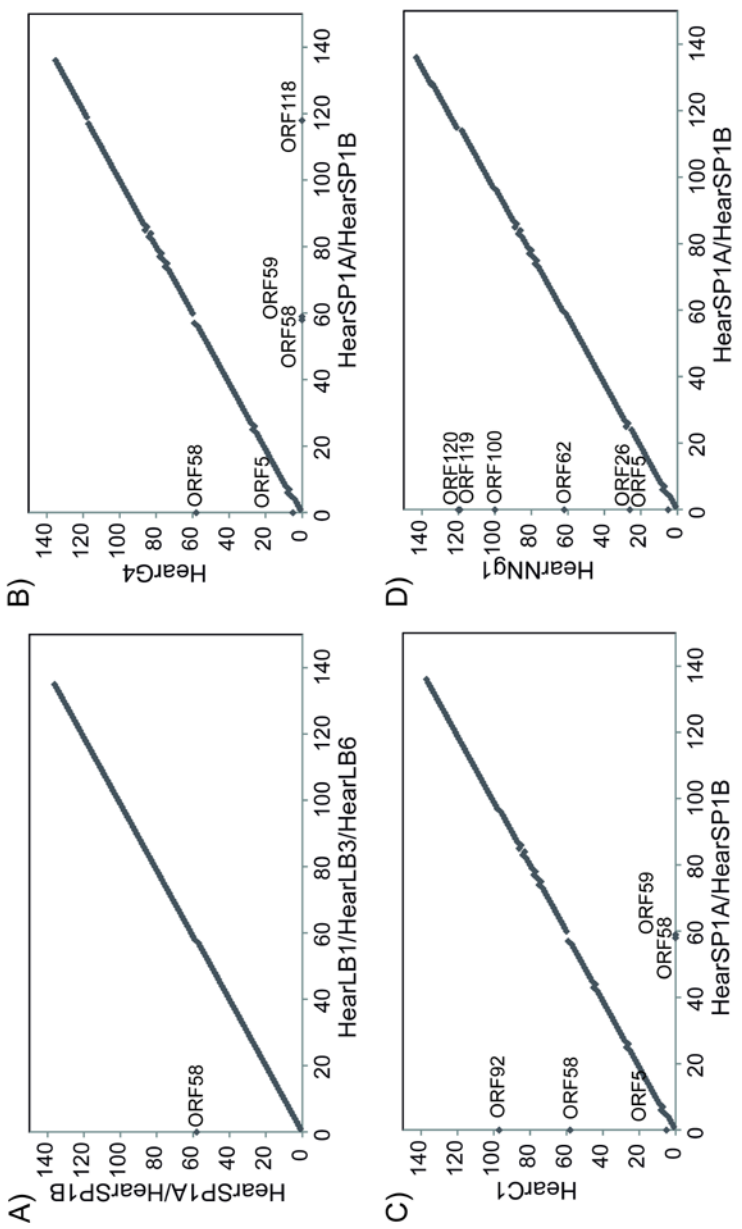
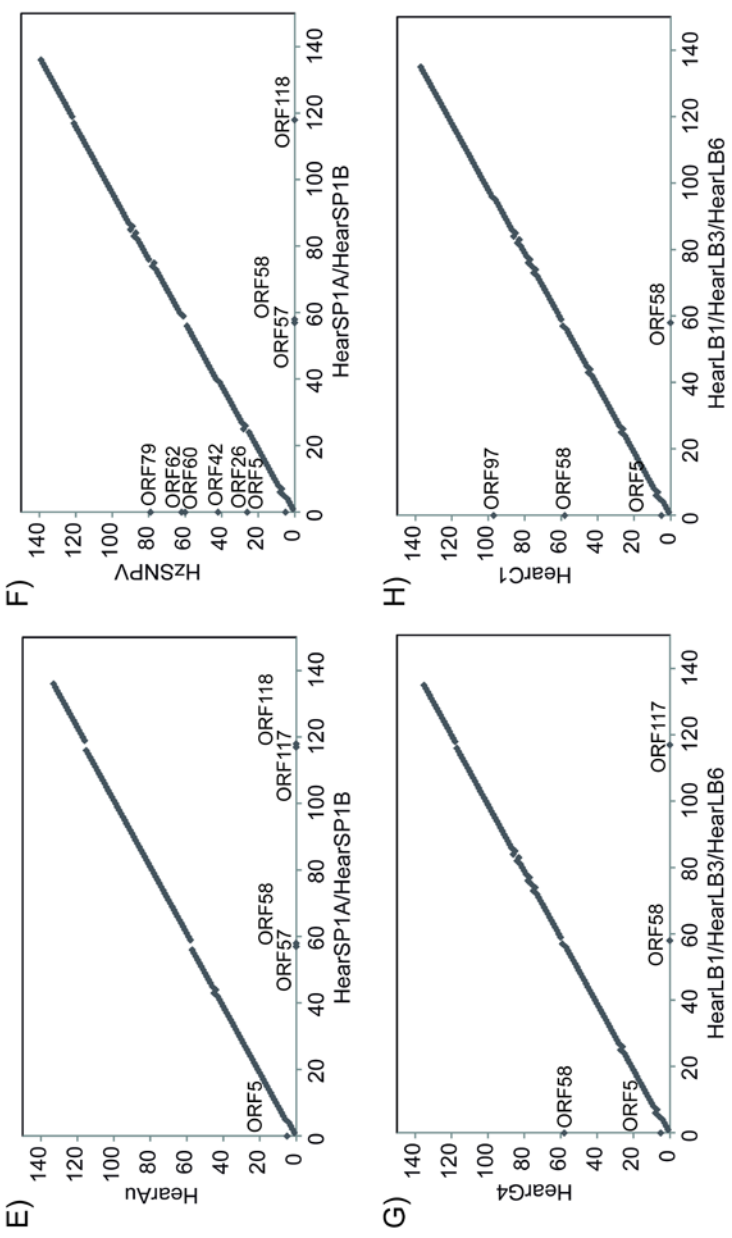


Figure 1: Maximum-likelihood phylogenetic tree of the complete genome sequences of *Helicoverpa* spp. SNPVs using MEGA5 software (Tamura et al., 2011) for alignment. Baculovirus sequences used in this phylogenetic analysis were GenBank (accession numbers indicated in parentheses): HearSP1A (KJ701032), HearSP1B (KJ701033), HearLB1 (KJ701029), HearLB3 (KJ701030), HearLB6 (KJ701031), HearG4 (AF271059), HearC1 (AF303045), HearNNg1 (AP010907), HearAu (JN584482) and HzSNPV (AF334030). Numbers on the nodes indicate bootstrap values (1,000 replicates).

3.2 Comparison of HearSNPV ORFs

Using computer assisted analysis 136 ORFs were identified in the HearSP1A and HearSP1B genomes, while 135 ORFs were present in the HearLB1, HearLB3 and HearLB6 genomes, due to a deletion found within the *hr* 2 and 3 genomic region that eliminated the *bro-b* gene present in the HearSP1A and HearSP1B genomes (Fig 2; Table 3). This number of ORFs was similar to those described in the Chinese genotypes, HearG4 (136 ORFs) and HearC1 (135 ORFs) (Chen et al., 2001; Zhang et al., 2005), and Australian genotype, HearAu (134 ORFs) (Zhang et al., 2014) (Table 1, Table 3), while in genotypes from Kenya, HearNNg1 (143 ORFs) and USA HzSNPV, 139 ORFs were identified (Chen et al., 2002; Ogembo et al., 2009).


A)
HearSP1A/HearSP1B
HearLB1/HearLB3/HearLB6
ORF58
B)
HearG4
HearSP1A/HearSP1B
ORF5
ORF58
ORF59
ORF118
C)
HearC1
HearSP1A/HearSP1B
ORF92
ORF58
ORF5
ORF59
ORF58
D)
HearNNg1
HearSP1A/HearSP1B
ORF120
ORF119
ORF100
ORF62
ORF26
ORF5
0
20
40
60
80
100
120
140


F)
HzSNPV
ORF79
ORF62
ORF60
ORF42
ORF26
ORF5
ORF58
ORF57
ORF118
HearSP1A/HearSP1B
H)
HearC1
ORF97
ORF58
ORF5
HearLB1/HearLB3/HearLB6
E)
HearAu
ORF5
ORF57
ORF58
ORF117
ORF118
HearSP1A/HearSP1B
G)
HearG4
ORF58
ORF5
ORF58
ORF117
HearLB1/HearLB3/HearLB6

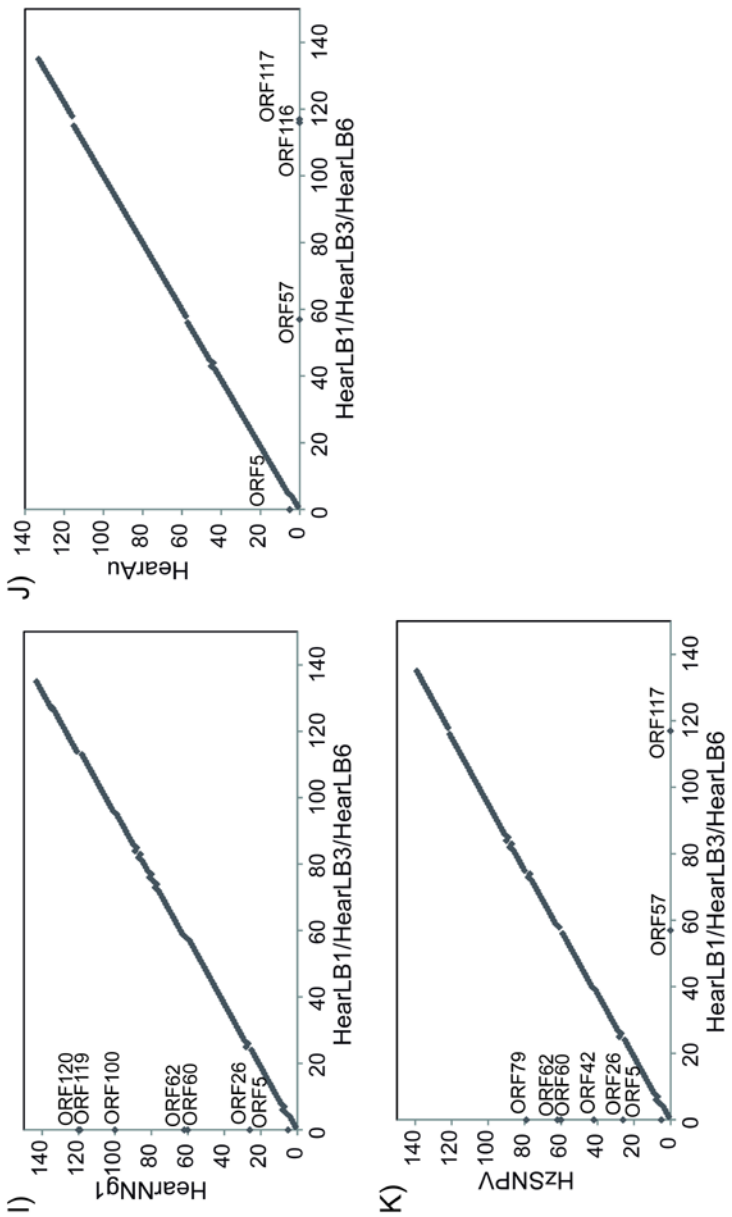


Figure 2: Gene parity plot comparing ORF content and order of A) the complete genome of HearSP1A and HearSP1B with HearLB1, HearLB3 and HearLB6, B) HearSP1A and HearSP1B with HearG4, C) HearSP1A and HearSP1B with HearC1, D) HearSP1A and HearSP1B with HearNNg1, E) HearSP1A and HearSP1B with HearAu, F) HearSP1A and HearSP1B with HzSNPV, G) HearLB1, HearLB3 and HearLB6 with HearG4, H) HearLB1, HearLB3 and HearLB6 with HearC1, I) HearLB1, HearLB3 and HearLB6 with HearNNg1, J) HearLB1, HearLB3 and HearLB6 with HearAu, and K) HearLB1, HearLB3 and HearLB6 with HzSNPV.

Among the 136 ORFs identified in the HearSP1A genome, 132 ORFs were present in all *Helicoverpa* spp. SNPVs, while the remaining 4 ORFs, ORF57 *(bro-a*), ORF58 (*bro-b*), ORF59 (*bro-c*) and ORF118 (unknown), were present at least in one of the previously sequenced HearSNPVs and HzSNPV (Fig 2; Table 3). The *bro-a* present in the five Spanish genotypes was also present in HearG4, HearC1 and HearNNg1 genomes (ORF59), whereas it was not detected in the HearAu or HzSNPV genomes, as this genomic region was considered part of the *hr2* (Chen et al., 2002; Zhang et al., 2014) (Fig 2; Table 3). The *bro-b* also present in HearNNg1 genome (ORF60) (Ogembo et al., 2009) was completely deleted in the other genomes (Fig 2; Table 3). Finally the *bro-c* present in the five Spanish genotypes was also present in the HearNNg1 (ORF61), HearAu (ORF58) and HzSNPV (ORF61) genomes (Chen et al., 2002; Ogembo et al., 2009; Zhang et al., 2014), while it was not identified in the Chinese genomes (Chen et al., 2001; Zhang et al., 2005), as the corresponding genomic region was considered part of *hr3* (Fig 2; Table 3). Moreover, ORF118 of unknown function, was present in all the Spanish genomes and in HearC1 (ORF119) (Zhang et al., 2005) and HearNNg1 (ORF124) (Ogembo et al., 2009) genomes, but was not initially described in the HearG4, HearAu and HzSNPV genomes (Chen et al., 2001, 2002; Zhang et al., 2014). However when this region was compared between the different genomes no nucleotide sequence variation was observed (Fig 2; Table 3) (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). The ORF118 overlapped 125 nucleotides with the ORF117 (lef-2), therefore the authors might not have considered ORF118 to be a putative gene in HearG4, HearAu and HzSNPV genomes (Chen et al., 2001, 2002; Zhang et al., 2014).

The 62 core genes conserved in all lepidopteran baculoviruses (Herniou et al., 2003) were present in the genomes of the five Spanish HearSNPVs genotypes, as well as in the previously sequenced *Helicoverpa* spp. NPVs (Table 3). However, the Spanish genomes lacked some of the ORFs present in the previously sequenced *Helicoverpa* spp. NPVs. ORF5 of unknown function found in all *Helicoverpa* spp. SNPVs (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014) was not present in the Spanish isolates, as these genomes presented a stop codon 94 nt downstream from the initial ATG, reducing the ORF length to 96 nt. Moreover, ORF58 (*bro-a*) of HearG4 and HearC1 genotypes (Chen et al., 2001; Zhang et al., 2005), was not identified in the Spanish genomes,

due to a double point mutation in the start codon (AGT). When comparing the ORFs with the most proximal genome HearNNg1, 10 of the 17 unique ORFs described by Ogembo et al. (2009), ORF6, ORF7, ORF17, ORF29, ORF40, ORF54, ORF105, ORF107, ORF111 and ORF143 (ORF5, ORF7, ORF16, ORF27, ORF38, ORF52, ORF101, ORF103, ORF107 and ORF136 in the HearSP1A genome), were present in the five Spanish genotypes and in the other *Helicoverpa* spp. SNPV genomes. Among the remaining seven ORFs, ORF26 of HearNNg1 and HzSNPV was absent due to a single adenine (A) deletion 26 nt downstream from the initial ATG, which changed the reading frame resulting in an earlier stop codon 51 nucleotides downstream from the start codon. Therefore, it was not considered a putative ORF in the Spanish genotypes, as only ORFs encoding more than 50 amino acids (150 nt) were considered to be putative proteins. ORF42 of HzSNPV was absent not only in the Spanish isolates but also in all HearSNPV genotypes. The Spanish genotypes also lacked ORF62 of HearNNg1 and HzSNPV, as did HearG4, HearC1 and HearAu, due to a guanine (G) insertion 113 nt downstream from the start codon between a thymine (T) and adenine (A), which resulted in an early stop codon (TGA), reducing its length to 114 nt (38 amino acids). Furthermore, ORF79 of HzSNPV was not described in all the HearSNPV isolates, neither in the Spanish isolates, due to a deletion of 16 nt located 36 nt downstream from the initial ATG, resulting in a change of the reading frame resulting in a stop codon 52 nucleotides downstream from the start codon. The Spanish isolates also lacked ORF100 of HearNNg1, homologous to ORF97 of HearC1, as these genotypes had a single nucleotide deletion 42 nucleotides downstream from the initial ATG, which changed the reading frame and resulted in an earlier stop codon 61 nucleotides downstream from the start codon. ORF119 of HearNNg1 was not present in the Spanish genotypes, due to a single nucleotide deletion 9 nt downstream from the ATG, which changed the reading frame and resulted in a stop codon 50 nucleotides downstream from the initial codon. Finally, the Spanish genotypes also lacked ORF120 of HearNNg1, as these genotypes had a 2 nucleotide deletion, guanine (G) and thymine (T), 135 nt downstream from the ATG, which resulted in an early stop codon at position 139. Experiments are in progress to determine the effect of the absence of these unique ORFs on the HearSNPV phenotype.

Table 3: Features of the HearSP1A genome in comparison to those of HearSP1B, HearLB1, HearLB3, HearLB6, HearG4, HearC1, HearNNg1, HearAu and HzSNPV.

Name	Dir.	HearSP1A	HearSP1B		HearLB1		HearLB3		HearLB6		HearG4		HearC1		HearNNg1		HearAu		HzSNPV	
		ORF (aa)	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID
polyhedrin	>	1 (246)	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100	1 (246)	100
p78/83	<	2 (413)	2 (413)	100	2 (413)	99.5	2 (411)	98.5	2 (413)	99.5	2 (413)	99.3	2 (414)	98.6	2 (411)	99.3	2 (413)	99.5	2 (413)	99.0
pk1	>	3 (267)	3 (267)	100	3 (267)	100	3 (267)	100	3 (267)	100	3 (267)	99.3	3 (267)	99.6	3 (267)	100	3 (267)	100	3 (267)	99.3
hoar	<	4 (746)	4 (746)	100	4 (759)	95.8	4 (750)	95.7	4 (750)	95.7	4 (756)	94.1	4 (755)	93.0	4 (769)	93.5	4 (753)	96.7	4 (756)	94.1
hypothetical	>										5 (59)		5 (63)		5 (53)		5 (59)		5 (59)	
hypothetical	>	5 (285)	5 (285)	100	5 (285)	100	5 (285)	90.2	5 (285)	100	6 (285)	100	6 (285)	99.0	6 (285)	100	6 (283)	99.0	6 (285)	100
ie-0	>	6 (285)	6 (285)	100	6 (285)	99.6	6 (285)	88.8	6 (285)	88.8	8 (285)	99.0	8 (285)	99.0	8 (285)	99.3	7 (285)	99.0	8 (285)	99.0
hypothetical	<	7 (53)	7 (53)	96.2	7 (56)	85.7	7 (56)	85.7	7 (56)	85.7	7 (56)	91.1	7 (58)	89.6	7 (81)	59.2	8 (51)	96.1	7 (51)	94.1
p49	>	8 (468)	8 (468)	100	8 (468)	99.8	8 (468)	100	8 (468)	100	9 (468)	99.6	9 (468)	99.8	9 (468)	99.8	9 (468)	99.8	9 (468)	99.8
odv-e18	>	9 (81)	9 (81)	100	9 (81)	100	9 (81)	100	9 (81)	100	10 (81)	98.8	10 (81)	100	10 (81)	100	10 (81)	100	10 (81)	72.0
odv-e27	>	10 (284)	10 (284)	100	10 (284)	100	10 (284)	100	10 (284)	100	11 (284)	100	11 (284)	99.6	11 (284)	100	11 (284)	99.6	11 (284)	100
hypothetical	<	11 (92)	11 (92)	100	11 (92)	100	11 (92)	100	11 (92)	100	12 (92)	100	12 (92)	100	12 (92)	100	12 (92)	100	12 (92)	100
ep23	>	12 (203)	12 (203)	99.5	12 (203)	99.5	12 (203)	99.0	12 (203)	99.0	13 (203)	99.5	13 (201)	97.5	13 (203)	99.5	13 (203)	99.5	13 (203)	99.5
ie-1	>	13 (655)	13 (655)	100	13 (655)	99.7	13 (655)	99.2	13 (655)	99.2	14 (655)	99.4	14 (661)	98.2	14 (655)	99.7	14 (655)	99.4	14 (655)	99.4
odv-e56	<	14 (354)	14 (354)	99.7	14 (354)	99.4	14 (354)	99.1	14 (354)	99.1	15 (354)	99.4	15 (354)	99.4	15 (354)	99.4	15 (354)	99.4	15 (354)	99.1
me53	>	15 (359)	15 (359)	100	15 (359)	100	15 (359)	100	15 (359)	100	16 (359)	99.4	16 (359)	99.4	16 (359)	100	16 (358)	99.4	16 (359)	100
hypothetical	>	16 (55)	16 (55)	100	16 (55)	100	16 (55)	100	16 (55)	100	17 (55)	100	17 (55)	100	17 (55)	100	17 (55)	100	17 (55)	100
hypothetical	<	17 (93)	17 (93)	98.9	17 (93)	98.9	17 (93)	98.9	17 (93)	98.9	18 (93)	93.6	18 (93)	93.6	18 (93)	100	18 (93)	97.9	18 (93)	100
p74	>	18 (686)	18 (686)	98.8	18 (686)	99.3	18 (688)	99.3	18 (688)	99.3	19 (688)	98.4	19 (688)	98.7	19 (686)	99.6	19 (688)	98.7	19 (688)	99.6
p10	<	19 (87)	19 (87)	100	19 (87)	100	19 (87)	100	19 (87)	100	20 (87)	100	20 (87)	100	20 (87)	100	20 (87)	100	20 (87)	98.9
p26	<	20 (267)	20 (267)	98.9	20 (267)	98.5	20 (267)	98.5	20 (267)	98.5	21 (267)	98.1	21 (267)	98.1	21 (267)	98.1	21 (267)	97.7	21 (267)	98.5
hypothetical	>	21 (67)	21 (67)	100	21 (67)	100	21 (67)	100	21 (67)	100	22 (67)	100	22 (68)	100	22 (67)	100	22 (67)	100	22 (67)	98.5
lef-6	<	22 (187)	22 (187)	100	22 (187)	100	22 (187)	100	22 (187)	100	23 (187)	99.5	23 (187)	99.5	23 (187)	100	23 (187)	100	23 (187)	100
dbp	<	23 (323)	23 (323)	100	23 (323)	100	23 (323)	100	23 (323)	100	24 (323)	100	24 (323)	100	24 (323)	100	24 (323)	100	24 (323)	100
hypothetical	>	24 (158)	24 (158)	99.4	24 (158)	99.4	24 (158)	99.4	24 (158)	99.5	25 (133)	82.3	25 (158)	98.1	25 (158)	99.4	25 (133)	82.3	25 (158)	99.4
hr1 (bp)		*hr1* (2067)	*hr1* (1885)	89.4	*hr1* (1853)	87.6	*hr1* (1749)	82.5	*hr1* (1833)	85.1	*hr1* (2282)	69.0	*hr1* (1969)	77.4	*hr1* (2001)	81.4	*hr1* (2321)	67.6	*hr1* (1926)	76.9
hypothetical	>														26 (50)				26 (50)	
ubiquitin	<	25 (83)	25 (83)	100	25 (83)	97.6	25 (83)	97.6	25 (83)	97.6	27 (83)	97.6	27 (83)	97.6	28 (83)	97.6	26 (83)	97.6	28 (83)	96.4
hypothetical	>	26 (255)	26 (255)	100	26 (255)	100	26 (255)	99.6	26 (255)	99.6	26 (255)	98.4	26 (255)	98.0	27 (255)	99.6	27 (255)	97.7	27 (255)	99.6
hypothetical	>	27 (168)	27 (168)	100	27 (168)	100	27 (168)	100	27 (168)	100	28 (168)	99.4	28 (168)	99.4	29 (168)	100	28 (168)	99.4	29 (168)	100
le25	>	28 (192)	28 (192)	100	28 (190)	98.4	28 (192)	99.5	28 (190)	98.4	29 (190)	95.9	29 (192)	97.4	30 (190)	97.9	29 (190)	97.4	30 (190)	97.9
39k/pp31	<	29 (311)	29 (311)	99.4	29 (311)	99.4	29 (310)	98.7	29 (310)	98.7	30 (311)	99.4	30 (312)	99.0	31 (312)	97.1	30 (311)	99.4	31 (311)	99.4
lef-11	<	30 (127)	30 (127)	100	30 (127)	100	30 (127)	100	30 (127)	100	31 (127)	100	31 (127)	100	32 (127)	100	31 (127)	100	32 (127)	100
bv-e31	<	31 (238)	31 (238)	99.2	31 (238)	99.2	31 (238)	99.6	31 (238)	99.6	32 (238)	98.7	32 (238)	98.7	33 (238)	99.2	32 (238)	98.7	33 (238)	98.7
hypothetical	>	32 (359)	32 (359)	100	32 (359)	100	32 (359)	100	32 (359)	100	33 (359)	99.4	33 (359)	99.2	34 (359)	99.7	33 (359)	99.7	34 (359)	99.7
p47	<	33 (412)	33 (412)	100	33 (412)	99.8	33 (412)	99.8	33 (412)	99.8	34 (333)	79.4	34 (412)	99.0	35 (412)	99.8	34 (412)	99.5	35 (412)	99.5
lef-12	>	34 (223)	34 (223)	100	34 (223)	99.6	34 (223)	100	34 (223)	100	35 (223)	99.6	35 (223)	99.6	36 (223)	100	35 (223)	99.6	36 (223)	100
hypothetical	>	35 (80)	35 (80)	100	35 (80)	98.75	35 (80)	100	35 (80)	100	36 (80)	98.8	36 (80)	98.8	37 (80)	97.5	36 (80)	98.8	37 (80)	98.8
lef-8	>	36 (901)	36 (901)	100	36 (901)	99.9	36 (901)	91.1	36 (901)	99.9	37 (901)	99.3	37 (901)	99.4	38 (901)	99.7	37 (901)	97.5	38 (901)	99.7
bJDP	>	37 (192)	37 (192)	99.5	37 (192)	100	37 (192)	99.5	37 (192)	99.5	38 (194)	99.0	38 (194)	99.0	39 (192)	100	38 (192)	99.5	39 (192)	99.5
hypothetical	>	38 (50)	38 (50)	98.0	38 (50)	98.0	38 (50)	98.0	38 (50)	98.0	39 (50)	94.0	39 (50)	98.0	40 (50)	100	39 (50)	92.0	40 (50)	94.0
chitinase	<	39 (575)	39 (575)	99.7	39 (575)	99.6	39 (575)	99.6	39 (575)	99.6	40 (570)	98.1	40 (584)	99.6	41 (575)	99.6	40 (589)	96.8	41 (575)	99.5
hypothetical	<																		42 (68)	

Table 3: continued.

Name	Dir.	HearSP1A		HearSP1B		HearLB1		HearLB3		HearLB6		HearG4		HearC1		HearNNg1		HearAu		HzSNPV	
		ORF (aa)		ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID
hypothetical	<	40 (181)		40 (181)	100	40 (181)	100	40 (181)	100	40 (181)	100	41 (180)	98.4	41 (180)	98.4	42 (181)	100	41 (180)	98.4	43 (181)	100
hypothetical	>	41 (136)		41 (136)	100	41 (136)	100	41 (136)	100	41 (136)	100	42 (136)	100	42 (136)	99.3	43 (136)	100	42 (136)	100	44 (136)	100
hypothetical	<	42 (378)		42 (378)	100	42 (378)	98.9	42 (378)	99.2	42 (378)	99.2	43 (378)	99.7	43 (378)	99.7	44 (378)	99.7	43 (378)	99.5	45 (378)	99.2
lef-10	>	43 (71)		43 (71)	100	43 (71)	100	43 (71)	100	43 (71)	100	44 (75)	100	45 (75)	98.6	45 (75)	100	45 (75)	100	46 (75)	100
hypothetical	<	44 (75)		44 (75)	100	44 (75)	100	44 (75)	100	44 (75)	100	45 (71)	100	44 (71)	100	46 (71)	100	44 (71)	100	47 (71)	100
vp1054	>	45 (349)		45 (351)	99.1	45 (351)	93.3	45 (349)	98.8	45 (349)	98.8	46 (351)	98.0	46 (351)	98.3	47 (351)	98.6	46 (351)	98.6	48 (351)	99.3
hypothetical	>	46 (68)		46 (68)	100	46 (68)	100	46 (68)	100	46 (68)	100	47 (68)	100	47 (68)	100	48 (68)	100	47 (68)	100	49 (68)	100
hypothetical	>	47 (64)		47 (64)	100	47 (64)	100	47 (64)	98.4	47 (64)	98.4	48 (64)	100	48 (64)	100	49 (64)	100	48 (64)	98.5	50 (64)	100
hypothetical	>	48 (163)		48 (163)	100	48 (163)	100	48 (163)	100	48 (163)	100	49 (171)	93.0	49 (171)	93.6	50 (163)	100	49 (171)	93.6	51 (163)	100
8.2 kDa	<	49 (154)		49 (156)	96.8	49 (153)	98.7	49 (153)	98.7	49 (153)	98.7	50 (160)	94.4	50 (159)	94.4	51 (159)	95.6	50 (160)	94.4	52 (155)	98.7
hypothetical	<	50 (88)		50 (88)	100	50 (88)	100	50 (88)	100	50 (88)	100	51 (88)	100	51 (88)	100	52 (88)	100	51 (88)	100	53 (88)	100
Fp25	<	51 (217)		51 (217)	100	51 (217)	99.5	51 (217)	100	51 (217)	100	52 (217)	99.5	52 (217)	99.5	53 (217)	99.5	52 (217)	98.6	54 (217)	100
hypothetical	>	52 (61)		52 (61)	100	52 (61)	100	52 (61)	100	52 (61)	100	53 (61)	98.4	53 (61)	98.4	54 (61)	100	53 (61)	100	55 (61)	100
lef-9	>	53 (519)		53 (519)	100	53 (519)	100	53 (519)	100	53 (519)	100	54 (519)	99.8	54 (519)	99.6	55 (519)	100	54 (519)	100	56 (519)	99.8
cathepsin	<	54 (367)		54 (367)	100	54 (367)	100	54 (367)	100	54 (367)	100	55 (365)	98.9	55 (365)	98.9	56 (367)	100	55 (367)	99.2	57 (367)	100
hypothetical	<	55 (195)		55 (195)	98.5	55 (195)	98.0	55 (195)	99.0	55 (195)	99.0	56 (195)	99.5	56 (195)	99.5	57 (195)	98.5	56 (195)	99.0	58 (195)	98.0
gp37	<	56 (279)		56 (279)	100	56 (279)	99.6	56 (279)	99.6	56 (279)	99.6	57 (279)	99.3	57 (279)	99.3	58 (279)	99.3	57 (279)	99.3	59 (279)	98.9
hr2 (bp)		*hr2* (1193)		*hr2* (1188)	100	*hr2* (1188)	96.2	*hr2* (780)	62.0	*hr2* (780)	62.0	*hr2* (1150)	47.2	*hr2* (907)	56.0	*hr2* (1396)	81.4	*hr2* (1150)	56.5	*hr2* (2378)	38.6
bro	>											58 (244)		58 (244)						60 (211)	
bro-a	>	57 (357)	66.4	57 (237)	66.4	57 (361)	91.2	57 (360)	90.6	57 (360)	90.6	59 (527)	51.5	59 (357)	71.8	59 (361)	91.4				
bro-b	>	58 (101)	72.5	58 (88)	72.5											60 (101)	100				
bro-c	>	59 (352)	58.1	59 (206)	58.1	58 (352)	82.6	58 (352)	82.8	58 (352)	82.8					61 (352)	82.8	58 (549)	47.3	61 (352)	76.3
hr3 (bp)		*hr3* (295)		*hr3* (296)	96.9	*hr3* (297)	97.3	*hr3* (295)	97.3	*hr3* (295)	97.3	*hr3* (759)	36.4	*hr3* (297)	92.6	*hr3* (133)	40.6	*hr3* (755)	36.6	*hr3* (482)	58.4
hypothetical	<															62 (59)				62 (59)	
he65	>	60 (236)	99.6	60 (236)	99.6	59 (236)	98.7	59 (236)	100	59 (236)	100	60 (236)	99.1	60 (236)	99.1	63 (236)	100	59 (236)	99.1	63 (236)	99.1
iap-2	<	61 (250)	98.4	61 (250)	98.4	60 (250)	98.4	60 (250)	99.6	60 (250)	99.6	61 (250)	98.4	61 (250)	97.6	64 (250)	98.8	60 (250)	98.8	64 (250)	98.8
met	<	62 (274)	98.9	62 (274)	98.9	61 (274)	98.9	61 (274)	99.5	61 (274)	99.5	62 (274)	94.3	62 (274)	94.3	65 (274)	94.0	61 (274)	94.0	65 (274)	94.3
hypothetical	<	63 (133)	99.2	63 (133)	99.2	62 (133)	99.2	62 (133)	100	62 (133)	100	63 (133)	100	63 (133)	100	66 (133)	100	62 (133)	99.2	66 (133)	99.2
lef-3	>	64 (379)	99.7	63 (379)	99.2	63 (379)	99.7	63 (379)	99.7	63 (379)	99.7	64 (379)	99.2	64 (379)	98.7	67 (379)	99.7	63 (379)	98.9	67 (379)	100
desmop	<	65 (785)	99.6	64 (785)	99.6	64 (785)	99.5	64 (785)	99.5	64 (785)	99.5	65 (785)	99.1	65 (785)	99.2	68 (785)	99.7	64 (785)	99.2	68 (785)	99.2
DNA polymerase	>	66 (1020)	99.8	65 (1020)	100	65 (1020)	99.7	65 (1020)	99.7	65 (1020)	99.7	66 (1020)	99.4	66 (1020)	99.3	69 (1020)	99.8	65 (1020)	99.2	69 (1020)	99.9
hypothetical	<	67 (152)	100	66 (152)	100	66 (152)	99.3	66 (152)	99.3	66 (152)	99.3	67 (152)	98.7	67 (152)	98.7	70 (152)	100	66 (152)	98.7	70 (152)	100
hypothetical	<	68 (127)	100	67 (127)	100	67 (127)	100	67 (127)	100	67 (127)	100	68 (127)	100	68 (127)	100	71 (127)	100	67 (127)	100	71 (127)	100
hypothetical	<	69 (85)	100	68 (85)	98.8	68 (85)	98.8	68 (85)	98.8	68 (85)	98.8	69 (85)	98.8	69 (85)	98.8	72 (85)	98.8	68 (85)	98.8	72 (85)	98.8
vlf-1	<	70 (415)	99.8	69 (415)	99.5	69 (415)	100	69 (415)	100	69 (415)	100	70 (412)	95.2	70 (413)	97.1	73 (415)	99.7	69 (414)	97.8	73 (414)	97.8
hypothetical	<	71 (110)	100	70 (110)	100	70 (110)	100	70 (110)	100	70 (110)	100	71 (110)	99.1	71 (110)	99.1	74 (110)	100	70 (110)	99.1	74 (110)	100
gp41	<	72 (322)	100	71 (322)	100	71 (322)	100	71 (322)	100	71 (322)	100	72 (322)	100	72 (322)	100	75 (322)	100	71 (322)	100	75 (322)	100
hypothetical	<	73 (241)	100	72 (241)	99.2	72 (241)	100	72 (241)	100	72 (241)	100	73 (241)	100	73 (241)	99.6	76 (241)	100	72 (241)	100	76 (241)	100
vp91capsid	>	74 (816)	99.9	73 (816)	99.9	73 (816)	99.6	73 (816)	99.9	73 (816)	99.9	75 (816)	96.6	75 (816)	96.6	78 (816)	97.0	73 (816)	96.4	78 (816)	96.6
tlp-20	<	75 (225)	100	74 (225)	99.1	74 (225)	100	74 (225)	100	74 (225)	100	74 (225)	99.6	74 (225)	99.6	77 (225)	100	74 (225)	99.6	77 (225)	100
hypothetical	<																			79 (58)	
gp30	<	76 (283)	100	76 (283)	100	75 (283)	100	75 (283)	100	75 (283)	100	76 (283)	98.9	76 (283)	98.9	79 (283)	99.6	75 (283)	98.9	80 (283)	99.3
lef-4	>	77 (461)	100	77 (461)	100	76 (461)	100	76 (461)	100	76 (461)	100	78 (461)	99.4	78 (461)	99.4	81 (461)	99.6	76 (461)	99.1	81 (461)	99.1

Table 3: continued.

Name	Dir.	HearSP1A	HearSP1B		HearLB1		HearLB3		HearLB6		HearG4		HearC1		HearNNg1		HearAu		HzSNPV	
		ORF (aa)	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID
vp39/capsid	<	78 (293)	78 (293)	100	77 (293)	100	77 (293)	100	77 (293)	100	77 (293)	100	77 (293)	100	80 (293)	100	77 (293)	100	82 (293)	99.7
p33	<	79 (254)	79 (254)	100	78 (254)	100	78 (254)	100	78 (254)	100	79 (254)	100	79 (254)	100	82 (254)	99.6	78 (254)	100	83 (254)	100
p18	>	80 (162)	80 (162)	100	79 (162)	100	79 (162)	100	79 (162)	100	80 (162)	100	80 (162)	100	83 (162)	100	79 (162)	100	84 (162)	100
odv-e25	> <	81 (232)	81 (231)	99.6	80 (232)	100	80 (232)	100	80 (232)	100	81 (230)	99.1	81 (230)	99.1	84 (231)	99.6	80 (230)	99.1	85 (230)	99.1
hypothetical	>	82 (166)	82 (165)	99.4	81 (165)	98.2	81 (165)	98.2	81 (165)	99.4	82 (165)	85.5	82 (165)	85.5	85 (167)	85.6	81 (165)	84.3	86 (165)	86.7
hypothetical	>	83 (173)	83 (173)	100	82 (173)	100	82 (173)	100	82 (173)	100	84 (173)	98.9	84 (173)	99.4	87 (173)	100	82 (173)	100	88 (173)	100
helicase	<	84 (1253)	84 (1253)	99.8	83 (1253)	99.8	83 (1253)	99.9	83 (1253)	99.8	83 (1253)	99.8	83 (1253)	100	86 (1253)	100	83 (1253)	99.8	87 (1253)	99.9
lef-5	>	85 (315)	85 (315)	100	84 (315)	99.4	84 (315)	99.7	84 (315)	99.7	86 (315)	100	86 (315)	100	89 (315)	99.7	84 (315)	100	90 (315)	99.1
38k	> <	86 (321)	86 (321)	100	85 (321)	100	85 (321)	100	85 (321)	100	85 (321)	99.1	85 (321)	99.1	88 (321)	99.4	85 (321)	99.4	89 (321)	99.1
p6.9	<	87 (109)	87 (109)	100	86 (116)	92.2	86 (109)	98.2	86 (109)	98.2	87 (109)	98.2	87 (109)	98.2	90 (109)	98.2	86 (109)	98.2	91 (109)	98.2
odv/bv-c42	<	88 (369)	88 (369)	100	87 (369)	99.7	87 (369)	100	87 (369)	100	88 (369)	99.5	88 (369)	99.5	91 (369)	100	87 (369)	99.5	92 (369)	99.7
p12	<	89 (122)	89 (122)	100	88 (122)	100	88 (122)	100	88 (122)	100	89 (122)	99.2	89 (122)	100	92 (122)	99.2	88 (122)	99.2	93 (122)	99.2
p48	<	90 (377)	90 (377)	99.7	89 (377)	100	89 (377)	99.5	89 (377)	99.5	90 (377)	99.5	90 (377)	99.7	93 (377)	99.7	89 (377)	99.7	94 (377)	99.5
vp80/capsid	>	91 (605)	91 (605)	99.7	90 (605)	99.5	90 (605)	99.8	90 (605)	99.8	91 (605)	99.7	91 (605)	98.0	94 (605)	99.3	90 (605)	98.5	95 (605)	99.5
hypothetical	>	92 (57)	92 (57)	100	91 (57)	100	91 (57)	100	91 (57)	100	92 (58)	98.3	92 (58)	98.3	95 (58)	100	91 (58)	98.3	96 (58)	100
odv-ec43	>	93 (361)	93 (361)	100	92 (361)	99.4	92 (361)	100	92 (361)	100	93 (361)	100	93 (361)	100	96 (361)	100	92 (361)	100	97 (361)	100
hypothetical	>	94 (94)	94 (94)	100	93 (94)	100	93 (94)	100	93 (94)	100	94 (94)	100	94 (94)	100	97 (94)	100	93 (94)	100	98 (94)	98.9
odv-e66	<	95 (672)	95 (672)	100	94 (672)	99.9	94 (672)	99.9	94 (672)	99.9	95 (672)	99.6	95 (672)	99.6	98 (672)	99.9	94 (672)	99.9	99 (672)	99.9
p13	<	96 (276)	96 (276)	100	95 (276)	100	95 (276)	99.6	95 (276)	99.6	96 (276)	98.9	96 (276)	98.9	99 (276)	100	95 (276)	98.6	100 (276)	100
hr4 (bp)		*hr4* (1728)	*hr4* (1728)	96.9	*hr4* (1729)	97.1	*hr4* (1300)	73.1	*hr4* (1300)	73.1	*hr4* (2304)	68.5	*hr4* (2253)	70.2	*hr4* (1734)	97.0	*hr4* (2598)	61.4	*hr4* (2177)	67.2
hypothetical	<												97 (55)		100 (55)					
pif-3	>	97 (199)	97 (199)	100	96 (199)	100	96 (199)	100	96 (199)	100	97 (199)	99.5	98 (199)	99.5	101 (199)	100	96 (199)	99.5	101 (199)	99.0
hypothetical	>	98 (118)	98 (118)	98.3	97 (118)	98.3	97 (118)	98.3	97 (118)	98.3	98 (118)	95.8	99 (118)	95.8	102 (118)	98.3	97 (118)	95.8	102 (118)	95.8
parg	>	99 (508)	99 (508)	99.8	98 (508)	100	98 (508)	100	98 (508)	100	99 (510)	99.0	100 (510)	99.0	103 (508)	100	98 (510)	99.4	103 (508)	99.0
hypothetical	>	100 (253)	100 (253)	100	99 (253)	99.6	99 (253)	100	99 (253)	100	100 (253)	99.6	101 (253)	99.6	104 (253)	100	99 (253)	99.2	104 (253)	99.2
hypothetical	>	101 (110)	101 (110)	100	100 (110)	98.2	100 (110)	99.1	100 (110)	99.1	101 (110)	99.1	102 (110)	99.1	105 (110)	100	100 (110)	99.1	105 (110)	99.1
iap-3	<	102 (268)	102 (268)	100	101 (268)	98.1	101 (268)	98.1	101 (268)	98.1	102 (268)	98.5	103 (268)	98.5	106 (268)	98.9	101 (268)	98.1	106 (268)	98.1
hypothetical	<	103 (51)	103 (51)	100	102 (51)	100	102 (51)	98	102 (51)	98	103 (51)	98.1	104 (51)	98.1	107 (51)	98.1	102 (51)	98.1	107 (51)	100
bro-d	<	104 (501)	104 (501)	99.8	103 (501)	99.8	103 (501)	99.8	103 (501)	99.8	104 (501)	98.2	105 (501)	99.2	108 (501)	99.8	103 (501)	99.4	108 (501)	99.8
sod	>	105 (159)	105 (159)	100	104 (159)	99.4	104 (159)	100	104 (159)	100	105 (159)	98.1	106 (159)	98.8	109 (159)	100	104 (159)	98.8	109 (159)	100
hypothetical	>	106 (457)	106 (457)	99.8	105 (457)	99.8	105 (457)	98.7	105 (457)	99.3	106 (457)	99.1	107 (457)	99.1	110 (457)	99.8	105 (457)	99.6	110 (457)	99.1
hypothetical	<	107 (192)	107 (192)	100	106 (192)	100	106 (192)	99.0	106 (192)	99	107 (192)	98.4	108 (192)	97.9	111 (192)	99.5	106 (192)	99.0	111 (192)	98.4
hypothetical	>	108 (118)	108 (118)	98.3	107 (118)	98.3	107 (118)	99.1	107 (118)	99.1	108 (118)	100	109 (118)	100	112 (118)	99.1	107 (118)	100	112 (115)	100
hypothetical	>	109 (102)	109 (102)	100	108 (102)	100	108 (102)	99.0	108 (102)	99	109 (88)	86.4	110 (88)	86.4	113 (88)	86.4	108 (88)	86.4	113 (99)	96.1
pif-1	>	110 (528)	110 (528)	99.4	109 (528)	99.6	109 (528)	99.6	109 (528)	99.6	110 (528)	99.2	111 (528)	99.2	114 (528)	99.8	109 (528)	99.4	114 (528)	99.4
hypothetical	> <	111 (78)	111 (78)	98.7	110 (78)	100	110 (78)	100	110 (78)	100	111 (78)	98.7	112 (78)	99.4	115 (78)	100	110 (78)	98.7	115 (78)	98.7
tgf	<	112 (301)	112 (301)	100	111 (301)	99.7	111 (301)	100	111 (301)	100	112 (301)	100	113 (301)	99.3	116 (301)	100	111 (301)	100	116 (301)	100
alk-exo	<	113 (428)	113 (427)	99.1	112 (428)	99.8	112 (430)	99.3	112 (430)	99.3	113 (428)	99.1	114 (428)	99.1	117 (428)	99.5	112 (428)	100	117 (428)	99.8
hypothetical	<	114 (129)	114 (129)	99.2	113 (129)	100	113 (129)	100	113 (129)	99.2	114 (129)	99.2	115 (129)	97.7	118 (129)	99.2	113 (129)	99.2	118 (129)	99.2
hr5 (bp)		*hr5* (2076)	*hr5* (2083)	95.9	*hr5* (2084)	97.9	*hr5* (2106)	96.0	*hr5* (2081)	95.4	*hr5* (2806)	50.8	*hr5* (899)	41.2	*hr5* (1135)	53.0	*hr5* (2806)	50.8	*hr5* (1385)	59.1
hypothetical	>														119 (65)					
hypothetical	<														120 (51)					
hypothetical	<	115 (308)	115 (308)	99.7	114 (308)	99.7	114 (308)	99.7	114 (308)	99.3	115 (308)	99.7	116 (308)	99.3	121 (308)	99.7	114 (308)	100	119 (308)	99.7

Table 3: continued.

Name	Dir.	HearSP1A	HearSP1B		HearLB1		HearLB3		HearLB6		HearG4		HearC1		HearNNg1		HearAu		HzSNPV	
		ORF (aa)	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID	ORF (aa)	%ID
hypothetical	>	116 (71)	116 (71)	98.6	115 (71)	98.6	115 (71)	98.6	115 (71)	98.6	116 (71)	97.2	117 (71)	98.6	122 (71)	98.6	115 (71)	97.2	120 (71)	97.2
lef-2	<	117 (238)	117 (238)	100	116 (238)	99.2	116 (238)	99.2	116 (238)	99.6	117 (241)	97.9	118 (242)	97.5	123 (226)	94.6	116 (238)	100	121 (238)	100
hypothetical	<	118 (131)	118 (131)	100	117 (131)	100	117 (131)	100	117 (131)	100			119 (135)	95.6	124 (119)	90.2				
p24 capsid	>	119 (248)	119 (248)	99.2	118 (248)	99.2	118 (248)	99.2	118 (248)	99.2	118 (248)	96.4	120 (248)	98.8	125 (248)	99.2	117 (248)	98.8	122 (248)	99.2
gp16	>	120 (94)	120 (94)	100	119 (94)	100	119 (94)	98.9	119 (94)	98.9	119 (94)	100	121 (96)	96.9	126 (96)	96.9	118 (96)	96.9	123 (94)	100
calyx/pep	>	121 (340)	121 (338)	99.1	120 (340)	100	120 (340)	100	120 (340)	99.7	120 (340)	99.7	122 (340)	99.7	127 (338)	99.1	119 (340)	100	124 (340)	100
hypothetical	>	122 (154)	122 (154)	100	121 (154)	100	121 (154)	100	121 (154)	100	121 (154)	100	123 (154)	100	128 (154)	100	120 (154)	100	125 (154)	100
ody-c21	>	123 (196)	123 (196)	100	122 (196)	99	122 (196)	99	122 (196)	100	122 (196)	98.5	124 (196)	97.5	129 (196)	98.5	121 (196)	99.0	126 (196)	99.0
38.7 kDa	<	124 (391)	124 (391)	100	123 (391)	98.0	123 (391)	100	123 (391)	98	123 (385)	95.4	125 (389)	96.9	130 (391)	97.7	122 (389)	96.9	127 (392)	98.2
lef-1	<	125 (245)	125 (245)	100	124 (245)	99.6	124 (245)	99.2	124 (245)	99.2	124 (245)	99.2	126 (245)	98.8	131 (245)	99.2	123 (245)	99.2	128 (245)	99.6
hypothetical	<	126 (144)	126 (144)	100	125 (144)	98.6	125 (144)	100	125 (144)	100	125 (144)	96.6	127 (142)	95.9	132 (142)	97.2	124 (142)	95.9	129 (144)	97.2
egf	>	127 (515)	127 (515)	100	126 (515)	99.4	126 (515)	99.6	126 (515)	99.6	126 (515)	98.6	128 (515)	99.2	133 (515)	99.2	125 (515)	99.0	130 (515)	99.2
hypothetical	>	128 (192)	128 (192)	98.4	127 (192)	98.4	127 (192)	97.9	127 (192)	98.4	127 (192)	98.4	129 (192)	97.9	134 (95)	82.9	126 (192)	98.4	131 (192)	98.4
hypothetical															135 (85)					
bv-ec31	>	129 (266)	129 (266)	99.6	128 (266)	99.2	128 (266)	99.2	128 (266)	98.5	128 (266)	98.1	130 (266)	98.1	136 (266)	99.6	127 (266)	98.5	132 (266)	97.7
hypothetical	<	130 (947)	130 (947)	100	129 (947)	99.2	129 (947)	99.4	129 (947)	99.3	129 (947)	98.4	131 (947)	98.1	137 (947)	98.9	128 (947)	98.9	133 (947)	99.1
pkip-1	>	131 (169)	131 (169)	100	130 (169)	100	130 (169)	100	130 (169)	100	130 (169)	97.1	132 (169)	99.4	138 (169)	100	129 (169)	100	134 (169)	100
arif-1	<	132 (265)	132 (265)	100	131 (265)	99.6	131 (265)	99.6	131 (265)	99.6	131 (265)	99.2	133 (265)	98.1	139 (265)	99.2	130 (265)	98.5	135 (265)	99.6
pif-2	>	133 (383)	133 (383)	100	132 (383)	100	132 (383)	100	132 (383)	100	132 (383)	99.7	134 (383)	99.5	140 (383)	99.5	131 (383)	99.5	136 (383)	99.7
f protein	<	134 (677)	134 (677)	100	133 (677)	100	133 (677)	100	133 (677)	100	133 (677)	99.7	135 (677)	99.3	141 (677)	99.7	132 (677)	55.0	137 (677)	99.7
hypothetical	<	135 (181)	135 (181)	100	134 (181)	98.3	134 (181)	97.2	134 (181)	97.2	134 (181)	100	136 (181)	99.5	142 (181)	99.5	133 (181)	99.5	138 (180)	95.6
hypothetical	>	136 (195)	136 (195)	100	135 (195)	100	135 (195)	100	135 (195)	100	135 (194)	94.9	137 (195)	96.4	143 (195)	100	134 (195)	98.5	139 (195)	100

Among the 135 ORFs found in the five Spanish genotypes, 43 showed 100% identity at the amino acid level (Table 3), which were distributed almost evenly along the genome. Additionally, 13 of these 43 ORFs were 100% identical to all *Helicoverpa* spp. SNPVs (Table 3). The remaining 92 ORFs, except the *bro* genes that showed greater variability, presented more than 85% amino acid sequence identity among them; however non-synonymous mutations were observed. A total of 37 of these 92 ORFs were ORFs of unknown function (Table 3), but as their function has not been determined, we were unable to associate these mutations with any specific change in phenotype. Additionally as the three *bro* genes common to the Spanish isolates showed a great number of mutations and deletions, they are described in more detail in the following section. Within the remaining 52 ORFs 179-non-synonymous single nucleotide polymorphisms were identified. However just 47 of these were associated with changes in amino acid polarity, which might have altered protein function (Thézé et al., 2014).

Overall, 19 of these non-synonymous mutations with polarity change were detected within three of the six ORFs associated with baculovirus DNA replication (Table 3), as *dbp* (ORF23 in HearSP1A genome) showed 100% identity at amino acid level among all the *Helicoverpa* spp. SNPVs (Table 3) and the mutations within the *ie-0* and *helicase* genes (ORF6 and ORF84, respectively in the HearSP1A genome) did not change the biochemical properties of the proteins. In contrast, the mutations found within *ie-1*, *lef-3* and *DNA polymerase* genes (ORF13, ORF64 and ORF66 in HearSP1A genome) produced changes in the amino acid polarity of the putative protein (Table 4). LEF-3 of HearLB1 had a mutation at position 34, arginine (R) to leucine (L), whereas IE-1 and DNA polymerase of HearLB3 and HearLB6 had a mutation at position 639, leucine (L) to lysine (K), and at position 171, aspartic acid (D) to asparagine (N), respectively. These mutations could be involved in the differences observed in OB productivity between HearSP1 and HearLB genotypes (Arrizubieta et al., 2015), as these genes are involved in DNA replication, influencing virion production and therefore, the spread of infection within the host (Thézé et al., 2014). Hence, the mutations that harbor HearSP1A and HearSP1B might improve the viral spread in the insect host resulting in altered productivity. In contrast, the mutations observed in HearLB1, HearLB3 and HearLB6 could be responsible for a slower virus spread of these genotypes (Arrizubieta et al., 2015).

Non-synonymous mutations were detected in 10 of the 16 ORFs associated with baculovirus transcription (Table 3), as the encoded proteins of *pk-1*, *lef-6*, *lef-11*, *lef-10*, *lef-9* and *lef-4* genes (ORF3, ORF22, ORF30, ORF43, ORF53 and ORF77, respectively in HearSP1A genome) showed 100% identity at the amino acid level among the Spanish genotypes (Table 3). Additionally mutations found in the proteins *p47*, *bjdp*, *he65*, *met* and *lef-5* (ORF33, ORF37, ORF60, ORF62 and ORF85 in HearSP1-A genome) did not change the amino acid polarity, and therefore may be less likely to affect protein function and virus phenotype. In contrast, the mutations that occurred within the encoded proteins of the *39k/pp31*, *lef-12*, *lef-8*, *lef-2* and *lef-1* genes (ORF29, ORF34, ORF36, ORF117 and ORF125 in HearSP1A genome) clearly affected the amino acid polarity of the encoded protein (Table 4). HearSP1B and HearLB1 had a mutation at position 137 within 39K/PP31 protein, asparagine (N) to aspartic acid (D), whereas HearLB3 and HearLB6 had a 4 amino acid deletion at position 238-241, alanine (A), asparagine (N), lysine (K) and threonine (T). Within LEF-12 HearSP1A, HearSP1B, HearLB3 and HearLB6 genomes had an arginine (R) at position 222 while HearLB1 had a leucine (L). In HearSP1A and HearSP1B LEF-8 an aspartic acid (D) was found at position 424, while in HearLB1, HearLB3 and HearLB6 genomes a glycine (G) was identified. Similarly to LEF-12, within LEF-2 HearSP1A, HearSP1B, HearLB3 and HearLB6 had a lysine (K) at position 146, while HearLB1 had an asparagine (N). Finally the LEF-1 protein of HearSP1A and HearSP1B had a lysine (K) at position 241, whereas HearLB1, HearLB3 and HearLB6 had a glutamine (Q). These ORFs are involved in late gene transcription, regulating the transcription of the structural proteins forming the ODVs. Therefore the correct assembly or formation of the ODVs could be affected, possibly influencing OB composition (Thézé et al., 2014). Previous studies demonstrated that deletions in early transcribed genes produced OBs with fewer ODVs with clear implications on OB pathogenicity (Simón et al., 2008). Although HearSP1B was more pathogenic than the other genotypes, HearSP1A, HearLB1, HearLB3 and HearLB6 showed similar pathogenicity (Arrizubieta et al., 2015), and therefore the changes observed in 39K/PP31, LEF-12 and LEF-2 might be silent or be involved in other functions. In contrast, the mutations of LEF-8 and LEF-1 proteins in HearLB genotypes could also be implicated in the improved OB productivity (Arrizubieta et al., 2015). Deletion

mutants are being constructed to determine the effect of *lef-8* and *lef-1* genes in late gene transcription and in ODV formation and OB yield.

Table 4: Non-synonymous mutations which resulted in amino acid polarity change detected in HearSP1B, HearLB1, HearLB3 and HearLB6 ORFs in comparison with the reference genotype HearSP1A.

General function	ORF (name)	Amino acid change*	Variant strains†
Replication	ORF6 (*ie-1*)	639 L→K	HearLB3, HearLB6
	ORF64 (*lef-3*)	34 R→L	HearLB1
	ORF66 (*DNApol*)	171 D→N	HearLB3, HearLB6
Transcription	ORF29 (*39k/pp31*)	137 N→D	HearSP1B, HearLB1
		238-241 ANKT→−	HearLB3, HearLB6
	ORF34 (*lef-12*)	222 R→L	HearLB1
	ORF36 (*lef-8*)	424 D→G	HearLB1, HearLB3, HearLB6
	ORF117 (*lef-2*)	146 K→N	HearLB1
	ORF125 (*lef-1*)	241 K→Q	HearLB1, HearLB3, HearLB6
Structure	ORF2 (*p78/83*)	333 Q→E	HearLB1
		333 Q→K	HearLB3, hearLB6
	ORF18 (*p74*)	348 K→E	HearLB3, HearLB6
	ORF20 (*p26*)	23 R→Q	HearLB1
		58 D→N	HearLB3, HearLB6
		101 K→E	HearSP1B, HearLB1, HearLB3, HearLB6
	ORF45 (*vp1054*)	between 55-56 −→D	HearSP1B, HearLB1
		94 V→E	HearLB1, HearLB3, HearLB6
	ORF65 (*desmop*)	625 D→N	HearSP1B
	ORF74 (*vp91 capsid*)	443 K→N	HearSP1B, HearLB1, HearLB3, HearLB6
	ORF75 (*tlp20*)	188 D→N	HearLB1
		201 D→G	HearLB1
	ORF87 (*p6.9*)	Between 91-92 −→GGGRRRS	HearLB1
	ORF93 (*odv-ec43*)	71 D→N	HearLB1
	ORF95 (*odv-e66*)	25 G→D	HearLB1, HearLB3, HearLB6
	ORF121 (*calyx/pep*)	160-161 RS→−	HearSP1B
	ORF123 (*odv-c21*)	94 D→N	HearLB1
	ORF129 (*bv-ec31*)	60 D→N	HearLB3, HearLB6
		187 Q→R	HearLB6
Auxiliary	ORF39 (*chitinase*)	526 V→E	HearSP1B, HearLB1, HearLB3, HearLB6
	ORF61 (*iap-2*)	158 H→D	HearSP1B, HearLB1
		183 N→D	HearSP1B, HearLB1
	ORF102 (*iap-3*)	110 K→M	HearLB1, HearLB3, HearLB6
	ORF113 (*alk-exo*)	51 K→E	HearSP1B
		347 D→−	HearSP1B
		between 352-353 −→DD	HearLB3, HearLB6
		404 D→G	HearSP1B, HearLB3, HearLB6
	ORF127 (*egt*)	278 D→N	HearLB1, HearLB3, HearLB6

*Amino acid change (original residue in the alignment) with respect to reference strain HearSP1A.
†Variant strains bearing mutated amino acids.

Among the 46 ORFs with structural functions, non-synonymous mutations were detected in 32 (Table 3). The deduced proteins of *polh* (ORF1 in all genotypes) and *gp41* (ORF72 in HearSP1A genome) were 100% identical at amino acid level among all *Helicoverpa* spp. SNPVs, while the other 12 ORFs (*odv-e18*, *odv-e27*, *me53*, *p10*, *vp39 capsid*, *p33*, *38K*, *p12*, *pif-3*, *pkip-1*, *pif-2* and *F-protein*; ORF9, ORF10, ORF15, ORF19, ORF78, ORF79, ORF86, ORF89, ORF97, ORF131, ORF133 and ORF134 in HearSP1A genotype) showed 100% identity among the five Spanish genotypes. Additionally, the mutations detected in 18 of these 32 ORFs, *p49*, *odv-e56*, *ubiquitin*, *bv-e31*, *fp25*, *gp37*, *vlf-1*, *cg30*, *odv/bv-e25*, *odv/bv-c42*, *vp80 capsid*, *parg*, *sod*, *pif-1*, *fgf*, *p24 capsid*, *gp16* and *arif-1* genes (ORF8, ORF14, ORF25, ORF31, ORF51, ORF56, ORf70, ORF76, ORF81, ORF88, ORF91, ORF99, ORF105, ORF110, ORF112, ORF119, ORF120 and ORF132 in the HearSP1A genome), might not affect the protein functions as the amino acid polarity was not modified. In contrast, the deduced proteins of 14 genes, *p78/83*, *hoar*, *p74*, *p26*, *vp1054*, *desmop*, *vp91 capsid*, *tlp20*, *p6.9*, *odv-ec43*, *odv-e66*, *calix/pep*, *odv-c21* and *bv-ec31* (ORF2, ORF4, ORF18, ORF20, ORF45, ORF65, ORF74, ORF75, ORF87, ORF93, ORF95, ORF121, ORF123 and ORF129, respectively in the HearSP1A genome) suffered mutations that changed the amino acid polarity, and therefore, may affect protein function. However, non-synonymous mutations located in 8 of these genes (*p74*, *p26*, *vp91 capsid*, *tlp20*, *p6.9*, *odv-ec43*, *odv-c21* and *bv-ec31*) did not appear to be associated with any specific phenotype as the overall phenotypic characteristics of genotypes harboring mutations in each protein were similar to those without mutations. As such, the lysine (K) found in the P74 protein at position 348 in HearSP1A, HearSP1B and HearLB1 genomes changed the amino acid polarity in comparison with the glutamic acid (E) of HearLB3 and HearLB6 genomes. In contrast in the P26 protein three mutations were observed at different positions. At position 23, HearLB1 had a glutamine (Q), whereas the other genotypes had an arginine (R). At position 58, HearSP1A, HearSP1B and HearLB1 had an aspartic acid (D), whereas HearLB3 and HearLB6 had an asparagine (N). Finally at position 101, HearSP1A had a lysine (K), compared to the glutamic acid (E) found in the other genotypes. Within VP91 HearSP1A had a lysine (K) at position 443 whereas HearSP1B, HearLB1, HearLB3 and HearLB6 had an asparagine (N). In the TLP20 protein HearLB1 had two aspartic acids (D) at positions 188 and 201, while the

other genotypes had an asparagine (N) and a glycine (G) at these positions. Within the P6.9 of HearLB1, an insertion of 7 amino acids (GGGRRRS) was found at position 91-97, which changed the polarity of the protein in comparison with that of HearSP1A, HearSP1B, HearLB3 and HearLB6 genotypes. In the ODV-EC43, HearSP1A, HearSP1B, HearLB3 and HearLB6 had an aspartic acid (D) at position 71, whereas HearLB1 had an asparagine (N). Within the ODV-C21 protein, at position 94, an aspartic acid (D) was found in HearLB1 genome, compared to the asparagine (N) of HearSP1A, HearSP1B, HearLB3 and HearLB6 genome. And finally in the BV-EC31 protein at position 60 HearLB3 and HearLB6 had an aspartic acid (D), whereas HearSP1A, HearSP1B and HearLB1 presented an asparagine (N), and at position 187, HearLB6 had a glutamine (Q), while the other genotypes had an arginine (R). In contrast, changes found in the encoded proteins of the *desmop*, *calyx/pep*, *p78/83*, *vp1054* and *odv-e66* genes might have clear effect on phenotype, as these genes are involved in ODV or budded virus (BV) formation (Braunagel et al., 2003; Cohen et al., 2009), and additionally the mutations clearly separated the genotypes with different phenotypes. The HearSP1B genotype had a non-synonymous mutation at position 625, aspartic acid (D) to asparagine (N), in the DESMOP protein and a two amino acid deletion after position 160, arginine (R) and serine (S), in the CALYX/PEP in comparison with the other Spanish isolates. DESMOP and CALYX/PEP are ODV structural proteins (Braunagel et al., 2003). DESMOP plays an important role in the efficient exit of nucleocapsids from the nucleus to the cytoplasm during BV synthesis as well as for pre-occlusion of virions and occlusion synthesis (Ke et al., 2008). In contrast, CALYX/PEP is involved in the morphogenesis of the polyhedral envelope of baculoviruses and is part of the carbohydrate envelope of occlusion bodies (Whitt and Manning, 1988). Therefore these mutations could affect ODV attachment to epithelial cells and in this way improve the adaptation of HearSP1B to the long-term insect host population used in the study, increasing its pathogenicity in comparison with the other genotypes (Arrizubieta et al., 2015). Similarly, the mutations found in P78/83, VP1054 and ODV-E66 clearly separated HearSP1 genotypes from HearLB genotypes. Specifically, HearSP1A and HearSP1B genotypes had glutamine (Q) at position 333 in P78/83 protein, in comparison with the glutamic acid (E) present in HearLB1 and the lysine (K) in HearLB3 and HearLB6 genomes. Similarly, within VP1054 protein a valine (V) was

found at position 94 in HearSP1A and HearSP1B in comparison with the glutamic acid (E) of HearLB genomes. Finally, HearSP1A and HearSP1B genomes presented a glycine (G) at position 25 in ODV-E66 protein, in comparison with the aspartic acid (D) of HearLB genomes. These mutations could influence the systemic infection within the host altering the time to death and the OB productivity (Thézé et al., 2014), as these proteins are involved in BV and ODV formation (Cohen et al., 2009). Therefore, they might be responsible of the differences observed in productivity between HearSP1 and HearLB genotypes (Arrizubieta et al., 2015).

However, among the structural proteins, HOAR was the most divergent (Fig. 3). HearSP1A and HearSP1B had the shortest HOAR (746 aa), both showing 100% identity, followed by HearLB3 and HearLB6 (750 aa) with 100% identity between both, while HearLB1 presented the largest HOAR protein (759 aa) (Table 3, Fig. 3). This difference in the protein length was attributed to several short deletions of 1 to 8 amino acids along the protein. The function of the *hoar* gene has not been determined but Le et al. (1997) suggested that it might be involved with virus adaptation, as it interferes with host anti-virus defenses or turns off host genes, which allows successful virus infection of different insect populations, thereby playing an important role in pathogenicity. The HearSP1A and HearSP1B genotypes, with a shorter version of the *hoar* gene, were isolated from HearSP1, the most widespread and predominant isolate during a period of a high *H. armigera* infestation in tomato fields in the Iberian Peninsula (Figueiredo et al., 1999), suggesting a high capacity for producing natural epizootics and for horizontal transmission. Therefore these two genotypes were considered as likely to be specialized in horizontal transmission. In contrast HearLB1, HearLB3 and HearLB6, with a larger version of the *hoar* gene, were obtained from larvae harboring a covert infection (Arrizubieta et al., 2015). Previous studies suggested that the *hoar* gene could be associated with the viral transmission strategy or with the ability to establish a covert infection in the insect hosts, as *Spodoptera exigua* MNPV (SeMNPV) genotypes specialized in vertical transmission had a single nucleotide polymorphism in the hoar gene compared to horizontally transmitted genotypes, which was associated with biochemical changes (Cabodevilla et al.; 2011; Thézé et al., 2014). However, unlike SeMNPV in which just a single nucleotide polymorphism seems to be implicated in the transmission strategy, in

the present study the hoar genes of HearSP1 genotypes were clearly shorter. Experiments are in progress to determine the transmission strategy of the HearSP1 genotypes compared to HearLB genotypes.

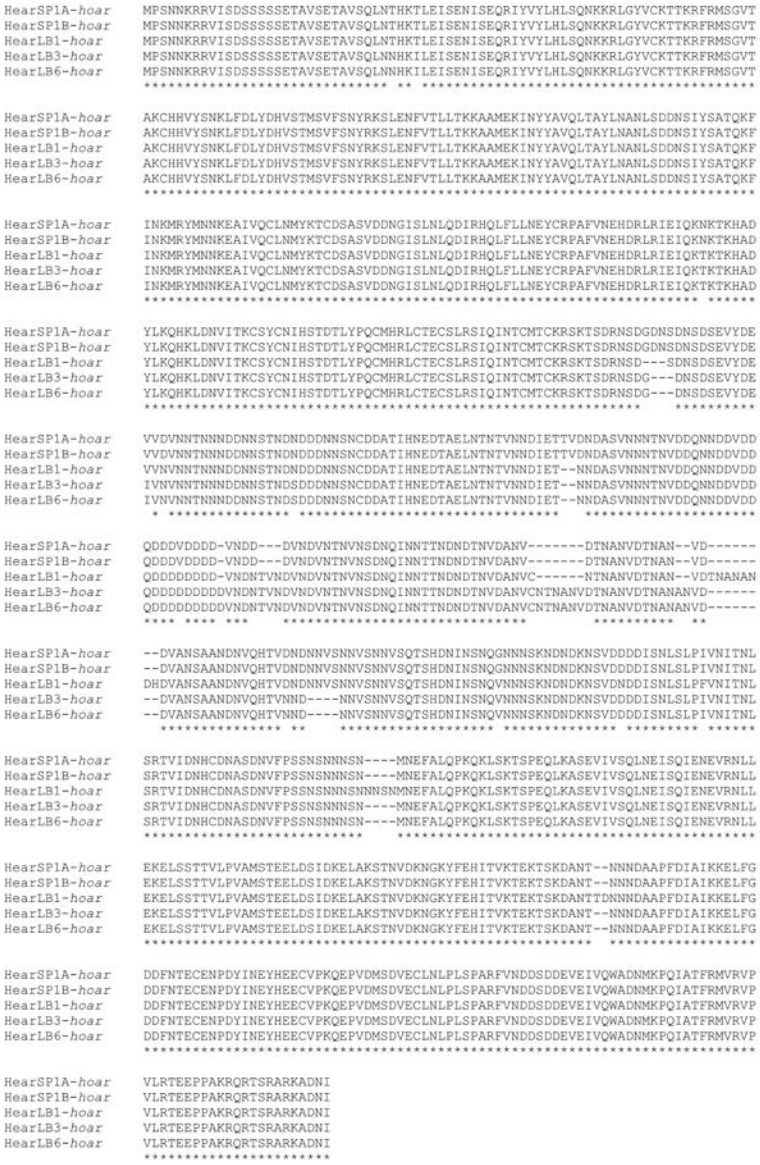


Figure 3: Sequence alignment of HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 HOAR protein.

Finally, non-synonymous mutations were also detected in five of the six ORFs with auxiliary functions, as the protein encoded by *cathepsin* (ORF54 in HearSP1A genome) showed 100% identity at the amino acid level among the five Spanish genotypes (Table 3). In contrast, the proteins encoded by *chitinase*, *iap-2*, *iap-3*, *alk-exo* and *egt* (ORF39, ORF61, ORF102, ORF113 and ORF127 in HearSP1A genome) harbored amino acid mutations that resulted in polarity changes (Table 4). The HearSP1A CHITINASE polarity was modified in comparison with those of the other four Spanish genotypes due to an amino acid mutation at position 526, changing the valine (V) to glutamic acid (E) (Table 4). CHITINASE is necessary for disruption of the chitin skeleton of the host (Hawtin et al., 1997). However HearSP1A infected larvae liquefied similarly to those infected by the other genotypes, suggesting that this mutation might not affect the HearSP1A phenotype in this sense. On the other hand, the inhibitors of apoptosis (IAP-2 and IAP-3 among them) are involved in the anti-apoptotic response induced by the host after infection (Clem and Miller, 1994; Katsuma et al., 2008). Therefore small changes could modulate the progression of the infection and also favor the establishment of covert infections, favoring vertical transmission (Thézé et al., 2014). The mutations observed in both HearSP1B and HearLB1 IAP-2 proteins at position 158 and 183, respectively, histidine (H) and asparagine (N) to aspartic acid (D), did not appear to be associated with any major phenotypic changes as the overall phenotype of HearSP1B and HearLB1 was similar to that of HearSP1A, HearLB3 and HearLB6 group. However the mutation at position 110 in IAP-3 in HearLB genomes, lysine (K) to methionine (M), might favor vertical transmission as this mutation clearly changed the polarity of IAP-3 in vertically transmitted genotypes. Thézé et al. (2014) suggested that slight changes in the apoptosis response might modulate systemic infection and also favor the establishment of covert infections in adults, resulting in efficient vertical transmission. Experiments are in progress to determine the role of *iap* genes in the transmission strategy.

Within ALK-EXO the HearSP1B genotype had a lysine (K) and an aspartic acid (D) at positions 51 and 347, respectively, whereas the other genotypes had a glutamic acid (E) at position 51, and an amino acid deletion at position 347. Moreover, HearLB3 and HearLB6 had two additional aspartic acids (D) at position 353 and 354 compared to HearSP1A, HearSP1B and HearLB1. Finally, HearSP1A and HearLB1 had an aspartic acid (D) at position 404, compared to the glycine (G)

found in HearSP1B, HearLB3 and HearLB6 at the same position. ALK-EXO is a protein involved in BV production and nucleocapsid maturation and therefore might be involved in virus spreading (Okano et al., 2004, 2007). However genotypes with similar speeds of kill could not be grouped together based on these mutations, suggesting that these mutations would not be associated with any specific phenotype identified to date. Finally, the *egt* encoded protein prevents insect molting, prolonging the larval stage and therefore the time to death, resulting in increased OB production (Cory et al., 2004). The EGT of HearSP1A and HearSP1B genotypes had a non-synonymous mutation at position 278 which changed the amino acid polarity, aspartic acid (D) to asparagine (N) (Table 4). However HearSP1A and HearSP1B infected larvae did not show a reduced larval stage period in comparison with those infected with HearLB genotypes, although the HearSP1 viruses were clearly less productive (Arrizubieta et al., 2015).

Major differences among Spanish genotypes and all others *Helicoverpa* spp. SNPVs were present in the baculovirus repeat ORFs (*bro*) genes and homologous regions (*hrs*) (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014), so in the following sections these regions are described in detail.

3.3 Comparison of HearSNPV *bro* genes

HearLB genomes contained one bro gene less (*bro-b*) than HearSP1 genomes, due to a mutation found between the *hr2* and *hr3* (Fig. 2A; Table 3). The three *bro* genes present between the *hr2* and *hr3* in HearSP1 genotypes, *bro-a*, *bro-b* and *bro-c* genes, showed major divergence among the different isolates, while *bro-d* was more conserved (Table 3). Previous studies reported that the most divergent ORFs between *Helicoverpa* spp. SNPVs are those located between *hr2* and *hr3* (Chen et al., 2002; Ogembo et al., 2009). Additionally, a variable number of *bro* genes have been described within the *hr2* and *hr3* genomic region (Table 3). The African genotype HearNNg1 has three *bro* genes (Ogembo et al., 2009), whereas only two were identified in the Chinese genotypes, HearG4 (Chen et al., 2001) and HearC1 (Zhang et al., 2005), and in HzSNPV (Chen et al., 2002), and just one in the Australian genotype, HearAu (Zhang et al, 2014). Therefore the *hrs* showed high variability among the different *Helicoverpa* spp. SNPV isolates, and they are considered sites of frequent recombination and

rearrangement (Chen et al., 2002; Harrison and Bonning, 2003; Hayakawa et al., 2000).

The *bro-a* gene, ORF57 in the five Spanish genotypes, was not only identified in the phylogenetically closest genotype, namely the Kenyan genotype HearNNg1, but also in the Chinese genotypes, HearG4 and HearC1, but was absent in the Australian, HearAu, and USA genotype, HzSNPV. The main differences in the *bro-a* gene between the Spanish isolates were present in the C-terminal region, which showed different mutations that resulted in changes in the amino acid composition of the putative protein (Fig. 4A). HearLB1 had the largest BRO-A protein (361 amino acids), whereas HearLB3 and HearLB6 lacked the proline (P) found at position 73 in the HearLB1 genome, resulting in 360 amino acids (Fig. 4A). In contrast, HearSP1A BRO-A comprised 357 amino acids, due to a 12 nucleotide deletion at positions 203-207 (CTGCG), 207 (G), 222 (AA) and 230 (CGAA) downstream from the initial ATG in comparison with the bro-a gene of HearLB1 genotype, resulting in four amino acid deletions and therefore in several changes in the amino acid composition (Fig. 4A). The HearSP1B genotype had the smallest BRO-A protein (237 amino acids), as this genotype had a mutation in the ATG codon found in the other genotypes (AGG), with a new start codon 358 nt downstream (Fig. 4A).

The *bro-b* gene, present in HearSP1 genomes (ORF58) and the Kenyan genotype HearNNg1, was absent in HearLB genotypes due to a 391 bp deletion found after nt 51,690, 51,113 and 51,180 in HearLB1, HearLB3 and HearLB6 genotypes, respectively, which corresponds with nt 51,861-52,251 in the HearSP1A genome. This *bro-b* gene was also absent in HearG4, HearC1, HearAu and HzSNPV genomes (Chen et al., 2001, 2002; Zhang et al., 2005, 2014). The presence of the *bro-b* gene in HearSP1 genotypes and its absence in HearLB genotypes could have resulted from virus-host co-evolution, as both groups were isolated from different geographical sources in the Iberian Peninsula and might be specialized in different routes of transmission (Arrizubieta et al., 2015). This coincides with previous results that suggest that the these genes resulted from the evolution of virus-host relationships, as in addition to their high variability, several baculovirus species lack *bro* genes (Bideshi et al., 2003; Cohen et al., 2009). Therefore their presence seems to be involved in the maintenance of the insecticidal activity against a specific host and to avoid the development of insect

resistance (Cohen et al., 2009). Moreover, this difference in the presence or absence of the *bro* genes among the different *Helicoverpa* spp. SNPV genomes suggests that *bro* genes might be involved in genetic recombination and evolution within isolates from different geographic regions (Zhang et al., 2014). Differences were also found in the size of the BRO-B protein of HearSP1B (88 amino acids), which was 13 amino acids shorter than that of HearSP1A (101 amino acids) (Fig. 4B), due to an insertion (G) found 218 nt downstream from the ATG initial codon, which changed the reading frame resulting in an early codon stop (TGA). Therefore the amino acid identity decreased to 72.5% in comparison with the 100% identity found between BRO-B proteins of HearNNg1 and HearSP1A (Table 3).

The encoded protein of the *bro-c* gene of HearSP1B was also smaller, due to a single nucleotide deletion (cytosine) 27 nt downstream from the ATG of the other Spanish genotypes, which resulted in an early stop codon delaying the start codon 437 nt and reducing it to 206 amino acids. In contrast, BRO-C of HearSP1A (ORF59), HearLB1 (ORF58), HearLB3 (ORF58) and HearLB6 (ORF58) presented the same size as that of HearNNg1 and HzSNPV genotypes (352 amino acids). Finally, the HearAu genotype presented the largest variant with 549 amino acids. (Fig. 4C; Table 3). The N-terminal region of the HearSP1B *bro-c* gene was more variable in comparison with the conserved C-terminal region, due to this mutation (Fig. 4C). Previous studies indicated that disruption of the N-terminal region of the unique *bro* protein of *Autographa californica* MNPV (AcMNPV), resulted in reduced OB production (Bideshi et al., 2003). However, HearSP1B was as productive as HearSP1A (Arrizubieta et al., 2015). Nevertheless, HearSP1B had four *bro* genes, whereas AcMNPV had only one, so the other three *bro* genes presented in the HearSP1B genome may have diluted the effect of the BRO-C protein in the reduction in OB progeny. Curiously, BRO-B showed high degree of amino acid identity with the N-terminal region of the BRO-C, suggesting that *bro-b* appeared as a result of *bro-c* gene duplication (Ogembo et al., 2009).

A)

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HearSP1A-bro-a   MSLTKIQFGDKEVETYTIDYNGEKWMVANPFAEALNYSKPNKAILEKVSQQNTRNLEQLRSYQIGTID--DSSL-
HearSP1B-bro-a   ----------------------------------------------------------------------------
HearLB1-bro-a    MSLTKIQFGDKEVETYTVDFNGEKWMVANPFAEALDYSRANKAIFEKVSAENQRTYDQIRSHRISATDCVTSPLP
HearLB3-bro-a    MSLTKIQFGDKEVETYTVDLDGEKWMVANPFAEALSYSNVNRAIRVHVSEKNQQNYEEFKSDRIGLTDCVTS-LP
HearLB6-bro-a    MSLTKIQFGDKEVETYTVDLDGEKWMVANPFAEALSYSNVNRAIRVHVSEKNQQNYEEFKSDRIGLTDCVTS-LP

HearSP1A-bro-a   -SLHPRTKFINRAGVFELINASDMPGAKRFQAWNNNDLLPTLCQEGEYKMARDAPANIAHGMNAVHVATNEGVAA
HearSP1B-bro-a   ----------------------------------------------MARDAPANIAHGMNAVHVATNEGVAA
HearLB1-bro-a    RNIQAKTKFINRAGVFELINASDMPGAKRFQAWNNNDLLPTLCQEGEYKMARDAPANIAHGMNAVHVATNEGVAA
HearLB3-bro-a    RNIQAKTKFINRAGVFELINASDMPGAKRFQAWNNNDLLPTLCQEGEYKMARDAPANIAHGMNAVHVATNEGVAA
HearLB6-bro-a    RNIQAKTKFINRAGVFELINASDMPGAKRFQAWNNNDLLPTLCQEGEYKMARDAPANIAHGMNAVHVATNEGVAA
                                                                 **************************

HearSP1A-bro-a   PWMKDLDHLKTAIVEKDRKIDDLTLALKSSNDELVKANAHLCDANKALVSFATEMISARRDCESARKDCEAARKE
HearSP1B-bro-a   PWMKDLDHLKTAIVEKDRKIDDLTLALKSSNDELVKANAHLCDANKALVSFATEMISARRDCESARKDCEAARKE
HearLB1-bro-a    PWMKDLDHLKTAIVEKDRKIDDLTLALKSSNDELVKANAHLCDANKALVSFATEMISARRDCESARKDCEAARKE
HearLB3-bro-a    PWMKDLDHLKTAIVEKDRKIDDLTLALKSSNDELVKANAHLCDANKALVSFATEMISARRDCESARKDCEAARKE
HearLB6-bro-a    PWMKDLDHLKTAIVEKDRKIDDLTLALKSSNDELVKANAHLCDANKALVSFATEMISARRDCESARKDCEAARKE
                 ********************************************************************************

HearSP1A-bro-a   TAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGEDQYAFLRPQKRSLKRSLDRLSVDEKDIVYKSDYVPNSMNV
HearSP1B-bro-a   TAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGEDQYAFLRPQKRSLKRSLDRLSVDEKDIVYKSDYVPNSMNV
HearLB1-bro-a    TAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGEDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMNV
HearLB3-bro-a    TAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGEDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMNV
HearLB6-bro-a    TAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGEDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMNV
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HearSP1A-bro-a   LNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATNNSVVGNKM
HearSP1B-bro-a   LNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATNNSVVGNKM
HearLB1-bro-a    LNKVKERLPKEKFKARHNRITLHEDLTREDLLEAIESTVSSRQVAIIVNKATNNSVVGNKM
HearLB3-bro-a    LNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNSVVGNKM
HearLB6-bro-a    LNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNSVVGNKM
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B)

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HearSP1A-bro-b   MAVTTVQFANSELEVISIKDDSGQLWMLANPFARILEYSNAPNAISTYVRVENQKYFEEIRSARYGQTCVIMRSN
HearSP1B-bro-b   MAVTTVQFANSELEVISIKDDSGQLWMLANPFARILEYSNAPNAISTYVRVENQKYFEEIRSARYGQTCVIMR--
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HearSP1A-bro-b   KVKVYQSRRPVRTDSGVANNSAINNS
HearSP1B-bro-b   ----------FKQSQSLSIAPACSN-
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C)

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HearSP1A-bro-c   MAVIKVQFANSELEVISIKDDNGELWMLANPFARILEYSNANRAVRVHVLEKNQCILEKIRPDHCGLDDVTLHPL
HearSP1B-bro-c   ----------------------------------------------------------------------------
HearLB1-bro-c    MAVIKVQFANSELEVISIKDDNGELWMLANPFARILEYSNANRAVRVHVLDKNQCILEKIRPDHCGLDDVTLHPL
HearLB3-bro-c    MAVIKVQFANSELEVISIKDDNGELWMLANPFARILEYSNANRAVRVHVLDKNQCILEKIRPDHCGLDDVTLHPL
HearLB6-bro-c    MAVIKVQFANSELEVISIKDDNGELWMLANPFARILEYSNANRAVRVHVLDKNQCILEKIRPDHCGLDDVTLHPL

HearSP1A-bro-c   SKFINRAGLFELIQASRMPKAQEFRDWINSDLLPKLCDDGKYDMATDAPVGIAVGMNAVHSITNEGEEAPWMKDL
HearSP1B-bro-c   ----------------------------------------------------------------------MKDL
HearLB1-bro-c    SKFINRAGLFELIQASRMPKAKEFRDWINSDLLPKLCDDGKYDMATDAPVGIAMGMNAVHAIANDGADAPWMKDL
HearLB3-bro-c    SKFINRAGLFELIQASRMPKAKEFRDWINSDLLPKLCDDGKYDMATDAPVGIAMGMNAVHAIANDGADAPWMKDL
HearLB6-bro-c    SKFINRAGLFELIQASRMPKAKEFRDWINSDLLPKLCDDGKYDMATDAPVGIAMGMNAVHAIANDGADAPWMKDL
                                                                                    ****

HearSP1A-bro-c   ARLKNAIVEKDQ--------------KIGTLTEALTQCNEKLVNFASALVQANNGLLEANRNAETARQDAERSRR
HearSP1B-bro-c   ARLKNAIVEKDQ--------------KIGTLTEALTQCNEKLVNFASALVQANNGLLEANRNAETARQDAERSRR
HearLB1-bro-c    HELRTAVVQKDKIIEAISYENKELSLSLRTSNEKLQGANDKLMYFASALVESNNGLMKANERIEN----------
HearLB3-bro-c    HELRTAVVQKDKIIEAISYENKELSLSLRTSNEKLQGANDKLMYFASALVESNNGLMKANERIEN----------
HearLB6-bro-c    HELRTAVVQKDKIIEAISYENKELSLSLRTSNEKLQGANDKLMYFASALVESNNGLMKANERIEN----------
                   *  * * **                    *  * *   * **  ******  ****  **   *

HearSP1A-bro-c   ETAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGGDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMN
HearSP1B-bro-c   ETAELANRMADIAQDVIAKPSDPQLLHSLAVCSMGGDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMN
HearLB1-bro-c    ----LANRMADIAQDVIAKPSDPQLLHSLAVCSMGGDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMN
HearLB3-bro-c    ----LANRMADIAQDVIAKPSDPQLLHSLAVCSMGGDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMN
HearLB6-bro-c    ----LANRMADIAQDVIAKPSDPQLLHSLAVCSMGGDQYAFLRPQKRSLKRSLDRLSVDEKDIVFKSDYVPNSMN
                     ********************************************************************************

HearSP1A-bro-c   VLNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNITSIGNNTTNK
HearSP1B-bro-c   VLNKVKERLPKEKFKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNITSIGNNTTNK
HearLB1-bro-c    VLNKVKERLPKEKFKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNITSIGNNTTNK
HearLB3-bro-c    VLNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNITSIGNNTTNK
HearLB6-bro-c    VLNKVKERLPKEKYKARHNRITLHEDLTREDLLQAIESTVSSRQVAIIVNKATSNITSIGNNTTNK
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D)

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HearSP1A-bro-d   MYLVNRKCKLGEVWITEIEENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVLNQHSLVTSSDSIE
HearSP1B-bro-d   MYLVNRKCKLGEVWITEIEENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVLNQHSLVTSSDSIE
HearLB1-bro-d    MYLVNRKCKLGEVWITEIEENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVLNQHSLVTSSDSIE
HearLB3-bro-d    MYLVNRKCKLGEVWITEIEENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVLNQHSLVTSSDSIE
HearLB6-bro-d    MYLVNRKCKLGEVWITEIEENRFLCSGHGVAEALGYKCPRRALYDHVKPQWRKTWAEIKGVLNQHSLVTSSDSIE
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HearSP1A-bro-d   LPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQSSTDNSTEVVSYDQKLAN
HearSP1B-bro-d   MPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQSSTDNSTEVVSYDQKLAN
HearLB1-bro-d    MPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQSSTDNSTEVVSYDQKLAN
HearLB3-bro-d    MPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQSSTDNSTEVVSYDQKLAN
HearLB6-bro-d    MPLNWQPNTLFITEAGIYALIMRSKLPAAEEFQSWLFEEVLPELRRTGKYSIENRRQSSTDNSTEVVSYDQKLAN
                  ********************************************************************************

HearSP1A-bro-d   VQMEALQLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMKDMAHQANMSMFQFAANALLAKDN
HearSP1B-bro-d   VQMEALQLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMKDMAHQANMSMFQFAANALLAKDN
HearLB1-bro-d    VQMEALQLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMKDMAHQANMSMFQFAANALLAKDN
HearLB3-bro-d    VQMEALQLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMKDMAHQANMSMFQFAANALLAKDN
HearLB6-bro-d    VQMEALQLKLQLSEANIKIAEWNTNMSEMKRNYEQQMSEYKEREFKMQLQMKDMAHQANMSMFQFAANALLAKDN
                 ********************************************************************************

HearSP1A-bro-d   IDENHRLRQTLEKISNRVVPVLTKQPEKEEYITGYERIVNGKRRIRMCRSQLYAIEMQDKVAKRYRDTLCTPKRF
HearSP1B-bro-d   IDENHRLRQTLEKISNRVVPVLTKQPEKEEYITGYERIVNGKRRIRMCRSQLYAIEMQDKVAKRYRDTLCTPKRF
HearLB1-bro-d    IDENHRLRQTLEKISNRVVPVLTKQPEKEEYITGYERIVNGKRRIRMCRSQLYAIEMQDKVAKRYRDTLCTPKRF
HearLB3-bro-d    IDENHRLRQTLEKISNRVVPVLTKQPEKEEYITGYERIVNGKRRIRMCRSQLYAIEMQDKVAKRYRDTLCTPKRF
HearLB6-bro-d    IDENHRLRQTLEKISNRVVPVLTKQPEKEEYITGYERIVNGKRRIRMCRSQLYAIEMQDKVAKRYRDTLCTPKRF
                 ********************************************************************************

HearSP1A-bro-d   KPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLRYTNKLCTEMEVLDETELRAKYRADVEMCQRNKT
HearSP1B-bro-d   KPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLRYTNKLCTEMEVLDETELRAKYRADVEMCQRNKT
HearLB1-bro-d    KPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLRYTNKLCTEMEVLDETELRAKYRADVEMCQRNKT
HearLB3-bro-d    KPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLRYTNKLCTEMEVLDETELRAKYRADVEMCQRNKT
HearLB6-bro-d    KPSPRYAWLCDSTKFLQLKCSNSVMVWCKIRADEPHVFYGLRYTNKLCTEMEVLDETELRAKYRADVEMCQRNKT
                 ********************************************************************************

HearSP1A-bro-d   VNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINAIVENIVEKMAKELVPSTPQRRHSNAGDVYSAQQVVHAM
HearSP1B-bro-d   VNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINAIVENIVEKMAKELVPSTPQRRHSNAGDVYSAQQVVHAM
HearLB1-bro-d    VNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINAIVENIVEKMAKELVPSTPQRRHSNAGDVYSAQQVVHAM
HearLB3-bro-d    VNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINAIVENIVEKMAKELVPSTPQRRHSNAGDVYSAQQVVHAM
HearLB6-bro-d    VNTKLIEEFKALDLIDEDDCVAKCLTQSVDAKDRINAIVENIVEKMAKELVPSTPQRRHSNAGDVYSAQQVVHAM
                 ********************************************************************************

HearSP1A-bro-d   NNCQNYFVKNVCNVRFYDESNIIPGNVPAIENVTTQKDITSATEATTSTNL
HearSP1B-bro-d   NNCQNYFVKNVCNVRFYDESNIIPGNVPAIENVTTQKDITSATEATTSTNL
HearLB1-bro-d    NNCQNYFVKNVCNVRFYDESNIIPGNVPAIENVTTQKDITSATEATTSTNL
HearLB3-bro-d    NNCQNYFVKNVCNVRFYDESNIIPGNVPAIENVTTQKDITSATEATTSTNL
HearLB6-bro-d    NNCQNYFVKNVCNVRFYDESNIIPGNVPAIENVTTQKDITSATEATTSTNL
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Figure 4: Sequence alignment of HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 BRO proteins: A) BRO-A, B) BRO-B, C) BRO-C and D) BRO-D.

This reduction in size in the three *bro* genes observed in HearSP1B might be related to its increased pathogenicity. Previous results showed that HearC1 genotype, with a shorter version of the BRO-A protein (357 aa) in comparison with that of HearG4 (527 aa), was ~3 fold more pathogenic against a Chinese *H. armigera* colony than HearG4 (Zhang et al., 2005). Additionally, HearNNg1, with a 361 aa BRO-A protein, was also ~300 fold more pathogenic than HearG4 against a Japanese colony (Ogembo et al., 2007). Furthermore, the presence of *bro-c* in the HearNNg1 genome and its absence in the Chinese genotypes, HearG4 and HearC1, could also be related with the ~300 fold increased OB pathogenicity compared to HearG4 (Ogembo et al., 2007).

In contrast, the encoded protein of the *bro-d* gene (ORF104 in HearSP1A genome), showed high amino acid sequence identity among the different Spanish HearSNPV isolates (Fig. 4D). In fact, the BRO-D of HearSP1B, HearLB1, HearLB3 and HearLB6 shared 100% identity (Table 3), and 99.8% with that of HearSP1A,

due to a point mutation (A to T) 225 nt downstream from the ATG initial codon, which resulted in a non-synonymous mutation, methionine (M) to leucine (L), but without amino acid polarity change. Moreover, BRO-D showed more than 99% identity at the amino acid level with that of previously sequenced *Helicoverpa* spp. SNPV (Table 3). This confirmed previous results that reported that *bro-d* was the most conserved *bro* gene among *Helicoverpa* spp. SNPV, sharing amino acid sequence identity higher than 99% (Chen et al., 2002; Ogembo et al., 2009).

3.4 Comparison of HearSNPV homologous regions

Regions with homologous repeats (*hrs*) occur at multiple locations along the genome and serve as putative origins of DNA replication and transcription enhancing regions (Chen et al., 2002). Additionally these regions are “hot spots” of frequent recombination and rearrangement in baculovirus genomes, which was supported by the observation that the spacing between the palindromic repeats is variable and alignments require multiple gaps. This suggests numerous deletions and insertions between the palindromic repeats in the *hrs* of these viruses (Chen et al., 2002; Harrison and Bonning, 2003), as occurs with the *hr2* and *hr3* of the HearSNPVs; originally a single *hr* divided in two by the insertion of the *bro* genes (Chen et al., 2001).

Five homologous regions (*hr*) were identified in the Spanish genotypes at the same positions as those present in the other HearSNPVs and HzSNPV genomes (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014) (Table 3). The *hr* lengths in the genomes of the Spanish HearSNPV genotypes were between 1,749-2,068, 780-1,193, 295-297, 1,300-1,729 and 2,076-2,106 bp for *hr1*, *hr2*, *hr3*, *hr4* and *hr5*, respectively, with total *hr* lengths of 7,367, 7,177, 7,151, 6,330 and 6,278 bp for HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6, respectively. The total lengths of the hrs in the Spanish genotypes were similar to the most closely related genotype HearNNg1 (6,399 bp) and to the Chinese genotype HearC1 (6,325 bp), however the other genotypes from China, HearG4 (9,301 bp), Australia, HearAU (9,430 bp), and USA, HzSNPV (8,348 bp) showed longer *hrs* (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014) (Table 1). As previously mentioned, the *hrs* within the HearSNPV Spanish genotypes were less conserved than the ORFs. Among them *hr3* and *hr5* were the least variable (95.4-97.9% identity), followed by *hr1* and *hr4* with

intermediate values (73.1-97.1%), and finally the *hr2* was the most variable (62.0-100%). HearSP1A showed the highest overall *hr* identity with that of HearSP1B (95.8%), whereas the *hrs* of HearLB6 were more divergent (with an overall identity of 82.6%). However compared with the *H. armigera* spp. SNPVs from other geographical regions, *hr1* and *hr4* were the least variable (61.4-97%). In contrast *hr5* was the most divergent (41.2-59.1%) (Table 3). Ogembo et al. (2009) identified *hr2* and *hr5* as the most divergent *hrs* among HearNNg1, HearG4, HearC1 and HzSNPV. In contrast, Chen et al. (2002) observed that the most divergent *hr* between HearG4 and HzSNPV was *hr1*. These differences highlight the diversity among *hrs* of the *H. armigera* spp. SNPVs. As expected, the Spanish *hrs* genotypes showed the highest identity with the *hrs* of the Kenyan genotype, HearNNg1 (70.7%), and the lowest identity with the *hrs* of the Chinese HearG4 genotype (54.4%). Additionally, all the genotypes contained different numbers of repeats, demonstrating the high structural plasticity of these genomic regions. Ogembo et al. (2009) suggested that this plasticity among the different *hrs*, not only in the sequence but also in the length, could be related to insecticidal activity and OB production. In fact, the total *hr* length of HearG4, which showed lower pathogenicity than HearC1 (Zhang et al., 2005) and HearNNg1 (Ogembo et al., 2007), was 2,976 and 2,902 nt shorter, respectively. In contrast, in the present study, the total *hr* length of the most pathogenic genotype, HearSP1B (Arrizubieta et al., 2015), was 26, 847 and 873 nt larger than those of HearLB1, HearLB3 and HearLB6, respectively, but 190 nt smaller than HearSP1A (Arrizubieta et al., 2005). Therefore, this difference in biological activity among the HearSNPV genotypes might not be related to differences in the *hr* length alone, but also with other differences in the *bro* genes or other genomic regions previously mentioned. However a correlation was observed with OB productivity as the highly productive HearLB genotypes (Arrizubieta et al., 2015), had a shorter total *hr* length. Previous studies have shown that the *hrs* are enhancers of *ie-0*/*ie-1* and *polyhedrin* transcription, which might influence BV, ODV and OB production (Carson et al., 1991; Habib and Hasnain, 1996; Habib et al., 1996; Schultz et al., 2009; Stewart et al., 2005). Additionally, the *hrs* are enhancers of early baculoviruses promoters (Alves et al., 2002; Guarino et al., 1986; Guarino and Summers, 1986) that play an important role in DNA replication (Pearson et al., 1992; Kool et al., 1993).

Experiments are in progress to determine if these differences in hrs lengths are related to the OB yield phenotype.

3.5 Analysis of selection on HearSNPV isolates genomes

Genomic regions that have been subjected to natural selection may show more functionally important molecular changes than those where there is no selection acting at present, resulting in new genetic variants (Nielsen et al., 2005). Selection pressure analysis (Yang, 1998) has been used to identify positively selected sites in a variety of genes and organisms, including vertebrate viruses involved in host immune recognition and receptor binding (Holmes et al., 2002; Twiddy et al., 2002; Woelk and Holmes, 2001; Woelk et al., 2001). Analysis of selection occurring on baculovirus genomes could identify genes involved in virulence or in adaptation to new insect hosts, even without previous knowledge of the mechanisms responsible for host range and virulence in these pathogens (Harrison and Bonning, 2003).

To detect instances of positive selection among HearSNPV genes, ω was calculated for all of the HearSNPV ORFs using maximum likelihood methods available in Paml4 (Yang, 2007) and the HyPhy package (Pond et al., 2005). Selection analysis performed on the ten HearSNPV genotypes identified eight positively selected ORFs (ω>1, P<0.05): *p78/83*, *ie-0*, *odv-e56*, *p74, bro-d*, *egt*, *bv-ec31* and ORF130 (unknown function) (Table 5). Positively selected sites detected in each ORF corresponded to non-synonymous mutations resulting in changes of specific amino acid residues (Table 5). These amino acid changes might potentially change protein function and be responsible for different co-evolutionary processes in host-pathogen interactions (Harrison and Bonning, 2003; Simón et al., 2011). The functions of five of these eight positively selected genes, *p78/83*, *ie-0*, *odv-e56*, *p74* and *egt* have been previously determined (Cohen et al., 2009). However, the functions of *bro-d*, *bv-ec31* and ORF130 remain unclear. This analysis suggests diversifying selection for proteins involved in primary infection, P78/83, ODV-E56 and P74 (Braunagel et al., 1996; Faulkner et al., 1997; Wang et al., 2008), which is the first barrier for the diversification of baculoviruses, for those involved in viral infection and replication, IE-0 (Stewart et al., 2005), or those with auxiliary function that facilitate virus transmission, such as EGT (Cory et al., 2004). Therefore, this diversifying selection may alter the activity

of the encoded protein, facilitating adaptation to a given host, and to overcome the immune response of the insect. Future experiments are required to determine the function of BRO-D, BV-EC31 and ORF130.

P78/83 (ORF2 in the Spanish genotypes) is an essential structural BV and ODV protein (Possee et al., 1991), which plays an important role in actin polymerization in the nucleus of infected cells (Goley et al., 2006; Machesky and Insall, 2001). Therefore, the alteration of *p78/83* ORF may modulate both virus primary infectivity and systemic infection and thereby modify speed of kill (Thézé et al., 2014). We found two positively selected amino acid sites in *p78/83* ORF for each genotype. At position 318, the HearSP1A, HearSP1B and HearLB1 genotypes had a positively selected valine (V), whereas HearLB3 and HearLB6 genotypes had an isoleucine (I) (Table 5). However, this mutation did not seem to alter the phenotype of the Spanish genotypes, as the overall biological activity, in terms of pathogenicity, virulence and productivity, of HearSP1A, HearSP1B and HearLB1 genotypes did not present large differences to that of the HearLB3 and HearLB6 genotypes (Arrizubieta et al., 2015). In contrast, the positively selected mutation at position 333 could be involved in the increasing OB productivity of HearLB genotypes compared to that of HearSP1 genotypes (Arrizubieta et al., 2015), as mentioned above.

The *ie-0* (ORF6 in the Spanish genotypes) transcript is the only known spliced baculovirus mRNA (Cohen et al., 2009). The *ie-0*/*ie-1* gene complex is essential for viral infection and replication, late gene expression, and BV and ODV production (Schultz et al., 2009; Stewart et al., 2005). One positively selected amino acid site was found in the *ie-0* ORF (Table 5) and might affect protein function or virus phenotype. However, this mutation did not modify the amino acid polarity, so it was not mentioned in the ORF comparison section described above. The HearLB1, HearLB3 and HearLB6 genotypes had a positively selected cysteine (C) at position 36, whereas the HearSP1A and HearSP1B genotypes had an arginine (R) (Table 5). The main biological difference was that HearSP1 genotypes were less productive than HearLB genotypes (Arrizubieta et al., 2015); therefore this point mutation might have an influence on ODV and OB production. Experiments are required to assess this hypothesis.

The *odv-e56* and *p74* (ORF14 and ORF18, respectively, in the Spanish genotypes) are late genes that encode for structural proteins present in the ODV

envelopes (Braunagel et al., 1996; Faulkner et al., 1997). Together with other ODV envelope proteins, including PIF-1, PIF-2, PIF-3 and PIF-4, they are essential for *per os* infection (Fang et al., 2006, 2009; Faulkner et al., 1997; Harrison et al., 2010; Kikhno et al., 2002; Pijlman et al., 2003), and are among the 30 core genes found in all baculovirus species sequenced to date (Braunagel and Summers, 2007; Fang et al., 2009; Harrison et al., 2010). One positively selected amino acid site that did not change the amino acid polarity was identified in *odv-e56* ORF, whereas four were identified in *p74* ORF (Table 5). The ODV-E56 of HearSP1A, HearSP1B and HearLB1 genotypes had a positively selected proline (P) site at position 46, whereas HearLB3 and HearLB6 a serine (S) (Table 5). The positively selected sites at positions 348, 356 and 644 of P74 might not be involved in any phenotypic differences, as the biological activity of the genotypes harbouring these positively sites was similar to that the other genotypes (Arrizubieta et al., 2015). Specifically, at position 348 the HearSP1A, HearSP1B and HearLB1 genotypes had a positively selected lysine (K), while HearLB3 and HearLB6 genotypes had glutamic acid (E). At position 356, a positively selected alanine (A) was detected in the HearSP1B and HearLB1, and a glutamine (Q) in HearSP1A, HearLB3 and HearLB6 genotypes. Finally, at position 644, a positively selected threonine (T) was identified in HearLB1, HearLB3 and HearLB6 genotypes, whereas a valine (V) was present in HearSP1A and HearSP1B (Table 5). In contrast, the positively selected isoleucine (I) found at position 281 in HearSP1B P74, compared to the valine (V) found in HearSP1A, HearLB1, HearLB3 and HearLB6 genotypes, might favour the primary infection of HearSP1B, and may be implicated in the increased pathogenicity of HearSP1B OBs (Arrizubieta et al., 2015).

In the *bro-d* gene (ORF104 in HearSP1A genome), the positively selected amino acid site found at position 76 of the putative protein resulted in a non-synonymous mutation without polarity change (Table 5). The HearSP1A, HearLB1, HearLB3 and HearLB6 presented a positively selected methionine (M), whereas HearSP1B had a leucine (L) (Table 5). This change could influence the function of this gene, and this mutation could be related again with improved OB pathogenicity of HearSP1B compared to the other genotypes (Arrizubieta et al., 2015).

Within the *egt* gene (ORF127 in HearSP1A genome), three positively selected amino acid sites were found (Table 5), but just one modified the amino

acid polarity as described above. Within those that did not modify the polarity at position 47 the HearLB1 genotype had a positively selected serine (S), while the HearSP1A, HearSP1B, HearLB3 and HearLB6 genotypes had a leucine (L) (Table 5), and at position 62 all the Spanish genotypes had a positively selected arginine (R) (Table 5). Finally, a positively selected asparagine (N) was identified in the HearLB1, HearLB3 and HearLB6 genotypes at position 278, whereas the HearSP1A and HearSP1B had an aspartic acid (D) (Table 5). This positively selected mutation in the EGT of HearLB genotypes could be involved in the increased productivity of these genotypes compared to HearSP1 genotypes, although no differences were found in the speed of kill among these genotypes (Arrizubieta et al., 2015). Previous studies demonstrated that changes in the EGT protein have clear effects on speed of kill, and therefore OB productivity, as the egt encodes an ecdysteroid UDP-glucosyl transferase (EGT), which prevents insect molting and increases the duration of the larval stage (O´Reilly and Miller, 1989), favouring the development of larger insects with increased OB yields (Cory et al., 2004). This was confirmed by the construction of recombinant HearSNPVs with deleted *egt* genes, that showed a faster speed of kill and therefore lower OB production values than the wild type isolate (Sun et al., 2005).

The *bv-ec31* (ORF129 in HearSP1A genome) encodes for a BV envelope protein (Thézé et al., 2014), which belongs to the baculovirus protein family DUF1251 of unknown function. Three non-synonymous mutations that did not alter the amino acid polarity but resulted in positively selected amino acid sites were found in BC-EC31 (Table 5). The HearLB1 and HearLB6 genotypes had a positively selected serine (S) at position 14, whereas the HearSP1A, HearSP1B and HearLB3 genotypes had a leucine (L) (Table 5). At position 22, a positively selected valine (V) was present in HearSP1B and HearLB1, while HearSP1A, HearLB3 and HearLB6 had an isoleucine (I) (Table 5). Finally, at position 187 the HearSP1A, HearSP1B, HearLB1 and HearLB3 genotypes had a positively selected glutamine (Q) compared to the arginine (R) present in the HearLB6 genotype (Table 5). Nevertheless, these mutations did not appear to be related with any specific phenotype, as no differences in the insecticidal activity were found among the genotypes with positively selected sites compared to those that did not (Arrizubieta et al., 2015). Additional studies are required to determine its

function as the presence of three positively selected sites within the same protein provides evidence of diversifying selection during BV formation.

Table 5: Positive selected ORFs and amino acid sites (ω>1, P<0.05) found in HearSNPV genotypes.

ORF name	Amino acid change*	Variant strains †
ORF2 (*p78/83*)	318 V→I	HearLB3, HearLB6, HearG4, HearAu, HzSNPV
	333 Q→K	HearLB3, HearLB6, HearC1
	333 Q→E	HearLB1, HearG4
ORF6 (*ie-0*)	36 R→C	HearLB1, HearLB3, HearLB6, HearG4, HearC1, HearNNg1
ORF14 (*odv-e56*)	46 P→S	HearLB3, HearLB6, HearC1, HearAu, HzSNPV
ORF18 (*p74*)	281 V→I	HearSP1B, HearNNg1, HzSNPV
	348 K→E	HearLB3, HearLB6, HearG4, HearC1, HearAu, HzSNPV
	356 Q→A	HearSP1B, HearLB1
	644 V→T	HearLB1, HearLB3, HearLB6, HearNNg1, HzSNPV
ORF104 (*bro-d*)	76 L→M	HearSP1B, HearLB1, HearLB3, HearLB6, HearG4, HzSNPV
ORF127 (*egt*)	47 L→S	HearLB1, HearG4, HearNNg1, HzSNPV
	62 R→K	HearG4, HzSNPV
	278 D→N	HearLB1, HearLB3, HearLB6, HearG4, HearC1, HearNNg1, HearAu
ORF129 (*bv-ec31*)	14 L→S	HearLB1, HearLB6, HearNNg1
	22 I→V	HearSP1B, HearLB1, HzSNPV
	187 Q→R	HearLB6, HearG4, HearNNg1, HearAu, HzSNPV
ORF130 (unknown)	136 T→A	HearLB1, HearNNg1, HzSNPV
	231 S→A	HearLB1, HearG4, HearC1
	704 S→F	HearLB1, HearLB3, HearLB6, HearAu, HzSNPV
	830 K→R	HearLB1, HearG4, HearC1, HearNNg1
	908 H→Y	HearC1, HearNNg1, HearAu
	919 T→N	HearC1, HearNNg1, HearAu
	925 Y→C	HearG4, HzSNPV

*Positively selected amino acid change (original residue in the alignment) with respect to reference strain HearSP1A.
†Variant strains bearing mutated amino acids.

Finally, within the ORF130 of unknown function seven non-synonymous mutations which resulted in positively selected amino acid sites were identified (Table 5). The HearLB1 genotype had a positively selected alanine (A) at position 136 and 231, and a positively selected arginine (R) at position 830, whereas the HearSP1A, HearSP1B, HearLB3, HearLB6, genotypes had a threonine (T) at position 136, a serine (S) at position 231 and a lysine (K) at position 830 (Table 5). However, these mutations did not seem to be related with any specific phenotype as the overall insecticidal characteristics of HearSP1A, HearSP1B, HearLB3 and HearLB6 did not clearly differentiate them from those of HearLB1 (Arrizubieta et al., 2015). In contrast, at position 704 HearLB genotypes had a positively selected phenylalanine (F), compared to the serine (S) found in HearSP1 genotypes. As previously mentioned, HearSP1 genotypes produced less OBs than did HearLB

genotypes (Arrizubieta et al., 2015), suggesting that this positively selected mutation could be involved in the increased OB production. Finally, all the Spanish genotypes, HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6 genotypes, had positively selected histidine (H), threonine (T), and tyrosine (Y) at the 908, 919 and 925 positions, respectively (Table 5). HearSNPV ORF130 deletion mutants should be constructed to determine the role of this unknown ORF in HearSNPV phenotype.

In summary, the phenotypic differences observed among the Spanish HearSNPV genotypes could be explained by the differences found in certain genes, specifically the *bro* genes and *hrs*. The reduced OB production of HearSP1 genotypes in comparison with HearLB genotypes might be related to several mutations found in genes involved in DNA replication (*ie-1*, *lef-3* and *DNA polymerase*), viral transcription (*lef-8* and *lef-1*) or structural genes (*p78/83* and *vp1054*). Additionally, the observed differences in the length of *hrs* might also influence OB production. Similarly, the increased pathogenicity of HearSP1B OBs might be influenced by mutations observed in the *desmop*, *calyx/pep* and *odv-e66* genes. Moreover, the difference in the length of HearSP1B *bro* genes might also be involved with increased pathogenicity. Finally, mutations identified in *iap-2*, *iap-3* and *hoar* genes could be related to the transmission strategy or the ability to establish a covert infection in the insect host, as the likely horizontally-transmitted genotypes, HearSP1A and HearSP1B, showed different mutations than the vertically-transmitted HearLB1, HearLB3 and HearLB6 genotypes. Finally, selection pressure analysis identified several genes that might be implicated in the increased productivity of HearLB genotypes, such as *ie-0* or the *orf130*, or the increased pathogenicity of HearSP1B genotype OBs, as the *p74* or *bro-d* genes. However additional studies are in progress, including the construction of specific HearSNPV deletion genotypes, for each of the mentioned genes and the determination of the phenotypic characteristics of each construct with the aim of identifying genes involved in specific pathogen phenotypes.

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CHAPTER VI

Increased host range of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) through co-envelopment with *H. armigera* multiple nucleopolyhedrovirus (HearMNPV)

Abstract

Helicoverpa armigera single nucleopolyhedrovirus (HearSNPV) is specific to species in the genus *Helicoverpa*. The aim of this study was to extend the host range of HearSNPV by applying co-occluded baculovirus technology which allows us to obtain virions derived from occlusion bodies (ODV), containing a mixture of genomes of both HearSNPV and the multiple nucleopolyhedrovirus, HearMNPV. For co-occlusion, *H. armigera* larvae were infected first with HearMNPV and at different time intervals with HearSNPV. When both viruses were administrated at the same time occlusion bodies (OBs) composition consisted of 97% HearSNPV, but when the second virus was administered 12 or 24 h later the genomes of both viruses (HearSNPV and HearMNPV) were present in the OBs in the same proportion (1:1). Both viruses were present in 55.6-58.1% of ODVs analyzed. Also, 1% of the HearSNPV nucleocapsids were present in multiple ODVs with nucleocapsids of HearMNPV. Nevertheless, the co-enveloped mixture did not present improved phenotypic characteristics against different susceptible species, but increased the HearSNPV host-range, as this virus was able to enter and infect previously non-susceptible species such as *S. frugiperda* and *M. brassicae*. Moreover, HearSNPV persisted in OB progeny during at least five serial passages in these two “non-permissive” insect species. In conclusion, co-envelopment of HearSNPV and HearMNPV genomes to produce mixed ODVs is a technology that can expand the narrow host range of HearSNPV. This technology has great potential for producing non-recombinant baculoviruses with a wider host range which are effective for controlling a broader number of insect pests than each virus alone.

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1. Introduction

Helicoverpa armigera (Hübner), the cotton bollworm, is an insect pest that causes important damage in many crops in Europe, Asia, Africa and Oceania (Fitt, 1989; Shepard et al., 2009), and recently has successfully invaded Brazil (Czepak et al., 2013). Control using chemical insecticides has generated numerous problems, including high levels of resistance. Alternative control methods include the use of baculovirus-based insecticides.

The *Baculoviridae* family include four genera: *Alphabaculovirus* (lepidopteran-specific nucleopolyhedrovirus, NPV), *Betabaculovirus* (lepidopteran-specific granulovirus, GV), *Gammabaculovirus* (hymenopteran-specific NPV) and *Deltabaculovirus* (dipteran-specific NPV) (Jehle et al., 2006). Viral occlusion bodies (OBs) of NPVs contain multiple virions that can either include one (single NPVs, SNPVs) or many nucleocapsids (multiple NPVs, MNPVs) (Funk et al., 1997). As such, ODVs can comprise identical or different genotypes of the same virus or even different virus species (Beperet, 2014a; Bernal et al., 2013; Clavijo et al., 2010). All known MNPVs are alphabaculoviruses, while SNPVs can also be gammabaculoviruses and deltabaculoviruses. As Lepidoptera is the most recently derived insect order, these host affinities suggest that MNPVs derived from SNPVs, and therefore, the MNPV phenotype may incur a selective advantage (Rohrmann, 1986; Washburn et al., 2003), which may favor the higher diversity of MNPVs compared to SNPVs (Vail et al., 1999; Volkman, 1997).

As baculoviruses are named after the host insect species, a virus such as *Autographa californica* NPV (AcMNPV), could be given different names, such as *Anagrapha falcifera* NPV and Rachiplusia ou NPV (Harrison and Bonning, 1999), which have been shown to be variants of AcMNPV, i.e. members of the same virus species. Alternatively, virus isolated from the same insect called equal, such as happened with *Spodoptera littoralis* NPVs (Kislev and Edelman, 1982) and *Mamestra configurata* NPVs (Li et al., 2002a, b), however they were after found to be very different virus species. MNPVs and SNPVs have also been characterized from a single host, such as *Orgyia pseudotsugata* (Zanotto et al., 1993) or *Helicoverpa armigera* (Gettig and McCarthy, 1982; Figueiredo et al., 1999; Ogembo et al., 2005; Zhang et al., 2005; Williams and Payne, 1984; Sun and Zhang, 1994). In the latter, all geographical isolates of HearSNPV are highly

similar at the genome and biological level (Arrizubieta et al., 2014, 2015; Figueiredo et al., 1999, 2009; Ogembo et al., 2007, 2009). Typically these HearSNPVs have a narrow host range (Gröner, 1986), which is limited to species belonging to the genus *Helicoverpa*/*Heliothis* (Gettig and McCarthy, 1982). By contrast, HearMNPV shows greater genetic diversity among geographical isolates (Sun and Zhang, 1994; Tang et al., 2012), and has a broader spectrum of insect hosts, including other species outside of the *Helicoverpa*/*Heliothis* complex (Rovesti et al., 2000; Tompkins et al., 1988). At the molecular level, HearSNPV and HearMNPV are quite distantly related (Tang et al., 2012). In terms of their insecticidal properties HearSNPV is more effective and kills insects faster than HearMNPV. Therefore according to the new classification of baculoviruses (Jehle et al., 2006), which is based on phylogenetic and biological characteristics, HearSNPV and HearMNPV although isolated from the same insect host, belong to different baculovirus species.

When considering developing a biological control agent against species in the *Helicoverpa*/*Heliothis* complex, HearSNPV should be used due to its greater virulence and pathogenicity against these species (Arrizubieta et al., 2014). However, in some circumstances, where a crop is also attacked by other lepidopteran pest species, HearSNPV specificity becomes a limiting factor for which the use of a baculovirus with a wider host range is desirable. The use of HearMNPV might address this problem but its pathogenicity is not comparable to that of HearSNPV or other baculoviruses developed as bioinsecticides (Caballero et al., 2009). Therefore, the present study aimed to generate OBs that co-enveloped both HearMNPV and HearSNPV into the same ODV, in order to combine the desirable insecticidal characteristics of HearSNPV with the broader host range of HearMNPV.

2. Experimental methods

2.1 Insect rearing, *H. armigera* NPV strains and cell line

H. armigera, *Spodoptera frugiperda* and *Mamestra brassicae* larvae were obtained from laboratory colonies reared at the Universidad Pública de Navarra (UPNA) (25±1ºC, 70±5% relative humidity and 16:8 h day:night photoperiod) on a semi-synthetic diet (Greene et al., 1976). The *H. armigera* population was

established with pupae received from a laboratory colony maintained in the Universidad Politécnica de Cartagena. The *S. frugiperda* population was initiated with pupae from a laboratory population maintained in Honduras and refresh periodically with pupae from southern Mexico, and the *M. brassicae* colony was originally established from pupae kindly provided by the Centre for Ecology and Hydrology, Oxford.

The *H. armigera* single nucleopolyhedrovirus (HearSNPV) used in this study was an isolate from the Iberian Peninsula selected due to its high pathogenicity (Arrizubieta et al., 2014), and was originally isolated from *H. armigera* larvae collected from tomato crops in Guadajira (Badajoz, Spain) which showed clear symptoms of baculovirus infection (HearSNPV-SP1) (Figueiredo et al., 1999). In contrast HearMNPV, originated from the former USSR (Williams and Payne, 1984) was kindly provided by D. Winstanley (Horticulture Research International, Wellesbourne, Warwick, UK). Amplification of these two viruses was performed by feeding forth instar (L_4) larvae of *H. armigera* with artificial diet contaminated with OBs of the corresponding virus. OBs extraction and purification was performed as described by Arrizubieta et al. (2014).

The HzAM1 cell line (Mclntosh and Ignoffo, 1981) was kindly provided by R.D. Possee (Centre for Ecology and Hydrology, Wallingford, UK) and maintained at 28±2ºC using TC100 medium (Lonza, Washington, USA) with 10% fetal bovine serum (FBS) (Lonza).

2.2 Biological activity of HearSNPV and HearMNPV OBs against *H. armigera* larvae

To design the co-occlusion strategy, firstly the concentration-mortality response for HearSNPV and HearMNPV was estimated by conducting insect per os bioassays to determine the 50% lethal concentration (LC_{50}), mean time to death (MTD) and OB productivity (OBs/larva). Groups of 30 recently molted second instars were starved for 12 h and allowed to drink OB suspensions containing 10% (w/v) sucrose, 0.05% (w/v) Fluorella Blue food dye and OBs (Hughes and Wood, 1981) in the concentrations that caused between 95% and 5% mortality; 5.7×10^5, 1.9×10^5, 6.3×10^4, 2.1×10^4 and 7.0×10^3 for HearSNPV, and 1.7×10^6, 5.7×10^5, 1.9×10^5, 6.3×10^4 and 2.1×10^4 for HearMNPV. Larvae that ingested the suspension in a 10 min period were individually transferred to 24-

well plates with semi-synthetic diet. Control larvae were allowed to drink a sucrose and food dye solution without OBs. Larvae were incubated at 25±1ºC and 70±5% relative humidity until death or pupation. Virus mortality was recorded every 24 h during 10 days. The experiment was performed in triplicate. Concentration-mortality data were subjected to Probit analysis using the POLO-Plus program (Le Ora Software, 1987).

Mean time to death and OB production were determined using the LC_{90} calculated in the pathogenicity bioassays, namely: 1.7 x 10^5 and 3.0 x 10^6 OBs/ml for HearSNPV and HearMNPV, respectively, which resulted in mortalities of 93% and 87%. Mortality was recorded every 8 h. Individuals showing terminal disease symptoms were transferred to microtubes and incubated at 25±1ºC until death. Time-mortality results of individuals that died due to lethal polyhedrosis disease were subjected to Weibull analysis, and the validity of the Weibull model was determined using the Kaplan-Meier survival analysis using GLIM program (Crawley, 1993).

Finally, for OB counting, infected cadavers from the mean time to death assay were thawed, individually homogenized in 1 ml of distilled water and OBs were counted in triplicate using a Neubauer improved hemocytometer. OB production values were normalized by log transformation and subjected to analysis of variance (ANOVA) using the SPSS 21.0 program.

2.3 Superinfection of *H. armigera* larvae with HearMNPV and HearSNPV at different time intervals

In order to obtain an OB sample that contained approximately 50% of HearSNPV and 50% of HearMNPV, 500 *H. armigera* fifth instar were firstly orally inoculated with HearMNPV, using the LC_{90} (2.5 x 10^8 OBs/ml) calculated in preliminary bioassays. Thereafter at 0, 12, 24, 48 and 72 hours post-infection (named T0, T12, T24, T48 and T72, respectively) 100 larvae were orally inoculated with HearSNPV using the LC_{90} of HearSP1 (2.5 x 10^7 OBs/ml) (Arrizubieta et al., 2014). As controls, 25 larvae were firstly fed with the same suspension but containing no OBs and 0, 12, 24, 48 and 72 h later were treated with the corresponding suspension of the LC_{90} of one of the two baculoviruses (HearMNPV or HearSNPV) involved in superinfection. Larvae were individually transferred to 12-well plates with semi-synthetic diet and incubated at 25±1ºC and

70±5% relative humidity until death. Cadavers were collected daily and OBs were purified as described previously (Arrizubieta et al., 2014).

2.4 Relative frequencies of HearMNPV and HearSNPV genomes in OB samples

The relative frequencies of HearMNPV and HearSNPV in the OB samples obtained after superinfection assays were determined by quantitative PCR (qPCR) using specific primers for HearSNPV and HearMNPV isolates. For HearSNPV, primers were designed in the unique *ha29* gene (Arrizubieta et al., 2015; Guo et al., 2005); ha29.1 (5´-CTCGTATCATGCAAAACGCC-3´; nucleotides 25,382 to 25,401 in the HearG4 genome, GenBank accession number AF271059) and ha29.2 (5´-GAATCTGGCTTCGACTGGC-3´; nucleotides 25,443 to 25,461), while for HearMNPV primers targeted the ORF63 which was not present in the HearSNPV genome (Arrizubieta et al., 2015; Tang et al., 2012) and which codes for the *nrk1* (*nicotinamide riboside kinase 1*) gene; MNPV.1 (5´-CGTCGACACTCCCAACTGG-3´; nucleotides 58,791 to 58,809 in the HearMNPV genome, GenBank accession number EU730893) and MNPV.2 (5´-CGTTGGACACATGCTGCTG-3´; nucleotides 58,851 to 58,869).

Genomic DNA was extracted from samples of 10^7 OBs by incubating with 100 µl of 0.5 M Na_2CO_3, 50 µl of 10% SDS and 250 µl of distilled water at 60ºC during 10 min and then pelleted at 6,000 x *g* for 5 min. Supernatants were incubated with 25 µl proteinase K (20 mg/ml) at 50ºC during 1 h. Viral DNA was extracted twice with 500 µl of phenol (pH 7.8) and once with 500 µl of chloroform. Finally, DNA was precipitated with 10% (v/v) 3 M sodium acetate (pH 5.2) and 2.5 volumes of ice-cold 96% ethanol at 12,000 x g for 10 min and washed with 70% ice-cold ethanol. DNA was resuspended in 50 µl 0.1% TE (10 mM Tris, 1 mM EDTA) and kept at 4ºC until use.

All reactions were performed using 5 µl SsoAdvanced SYBR Green Supermix (Bio-Rad, Berkeley, California, USA), forward and reverse primers at 0.2 µM final concentration, and 1 µl of template DNA in a total reaction volume of 10 µl. Non-template controls (NTCs), standard curves (30 to 1.9 x 10^{-3} ng/µl of serial 5-fold dilutions of template DNA of HearSNPV genotype for ha29.1 and ha29.2 primers, and of HearMNPV for MNPV.1 and MNPV.2 primers), and samples were analyzed in duplicate. All qPCR reactions were performed in a CFX96 Touch Real-

Time PCR Detection System (Bio-Rad). The program used was: 2 min 30 sec at 95ºC; 45 cycles of 98ºC for 15 sec and 60ºC for 30 sec; followed by a melting curve (60ºC-95ºC). Data were analyzed using Bio-Rad CFX Manager software (Bio-Rad).

2.5 Co-envelopment of HearSNPV and HearMNPV within the same ODV

First, the presence of HearSNPV within multiple HearMNPV ODVs was determined by PCR using as template the ODV bands extracted from sucrose gradients. For this, ODVs were released from 10^9 OBs of superinfection samples at different time intervals (T0, T12, T24, T48 and T72) by incubating with 0.1 M Na_2CO_3 for 30 min at 28ºC. Debris was removed by low speed centrifugation (2,500 x *g*) during 5 min. The ODV containing supernanants were separated by density equilibrium centrifugation at 30,000 x *g* during 1 h on 30-70% (w/w) continuous sucrose gradient. The banding patterns were visually inspected and photographed. The upper band, which comprised ODVs with single nucleocapsid, was extracted by puncturing the tube at the height of the band with a syringe needle and transferred to a 2 ml sterile tube. In addition, the two or three lower bands, comprising ODVs with multiple nucleocapsids, were extracted separately. A 100 µl volume of each ODV sample was treated with 3 µl proteinase K (20 mg/ml) at 50ºC during 30 min and boiled at 100ºC during 10 min, and used as template in PCR amplification using Taq DNA polymerase (Bioline, London, UK) and specific primers for the HearSNPV or HearMNPV genomes. Specific primers for HearSNPV were also designed in the unique *ha29* gene (Arrizubieta et al., 2015; Guo et al., 2005); ha29.3 (5´-ATCGCACCATACCATGTATC-3´; nucleotides 25,251 to 25,270 in the HearG4 genome) and ha29.4 (5´-ATATCGCGATAACTAGTGGC-3´; nucleotides 25,639 to 25,658), while for HearMNPV primers targeted the ORF2, that encodes the viral capsid associated protein, absent in HearSNPV (Arrizubieta et al., 2015; Tang et al., 2012); MNPV.3 (5´-GGTAAGAAAGATCCAGACG-3´; nucleotides 1,529 to 1,557 in the HearMNPV genome) and MNPV.4 (5´-CGTCCAAAATTGCTATTCTTG-3´; nucleotides 2,082 to 2,102). PCR products were separated by electrophoresis in 1% TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA) agarose gels containing 0.25 µg/ml ethidium bromide, at 80 V for 1 h and visualized on a GeneSnap (Syngene) UV-transiluminator. DNA fragment sizes were estimated by comparison to a standard

molecular weight marker (100 bp NIPPON Genetics, Europe GmbH). Relative proportions of HearSNPV and HearMNPV in the multiple ODV fractions were also determined by qPCR analysis as described above.

Second, co-envelopment of HearSNPV and HearMNPV genomes within the same ODV was verified by end point dilution assays (Beperet, 2014a; Clavijo et al., 2010). ODVs from 10^8 OBs of T12 and T24 samples were released by incubation with 0.1 M Na_2CO_3 for 30 min at 28ºC, then centrifuged at 2,500 x g during 5 min, and ODVs containing supernatants were serially diluted by a factor of 10 (10^{-1} to 10^{-5}) in TC100 medium (Lonza) supplemented with 1% antibiotics (penicillin, streptomycin) (Lonza). 100 µl of each dilution were mixed with 900 µl of 2 x 10^5 HzAM1 cells/ml. A 100 µl volume of each virus-cell suspension was added to each well of a 96 well plate, leaving the last well as a negative control, with cells but without virus. The assay was replicated three times. Plates were incubated at 28ºC. After 7 days all wells were examined to determine the presence of infected cells with OBs in the nucleus. Infected wells resulting from plates with less than 15% of infection were extracted individually using a sterile Pasteur pipette and used as template in PCR and qPCR amplifications as described above, after treatment with 5 µl proteinase K (20 mg/ml) at 50ºC during 30 min followed by 10 min at 100ºC.

2.6 Biological activity of co-enveloped mixed virus OBs against *H. armigera*, *S. frugiperda* and *M. brassicae*

The biological activity of the co-enveloped mixtures obtained after inoculation with HearMNPV and HearSNPV at intervals of 12 and 24 hours (T12 and T24 samples) was determined in terms of pathogenicity (LC_{50}) against three different insect species: *H. armigera*, *S. frugiperda* and *M. brassicae*. All these species are susceptible to infection by HearMNPV, whereas HearSNPV is only infective for *H. armigera*. Pure HearMNPV and HearSNPV, and the OB mixture in a 1:1 proportion of both isolates (50% HearSNPV + 50% HearMNPV) were included as reference treatments.

The LC_{50} values were estimated using five different OB concentrations as described above. OB concentrations used to treat *H. armigera* larvae were 5.7 x 10^5, 1.9 x 10^5, 6.3 x 10^4, 2.1 x 10^4 and 7.0 x 10^3 OBs/ml for most viral inocula, HearSNPV, T12, T24 and the mixture 50% HearSNPV + 50% HearMNPV, while

for larvae treated with HearMNPV the following concentrations were used: 1.7 x 10^6, 5.7 x 10^5, 1.9 x 10^5, 6.3 x 10^4 and 2.1 x 10^4 OBs/ml. In the case of *S. frugiperda*, OB concentrations used for viral inocula HearSNPV, T12, T24, and the mixture 50% HearSNPV + 50% HearMNPV, were 1.5 x 10^9, 3.1 x 10^8, 6.2 x 10^7, 1.2 x 10^7 and 2.5 x 10^6 OBs/ml and for HearMNPV were 3.1 x 10^8, 6.2 x 10^7, 1.2 x 10^7, 2.5 x 10^6 and 5.0 x 10^5 OBs/ml. Finally, in *M. brassicae* the same range of concentrations, 3.1 x 10^6, 6.2 x 10^5, 1.2 x 10^5, 2.5 x 10^4 and 5.0 x 10^3 OBs/ml, was used in all treatments. The bioassays were performed in three occasions. Interactions between both viruses in T12, T24 and the 50% HearSNPV + 50% HearMNPV mixture treatments were evaluated using the method previously described by Tabashnik (1992). In this method, the expected LC_{50} value of the mixture (LC_{50m}) is given by the relative proportions ($r_{HearSNPV}$, $r_{HearMNPV}$) of HearSNPV and HearMNPV and the LC_{50} values of each component following the equation $LC_{50m}=[r_{HearSNPV}/LC_{50HearSNPV}+r_{HearMNPV}/LC_{50HearMNPV}]^{-1}$ (Tabashnik, 1992). Hence, a lower LC_{50m} value than that predicted by Tabashnik formula would mean a synergistic interaction, whereas higher value indicates an antagonistic interaction.

2.7 Mixture stability during successive passage *in vivo*

Larvae that died after infection with the highest concentration of T12, T24 and the 50% HearSNPV + 50% HearMNPV mixture used in pathogenicity bioassays were collected in groups. These OBs, representing the total production of the infected larvae, were considered passage one (P_1) and were used as inoculum to infect the subsequent group of larvae (24 second instars for each passage). The virus populations were followed for four additional passages (P_2, P_3, P_4 and P_5). Additionally, as controls, groups of 24 second instar *H. armigera*, *S. frugiperda* and *M. brassicae* were inoculated with the superinfection mixtures composed mostly by HearSNPV (T0) and HearMNPV (T48 and T72) with the LC_{90} calculated in preliminary bioassays. Larvae of *H. armigera* were inoculated with 5 x 10^5, 2 x 10^6 and 2 x 10^6 OBs/ml of T0, T48 and T72 samples, respectively. In *S. frugiperda*, larvae were inoculated with 2 x 10^9 OBs/ml of T0, or 3 x 10^8 OBs/ml for T48 and T72 samples. Finally, *M. brassicae* were inoculated with 2 x 10^9, 3 x 10^6 and 3 x 10^6 OBs/ml of T0, T48 and T72 samples, respectively. Cadavers were

collected in groups and the OBs were designated as passage one (P_1). The entire experiment was performed in triplicate.

DNA extracted from purified OBs at each successive passage was subjected to PCR and qPCR amplifications as described above, to determine the relative frequencies of HearSNPV and HearMNPV genomes in the samples.

3. Results

3.1 Insecticidal characteristics of HearSNPV vs. HearMNPV OBs

HearSNPV OBs (LC_{50}=2.8 x 10^4 OBs/ml), was ~6.4-fold more pathogenic than HearMNPV OBs (LC_{50}=1.8 x 10^5 OBs/ml) (Table 1). The *H. armigera* larvae treated with HearSNPV died on average at 130 hours post infection (hpi), while those treated with HearMNPV died ~13 hours later (Table 1). Furthermore, HearMNPV although being less virulent was almost two-fold less productive (6.1 x 10^7 OBs/larva) than HearSNPV (1.1 x 10^8 OBs/larva) (Tukey, P<0.05) (Fig 1).

Table 1: LC_{50} values, relative potencies and mean time to death (MTD) values of HearMNPV and HearSNPV against second instar *H. armigera* larvae.

	LC_{50}	Relative	95% Fiducial limits			95% Fiducial limits	
Virus	(OBs/ml)	Potency	Low	High	MTD (h)	Low	High
HearMNPV	1.8 x 10^5b	1	-	-	143.4b	140.4	146.5
HearSNPV	2.9 x 10^4a	6.3	4.0	10.3	130.0a	127.3	132.7

Probit regressions were fitted in POLO-Plus. A test for non-parallelism was significant (χ^2=5.94, df=1, P=0.015). Relative potencies were calculated as the ratio of LC_{50} values relative to the HearMNPV. Mean time to death (MTD) values were estimated by Weibull analysis (Crawley, 1993). Values followed by different letters are significantly different (t-test, P<0.05).

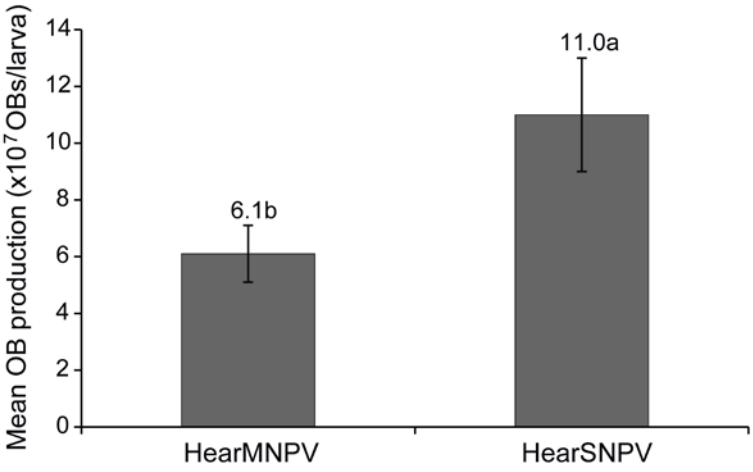

Figure 1: Median OB production (x 10^7 OBs/larva) in *H. armigera* second instars infected with the LC_{90} of HearMNPV and HearSP1. Vertical lines indicate standard error. Values followed by different letters differed significantly by ANOVA and Tukey test (P<0.05).

3.2 Relative frequencies of HearSNPV and HearMNPV in experimental mixtures

qPCR analysis indicated that when *H. armigera* larvae were inoculated at the same moment with HearMNPV and HearSNPV (T0), HearSNPV was the most frequent virus (96.8%) in OBs produced in co-infected larvae (Fig. 2). However, the frequencies of both viruses in OBs were similar when HearSNPV OBs were ingested by the larvae 12 h (T12) (41.0% of HearSNPV:58.9% of HearMNPV) or 24 h (T24) (57.3% of HearSNPV:42.7% of HearMNPV) later than HearMNPV OBs (Fig. 2). By contrast, HearMNPV was the major component in the OBs produced in larvae fed with HearSNPV OBs 48 h (T48) (96.8%) or 72 h (T72) (95.6%) hours later (Fig. 2).

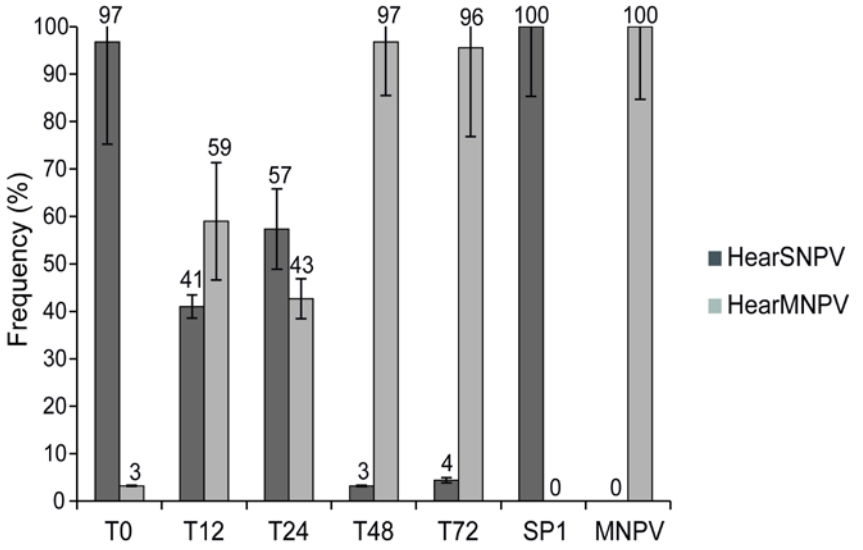

Figure 2: Relative frequencies of HearSNPV and HearMNPV determined by qPCR in mixtures obtained after infection with the LC_{90} of HearMNPV and HearSNPV at 0 h (T0), 12 h (T12), 24 h (T24), 48 h (T48) and 72 h (T72) time intervals. Vertical lines indicate standard error.

3.3 HearSNPV and HearMNPV genomes are co-enveloped within the same ODV

OBs recovered from treated larvae that ingested both viruses (HearSNPV and HearMNPV) simultaneously consisted mainly of virions (ODVs) containing a single nucleocapsid as they generated a single band when subjected to centrifugation through a continuous sucrose gradient (Fig. 3). Other bands were not seen on the sucrose gradient. However, by qPCR was possible to detect the presence at low frequency of multiple ODVs (Fig. 2). OB samples obtained from larvae inoculated first with HearMNPV and 12, 24, 48 and 72 h later with HearSNPV (T12, T24, T48 and T72 samples) were composed of ODVs with multiple nucleocapsids, as multiple bands were visible (Fig. 3), but in all the cases the single nucleocapsid ODVs were also detected as the clearest and most dense band (Fig. 3).

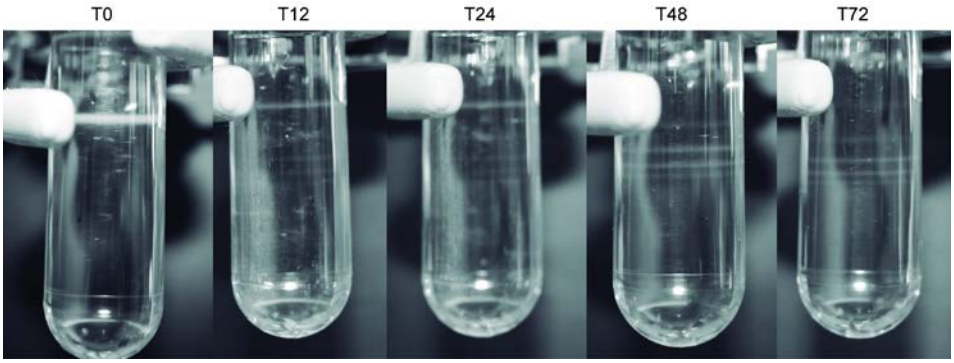

Figure 3: ODV banding patterns of T0, T12, T24, T48 and T72 OB samples after continuous sucrose gradient separation of ODVs obtained from 10^9 OBs.

When analyzing the composition of the different ODV bands from each sample, the presence of HearSNPV in multiple ODVs in T12 and T48 samples was demonstrated (Fig. 4A). In T0 OB samples, PCR analysis of the unique band visible revealed the presence of both viruses (Fig. 4A and 4B), although HearSNPV was clearly the most abundant (99.8%) (Table 2). In OB samples corresponding to T12 and T24, the top band of the continuous sucrose gradient was composed of ODVs corresponding to both viruses (Fig. 4A and 4B) but the majority (97-98%) of these comprised HearSNPV (Table 2). Bands corresponding to ODVs with two or more nucleocapsids were also tested and in all of them the presence of the HearSNPV virus genome was detected (Fig. 4A), although its relative proportion was low in both samples T12 (0.47%) and T24 (0.88%) (Table 2). These results indicate that a single nucleopolyhedrovirus was present within multiple nucleocapsid ODVs, suggesting its co-occlusion with a multiple nucleocapsid virus. Also, although the proportion of both viruses in the OB samples was almost 50%, most of the HearSNPV genomes (~99%) were enveloped as single ODVs. Finally, in the upper band of T48 and T72 samples, which corresponded to single nucleocapsid ODVs, both viruses were detected at similar frequencies, 55.8% of HearSNPV:44.2% of HearMNPV and 47.7% of HearSNPV:52.3% of HearMNPV, respectively (Fig. 4A and 4B) (Table 2). However, the lower bands, representing multiple ODVs only comprised HearMNPV genomes (100%) (Fig. 4A and 4B, Table 2).

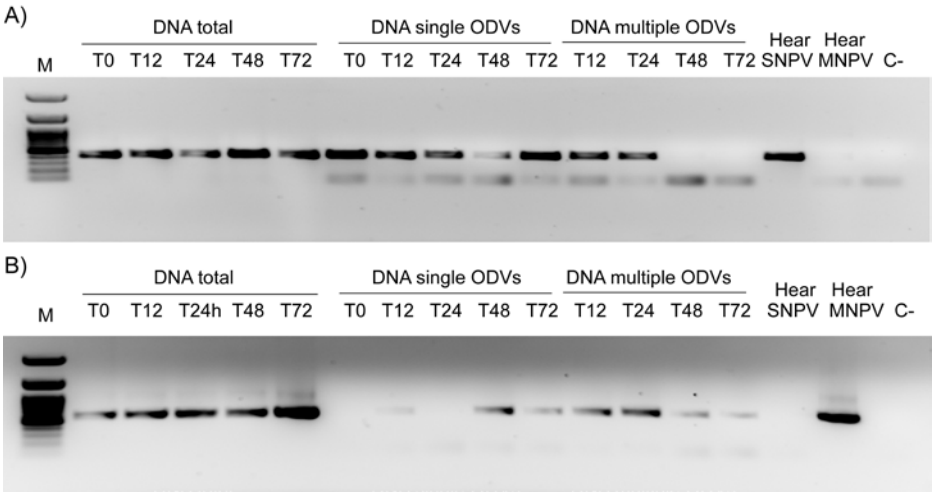

Figure 4: PCR amplifications using A) HearSNPV specific primers and B) HearMNPV specific primer on total DNA and DNA extracted from ODVs of the upper band comprising a single nucleocapsid (single ODVs) and ODVs of lower bands with multiple nucleocapsids (multiple ODVs) after continuous sucrose gradient separation of ODVs obtained from 10^9 OBs of T0, T12, T24, T48 and T72 samples.

Table 2: Relative frequencies of HearSNPV and HearMNPV in single and multiple ODVs obtained from OBs of T0, T12, T24, T48, T72 samples determined by qPCR using virus-specific primers.

	Single ODVs		Multiple ODVs	
	HearSNPV	HearMNPV	HearSNPV	HearMNPV
T0	99.78%	0.22%	-	-
T12	97.09%	2.91%	0.47%	99.53%
T24	98.73%	1.27%	0.88%	99.12%
T48	55.77%	44.23%	0.00%	100.00%
T72	47.73%	52.27%	0.00%	100.00%

Finally, co-envelopment of HearSNPV and HearMNPV in the same ODV was examined by end point dilution assay of the ODVs from T12 and T24 samples. In both cases, a dilution of 10^{-3} resulted in approximately 90% of uninfected wells, which reflected the situation in which 10% of the wells are infected by a single ODV and less than 1% by two or more ODVs (Table 3). From a total of 27 wells analyzed in T12 samples, 12 comprised HearSNPV alone (44%) and 15 comprised a mixture of both isolates (56%) (Fig. 5). Similarly from the 31 wells analyzed in T24 sample, 18 comprised HearSNPV alone (58%) and 13 comprised a mixture of both isolates (42%) (Fig. 5). However, in those cells where

both viruses were detected, 88.4±18.8 and 69.4±39.3 of the viral DNA was HearSNPV in T12 and T24 samples, respectively, although only 0.47-0.88% of the genomes found within multiple ODVs comprised HearMNPV. These results indicate that HearSNPV could be co-enveloped within HearMNPV ODVs, although at low proportions.

Table 3. Probabilities of infection by 0 (Prob. (0)), 1 (Prob. (1)), 2 (Prob. (2)) or 3 (Prob. (3)) occlusion derived virions in each well following end point dilution assay.

	T12			T24		
	Repetition 1	Repetition 2	Repetition 3	Repetition 1	Repetition 2	Repetition 3
Positive/Total	11/88	8/88	8/88	7/88	11/88	13/88
Prob. (0)	0.875	0.909	0.909	0.920	0.875	0.852
Prob. (1)	0.117	0.0866	0.0866	0.0763	0.117	0.136
Prob. (2)	0.00780	0.00413	0.00413	0.00316	0.00780	0.0109
Prob. (3)	0.000347	0.000131	0.000131	0.0000874	0.000347	0.000580

Positive wells were those contained at least one cell with pathological signs of baculovirus infection (OBs in the nucleus). Prob. (0) is the calculated probability in each repetition of having a well without any signs of infection. Prob. (1-3) refer to the Poisson distribution probability of the cells in a particular well being infected by one, two or three ODVs, respectively.

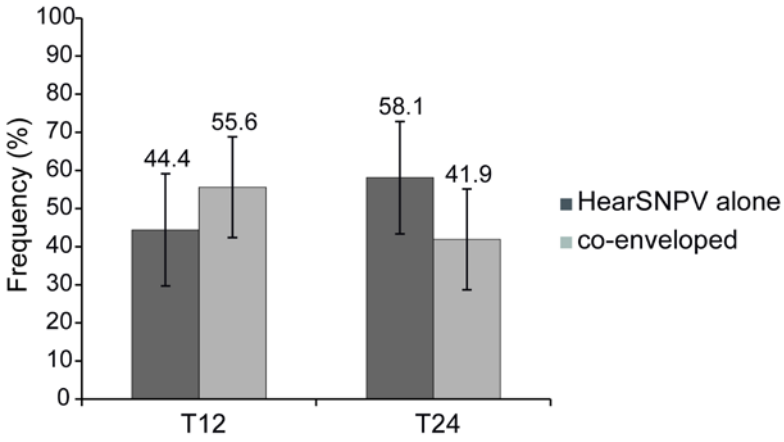

Figure 5: Frequency of HearSNPV and HearMNPV determined by PCR using virus-specific primers in the 27 and 31 wells that resulted infected after infection with a 10^{-3} dilution of the ODVs released from the T12 and T24 OBs.

3.4 Biological activity of co-occluded mixtures against *H. armigera*, *S. frugiperda* and *M. brassicae*

Bioassays were performed with the mixtures that comprised 50% of each isolate (T12 and T24 samples) with the aim of detecting any advantage compared with each virus alone with respect to their insecticidal properties including pathogenicity, virulence, productivity, and host range. For this, bioassays were performed with three different host species: *H. armigera* is more susceptible to HearSNPV than to HearMNPV, whereas *S. frugiperda* and *M. brassicae* are only permissive to HearMNPV (Gettig and McCarthy, 1982; Gröner, 1986; Rovesti et al., 2000; Tompkins et al., 1988).

For *H. armigera* larvae, the LC_{50} values of the OBs from T12 (2.2×10^4 OBs/ml), T24 (3.5×10^4 OBs/ml), and the mixture 50% HearSNPV + 50% HearMNPV (3.0×10^4 OBs/ml) showed no significant differences from the LC_{50} value of HearSNPV OBs (1.5×10^4 OBs/ml); however, all were 3-5 times significantly more pathogenic than HearMNPV OBs (1.0×10^5 OBs/ml) (Table 4). Interestingly, when larvae of *S. frugiperda* and *M. brassicae* ingested co-occluded mixtures of viruses, from T12 and T24, or the mixture of OBs from 50% HearSNPV + 50% HearMNPV, HearSNPV was able to infect and replicate in these host species, which are non-permissive to HearSNPV OBs alone. This indicates that the host range of HearSNPV was expanded in the presence of HearMNPV. The LC_{50} values of mixed OBs, from T12 and T24, or the co-occluded mixture 50% HearSNPV + 50% HearMNPV, were similar to one another, both in the case of *S. frugiperda* larvae ($3.6\text{-}4.2 \times 10^7$ OBs/ml) and in the case of *M. brassicae* ($3.9\text{-}5.5 \times 10^5$ OBs/ml). However, these LC_{50} values were in each case about 2-fold lower than the LC_{50} values of HearMNPV in *S. frugiperda* (2.0×10^7 OBs/ml) and *M. brassicae* (2.0×10^5 OBs/ml) (Table 4).

Table 4: LC_{50} values and relative potencies of HearMNPV, HearSNPV, T12 and T24 co-enveloped mixtures and the OB mixture of HearSNPV and HearMNPV at 1:1 proportion (50% HearSNPV + 50% HearMNPV) against second instar *H. armigera, S. frugiperda* and *M. brassicae* larvae. The expected $LC_{50\,m}$ values were calculated using the Tabashnik method (Tabashnik, 1992).

Insect	Virus	LC_{50} (OBs/ml)	Relative potency	95% Fiducial limits		Expected LC_{50m}
				Low	High	
H. armigera	HearMNPV	1.0×10^5	1	-	-	-
	HearSNPV	1.5×10^4	6.6	2.5	17.2	-
	T12	2.2×10^4	4.5	1.9	10.8	3.1×10^4
	T24	3.5×10^4	2.9	1.5	5.9	2.4×10^4
	50% HearSNPV + 50% HearMNPV	3.0×10^4	3.4	1.6	7.1	2.7×10^4
S. frugiperda	HearMNPV	2.0×10^7	1	-	-	-
	HearSNPV	-	-	-	-	-
	T12	3.8×10^7	0.5	0.3	0.9	3.4×10^7
	T24	3.6×10^7	0.6	0.3	0.9	4.7×10^7
	50% HearSNPV + 50% HearMNPV	4.2×10^7	0.5	0.3	0.8	4.0×10^7
M. brassicae	HearMNPV	2.0×10^5	1	-	-	-
	HearSNPV	-	-	-	-	-
	T12	3.9×10^5	0.5	0.3	0.9	3.3×10^5
	T24	4.1×10^5	0.5	0.2	0.9	4.6×10^5
	50% HearSNPV + 50% HearMNPV	5.5×10^5	0.4	0.2	0.7	3.9×10^5

Probit regressions were fitted in POLO-Plus. Relative potencies were calculated as the ratio of LC_{50} values relative to the HearMNPV.

The expected LC_{50} values of these mixtures against *H. armigera*, *S. frugiperda* and *M. brassicae* larvae calculated according to Tabashnik method (Table 4) were clearly within the 95% fiducial limits of each of these LC_{50} values, indicating the absence of synergistic or antagonistic interactions between HearSNPV and HearMNPV in the mixtures against the different insect species.

3.5 Mixtures stability throughout successive passages *in vivo*

The stability of the mixture of viruses co-occluded in OBs from T12 and T24 and the mixture OBs 50% HearSNPV + 50% HearMNPV was determined by

measuring the relative proportions of the viruses (HearSNPV and HearMNPV) in different experimental populations that were subjected to five successive passages *in vivo*. These experiments were performed in parallel in *H. armigera*, *S. frugiperda*, and *M. brassicae* larvae which showed different degrees of susceptibility to both viruses.

As expected, the relative proportions of HearSNPV increased rapidly after a single pass (P_1) in larvae of *H. armigera* in both mixed OBs originally obtained in T12 (from 41.0% to 99.9%) and T24 (from 57.3% to 96.5%) and the mixture 50% HearSNPV + 50% HearMNPV (from 50% to 97.8%) (Fig. 6A). The same pattern was observed in populations of OBs from T48 and T72, in which the relative proportion of HearSNPV was clearly in a minority, at 3.2 and 4.4%, respectively. In the progeny of these OBs, after one passage in *H. armigera* larvae, the relative proportion of HearSNPV increased markedly from 3.2 to 63.9% and those from T72 increased similarly from 4.4 to 88.4% (Fig. 6A). The presence of HearMNPV was not detected in populations of OBs generated in the following successive passages (P_2-P_5) suggesting that HearMNPV was completely displaced by HearSNPV (Fig. 6A). In the T0 sample the frequency of HearSNPV was maintained at 98.9% (Fig. 6A).

In contrast, the relative proportion of HearSNPV decreased after one passage in larvae of *S. frugiperda* or *M. brassicae*, both in the descendants of the OBs from T12 (from 41% to less than 0.01%) and the OBs from T24 (from 57.3% to less than 0.01%). This decrease was even more pronounced in the T0 mixture in which the relative proportion of HearSNPV decreased from 96.7% to 0.00008% in *S. frugiperda*, and to 0.00084% in *M. brassicae* (Fig 6B and 6C). Although a very marked decrease in HearSNPV proportion was observed in the mixtures throughout passages in *S. frugiperda* and *M. brassicae* larvae, HearSNPV was maintained at detectable levels in the OB progeny at least during five passages in T12, T24 and the 50% HearSNPV + 50% HearMNPV mixtures (Fig. 6B and 6C). In T48 and T72 the decrease was not less dramatic due to the low proportion of HearSNPV present at P0 (Fig 6B and 6C).

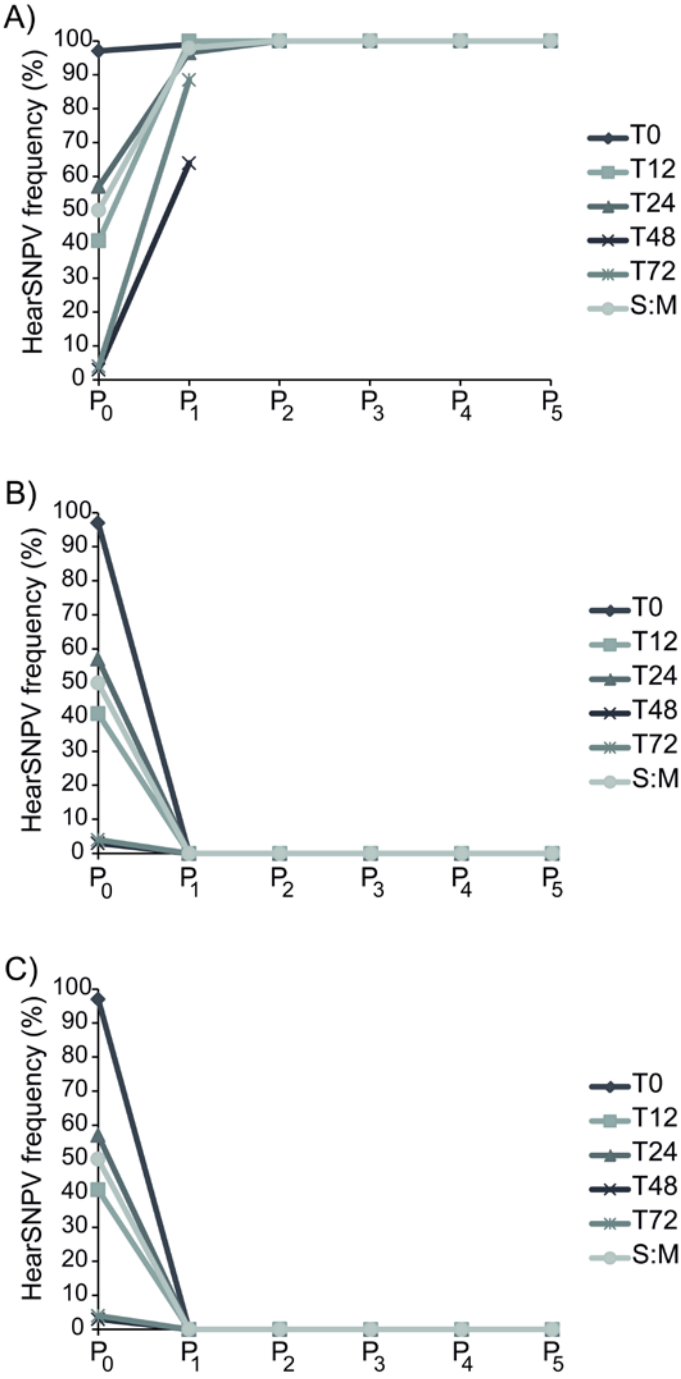

Figure 6: Relative frequencies of HearSNPV quantified by qPCR in OB progeny after *per os* serial passages (P_0-P_5) of T0, T12, T24, T48, T72 and 50% HearSNPV + 50% HearMNPV OB samples in A) *H. armigera*, B) *S. frugiperda* and C) *M. brassicae* larvae.

4. Discussion

This study demonstrates for the first time that by coinfecting *H. armigera* larvae with a single (HearSNPV) and a multiple (HearMNPV) NPV, under certain conditions, it is possible to obtain ODVs comprising co-enveloped genomes of both viruses. This naturally results in the co-occlusion of both viruses in mixed-virus OBs. The formation of mixed-virus ODVs and mixed-virus OBs requires that both viruses infect and replicate in the same host cell nucleus. Previous studies have shown that a single host cell can be infected at least by two different genotypes of a multiple-nucleocapsid alphabaculovirus (Clavijo et al, 2010). Furthermore, it has been shown that a single insect cell can be co-infected with at least two genotypes from different multiple-nucleocapsid alphabaculovirus species, provided that co-infection occurs within a given time window (Beperet, 2014b). It is foreseeable that this time window may vary depending on the insect and the viruses used in the co-infection. For example, when *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) are used to co-infect Sf9 cells, the second virus infection is only possible within 20 h following infection was initiated by the first virus. After this time an absolute block prevents productive co-infection by the second virus (Beperet, 2014b). These authors also observed that in *S. frugiperda* larvae co-infections occurred with greater frequency during the temporal window of co-infection, but after this time superinfection by a second virus was still possible, albeit at a reduced prevalence (Beperet, 2014b). We observed similar results when larvae of *H. armigera* were first infected by the less pathogenic and less virulent HearMNPV, no exclusion to subsequent superinfection by HearSNPV was observed. Our results shown that when *H. armigera* larvae were simultaneously co-infected with both alphabaculoviruses, the single type (HearSNPV) and the less pathogenic and less virulent multiple type (HearMNPV), progeny OBs contained mostly HearSNPV genomes (97%), probably due to the higher capacity for replication of HearSNPV in *H. armigera*. However, the OBs recovered from virus-killed larvae of *H. armigera* which were first inoculated with HearMNPV and then superinfected with HearSNPV at 48 or 72 h later, mostly contained HearMNPV genomes (96-97%). This suggests that the period of 48-72 h is sufficient for: 1) the HearMNPV to infect most susceptible

insect cells, and 2) the infected cells are not receptive to infection by a second virus (HearSNPV). The limited presence of HearSNPV genomes observed in OBs from infected larvae may be due to the ability of this virus to infect cells that had escaped the first HearMNPV infection. Another possibility is that a fraction of the HearMNPV infected cells were within the temporal window of susceptibility to superinfection by HearSNPV. This partial superinfection exclusion was previously observed in *S. frugiperda* larvae that were first inoculated with SfMNPV OBs and subsequently inoculated with AcMNPV OBs (Beperet, 2014b). However, when *S. frugiperda* larvae were initially inoculated with the less pathogenic virus, AcMNPV, and subsequently inoculated with the homologous virus, SfMNPV, total superinfection exclusion was observed (Beperet, 2014b). These differences might be specific to the host-pathogen system used. Therefore each host-pathogen system might require separate study when constructing different OB mixtures; at this point in time it is not possible to draw firm conclusions due to the novelty of these observations and the paucity of information on baculovirus superinfection dynamics from other baculovirus pathosystems.

In contrast, when HearSNPV was inoculated 12 or 24 hours later than HearMNPV, the proportions of both viruses in OBs obtained from co-infected larvae were similar, suggesting that this time interval could offset the replication disadvantage of HearMNPV in *H. armigera*. The ODVs released from these OBs resolved into several bands by ultracentrifugation, each corresponding to ODVs with a specific number of nucleocapsids. HearSNPV was detected by PCR in multiple ODV bands, suggesting that HearSNPV nucleocapsids were enveloped within HearMNPV ODVs. This was confirmed by end point dilution assays performed with serial dilutions of ODVs released from the OB samples. In cells infected by a single ODV both HearSNPV and HearMNPV were detected, demonstrating that nucleocapsids of HearSNPV and HearMNPV were enveloped in the same ODV. However, the frequency at which HearSNPV was co-enveloped was very low (<1%). The co-envelopment of different alphabaculovirus species has been demonstrated previously (Beperet, 2014a). However this is the first time that a SNPV has been shown to be co-enveloped with a MNPV in multiple-nucleocapsid ODVs. A higher proportion of ODVs containing both viruses were obtained when larvae were initially inoculated with HearMNPV and superinfected 12 hours later with HearSNPV, than with the 24 hour interval, for reasons that are

presently unclear. However, T12 and T24 samples comprised ~50% of HearSNPV genomes in of which ~1% was co-enveloped, indicating that most of the HearSNPV genomes were present as single-nucleocapsid ODVs and most of the multiple-nucleocapsid ODVs (99%) comprised HearMNPV genomes. This lower co-envelopment proportion might explain the absence of wells infected by HearMNPV alone, as HearMNPV would not be able to initiate the infection in cells due to its lower replication capacity in the HzAM1 cell line compared with HearSNPV. However, when both viruses are co-enveloped in the same ODV, HearSNPV could act as a helper virus facilitating the entry and replication of HearMNPV in cells, as previously observed with other alphabaculoviruses (Clavijo et al., 2010; López-Ferber et al., 2003), and also with pathogenic viruses in plants (Stenger, 1998) and mammals (Eckner, 1973).

The co-envelopment of HearSNPV and HearMNPV could potentially have effects on the pathogenicity of mixed virus OBs. However T12 and T24 OB mixtures were as pathogenic as the 50% HearSNPV + 50% HearMNPV OB mixture against the three host species. Both viruses acted independently, indicating that virions of the co-enveloped mixtures and the OB mixture were transmitted in a similar manner. Similar results were observed in *S. frugiperda* and *S. exigua* larvae after inoculation with co-enveloped ODVs and OB mixtures of AcMNPV and SfMNPV, as both viruses were transmitted independently, with no apparent interaction in terms of OB pathogenicity (Beperet, 2014b). In contrast, these mixtures showed a lower pathogenicity than HearSNPV against *H. armigera*, which is more permissive to HearSNPV. The mixtures were also slightly less pathogenic than HearMNPV against *S. frugiperda* and *M. brassicae* which are both more permissible to HearMNPV. This lower infectivity might be explained because T12 and T24 OB samples contained 50% of ODVs comprising single-nucleocapsid HearSNPV ODVs and the other 50% of multiple ODVs composed mainly by HearMNPV, as only ~1% of HearSNPV genomes were found in these multiple ODVs. These effects seem to due to the fact that these mixtures comprised 50% of HearSNPV and 50% of HearMNPV genomes, which reduced the presence of the more pathogenic virus by 50% in each case.

However, co-envelopment might have clear implications in insect control programs, as these mixtures increased the host range of HearSNPV and improved the phenotypic characteristics of HearMNPV. The mixtures were more effective

controlling all the species studied than either of the viruses alone, because the mixture extended the host range of HearSNPV and increased its biological activity against *S. frugiperda* and *M. brassicae*. This suggests that such mixtures could form the basis for products that could be used in pest control programs targeted at two or more insects in a given crop. For example, although *H. armigera* is the most important pest of tomato crops in Spain (Torres-Vila et al., 2003), *M. brassicae* is also present (Rojas et al., 2000). In addition, *M. brassicae* is the key pest of cabbage in Spain (Cartea et al., 2010), and *H. armigera* may also cause damage in this and other brassica crops (CABI, 2014). Moreover, *S. frugiperda* is an important pest in several crops in the American continent, such as maize, soybean, sorghum, cotton and rice (Casmuz et al., 2010) and recently, *H. armigera* has been introduced in this continent via Brazil in soybean and cotton crops (Czepak et al., 2013). In these cases, applications of mixtures including HearSNPV and HearMNPV might allow the simultaneous control of both pests.

The co-occluded virus mixtures were not stable and the relative proportion of each virus varied markedly depending on the host species in which the mixture was replicated. When mixtures were passaged in *H. armigera* larvae the prevalence of HearMNPV genomes decreased from ~50% to less than 4%, and then disappeared after two passages. Conversely, when mixtures were passaged in *S. frugiperda* and *M. brassicae*, the relative proportion of HearMNPV increased form ~50% to 99.9% in just one passage. However, in this case HearSNPV persisted at a similar frequency during at least five serial passages. It is unclear why HearMNPV, although infective to *H. armigera*, disappeared and HearSNPV, although usually infective to *S. frugiperda* and *M. brassicae*, managed to persist in the mixed virus population albeit at a low level. One possibility is that mixed virus populations might be more efficient at transmission in heterologous hosts. This issue remains to be resolved.

In conclusion, we have demonstrated for the first time that nucleocapsids from a single-nucleocapsid alphabaculovirus can be enveloped within multiple-nucleocapsid ODVs. In addition, the HearSNPV increased its host range when is co-enveloped with HearMNPV and persisted in progeny OB populations for at least after five serial passages in *S. frugiperda* and *M. brassicae* larvae, whereas when present in mixtures with HearSNPV, HearMNPV improved its pathogenicity towards *H. armigera*. The co-envelopment of HearSNPV and HearMNPV has

potential to broaden the narrow host range of HearSNPV, favoring the control of specific complexes of agricultural pests using unique mixtures of baculoviruses as the basis for novel biological insecticides.

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CHAPTER VII

Determinant factors in the production of a co-occluded binary mixture of *Helicoverpa armigera* alphabaculovirus (HearSNPV) genotypes with desirable insecticidal characteristics

Abstract

The effects of larval instar, density, inoculum concentration and temperature on the production of HearSP1B+HearLB6 were evaluated. The high prevalence of cannibalism indicated that insects should be reared individually. Larvae inoculated one day after molting to L_4 and L_5, and recently molted L_5 produced the greatest quantities of OBs (6.7-9.1 x 10^9 OBs/larva). However, the prevalence of mortality (85.7%) was higher in recently molted L_5 larvae than in those inoculated one day after molting (19.5%), resulting in a greater overall OB yield (6.0 x 10^{11} OBs/100 inoculated larvae). L_5 larvae inoculated with the LC_{80} produced similar OB yield to that inoculated with higher OB concentrations. Incubation temperature did not influence OB yield, although larvae maintained at 30ºC died 13 and 34 hours earlier than those incubated at 26ºC and 23ºC, respectively. The efficient production of HearSP1B+HearLB6 OBs in *H. armigera* larvae involves inoculation of recently molted L_5 with the LC_{80} followed by individual rearing at 30ºC.

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1. Introduction

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a mayor polyphagous insect pest in a wide range of crops in many parts of the world (Czepak et al., 2013; Fitt, 1989). Originally native to the Old World, this pest has been introduced to Asia and Oceania, and has recently invaded Brazil and Argentina (Murúa et al., 2014). Its recent appearance in Puerto Rico (http://www.pestalert.org/oprDetail.cfm?oprID=600) also represents a threat to the United States, Mexico, Central America and the Islands of the Caribbean.

Spain is a major producer of tomatoes for export to European countries and elsewhere, with an area of 59,300 hectares (ha) in production that yields over 4.3 million tonnes per year (MAGRAMA, 2013). In this region, *H. armigera* is the most important pest in field-grown tomato crops (Torres-Vila et al., 2002). Infestations of *H. armigera* are controlled by applying synthetic broad-spectrum insecticides and, more recently, using newer biorational products such as *Bacillus thuringiensis*. However, repeated use of these products has led to the appearance of insecticide-resistant pest biotypes (Torres-Vila et al., 2000), and the need for careful monitoring of pesticide residues in produce from this region in order to comply with European Union regulations (EFSA, 2014). This has highlighted the need to develop effective alternative control methods that are economically-viable and which do not contribute to the presence of xenobiotic residues in food produce (Chandler et al., 2011; Torres-Vila et al., 2000, 2002).

The *H. armigera* single nucleopolyhedrovirus (HearSNPV) (genus Alphabaculovirus, family Baculoviridae) has proved effective as the basis for biological insecticide products targeted at this pest (Zhang, 1994; Jones et al., 1998). In previous studies conducted in our laboratory, a binary mixture of HearSNPV genotypes, named HearSP1B+HearLB6, was co-occluded into virus occlusion bodies (OBs) in equal proportions using previously established co-infection techniques (Bernal et al., 2013). The binary mixture of genotypes had insecticidal properties that were greater than those of the component genotypes, and this was the subject of a patent application for the development of a virus-based biological insecticide (Arrizubieta et al., 2015; Caballero et al., 2014).

As baculoviruses are obligate pathogens, these viruses can only be produced in the larvae of susceptible species of insects (*in vivo*) or in cell culture

systems (*in vitro*). However, *in vitro* production is expensive (Inceoglu et al., 2001) and a number of technical issues limit its use as a method for the large-scale production of these viruses (Pijlman et al., 2004). For example, in cell culture virus populations rapidly accumulate defective particles with reduced insecticidal properties or reduced persistence on plant surfaces (Nguyen et al., 2011; Pedrini et al., 2006, 2011). For this reason, all current baculovirus-based bioinsecticides are produced using *in vivo* systems, involving the infection of large colonies of the host insect, or an alternative but susceptible host species. Given the limited host range of HearSNPV, which is only infective for *Helicoverpa* spp. (Ignoffo and Couch, 1981), the production of this virus is achieved using its homologous host, *H. armigera*.

In vivo production systems require optimization studies that take into account the biology and behavior of the host and the particular characteristics of each host-virus pathosystem. Factors such as quantity of inoculum, method and timing of inoculation, insect diet, rearing conditions, and virus harvesting have to be addressed. These issues are usually established first at the laboratory scale and then suitably modified and standardized for pilot-plant or industrial scale production (Shapiro et al., 1981; Shieh, 1989).

Like many species of Lepidoptera, *H. armigera* exhibit cannibalistic behavior during the larval stage (Dhandapani et al., 1993). Frequent cannibalism among larvae is undesirable in virus production systems since it adversely affects the quantity of occlusion bodies (OBs) produced in each container of larvae or, in extreme cases, means that larvae have to be individualized post-inoculation, resulting in increases in the costs of OB production. Factors such as larval density or larvae stage directly influence the prevalence of cannibalism behavior in virus-infected insects (Chapman et al., 1999; Elvira et al., 2010), even when there is no competition for food (Joyner and Gould, 1985). OB production is particularly affected by larval growth rate following inoculation, larval age at inoculation, inoculum concentration and incubation temperature (Ignoffo and Couch, 1981). Incubation temperature is relevant because it influences both the rate of growth of the infected host and the speed of kill of the virus (Johnson et al., 1982). As such, efficient OB production requires defining a balance between the conditions that result in maximum larval growth and a high incidence of virus-induced mortality in a short time period (Shieh, 1989; Subramanian et al., 2006). In the present study,

we examined how these factors could be established to improve the efficiency of production of HearSP1B:LB6 OBs in the homologous host, *H. armigera*.

2. Experimental methods

2.1 Insect rearing and virus strain

A laboratory colony of *H. armigera* was maintained in the Universidad Pública de Navarra (UPNA) at 25±2ºC, 70%-80% relative humidity and 16:8h day:night photoperiod on an artificial diet (Greene et al., 1976). The colony was established with pupae received from the Centre for Ecology and Hydrology (CEH), Oxford, United Kingdom.

The virus used in this study was the co-occluded binary genotypic mixture HearSP1B:LB6 (in a 1:1 ratio), which was developed in a previous study and which possessed insecticidal properties better than those of individual component genotypes, as well as any other of the genotypes or genotype mixtures tested (Arrizubieta et al., submitted).

2.2 Effects of larval stage and density on OB production

The effects of larval stage and density on OB production were evaluated in the final three larval instars, L_3, L_4 and L_5, at rearing densities of 5, 10 and 20 larvae in 500 ml transparent plastic tubs (10 cm diameter at the base x 4 cm height) with a cardboard lid. Each tub contained a layer of artificial diet, 0.5 cm in depth at the bottom of the tub (Greene et al., 1976). In addition, 5 larvae of each stage were incubated individually in 30 ml transparent plastic cups (2.7 cm diameter at the base x 4.2 cm height), with a plastic cap. Newly molted larvae that had been starved for 12 hours were used. Larvae were inoculated with an OB suspension containing 100 mg/ml sucrose, 0.05 mg/ml Fluorella Blue food dye and the corresponding 90% lethal concentration (LC_{90}) of OBs: 6.1×10^5, 2.4×10^6 and 2.5×10^7 OBs/ml for L_3, L_4 and L_5, respectively (Arrizubieta et al., 2014) using the droplet-feeding method (Hughes and Wood, 1981). As controls, identical numbers of larvae were inoculated with food dye and sucrose solution, without OBs. Larvae that drank the suspension in a 10 min period were transferred to the corresponding 500 ml plastic tubs for treatments involving densities of 5, 10 and 20 larvae/tub or 30 ml cups for individualized larvae. Tubs containing inoculated

larvae were incubated at 26±1ºC, 70±5ºC relative humidity and darkness in a growth chamber. The numbers of larvae or infected corpses per tub was noted daily until all insects had either died or pupated. Insects showing signs of the final stages of polyhedrosis disease were individually transferred to Eppendorf tubes, incubated at for up to 6 h at 26ºC until death and subsequently stored at -20ºC. Pupae were discarded. The experiment was performed on three occasions.

For OB yield measurements, larvae were thawed, individually homogenized in 1 ml of distilled water, and OBs were directly counted in triplicate using a Neubauer improved hemocytometer. The numbers of pupae, cannibalized larvae (larvae that disappeared or were partially devoured) and virus-killed larvae were averaged for each replicate and subjected to analysis of variance (ANOVA) and Tukey Test using the SPSS 15.0 program (IBM SPSS Statistics). Average OB production values were normalized by log transformation and subjected to ANOVA and Tukey Test ($P<0.05$) using the SPSS 15.0 program. The correlation between OB production and larval density was examined by Pearson coefficient as both variables were normally distributed.

2.3 Effect of inoculation time

Previous studies indicated that larvae of other lepidopteran species inoculated one day after molting produced significantly more OBs than recently molted larvae (Bernal et al., submitted). Therefore, to determine the optimal inoculation time for production of HearSP1B:LB6 OBs, groups of 24 larvae L_3, L_4 and L_5 were inoculated either as recently molted larvae (1 - 8 h post-molting) or as larvae at one day after molting (22 – 28 h post-molting). For this, larvae were inoculated with 90% lethal concentrations (LC_{90}) of OBs for each instar (6.1×10^5, 2.4×10^6 and 2.5×10^7 OBs/ml for L_3, L_4 and L_5 newly molted, respectively), using the droplet feeding method (Arrizubieta et al., 2014). Following inoculation, insects were individually transferred to 12-well plates containing artificial diet and incubated at 26±1ºC, 70±5% relative humidity and darkness in a growth chamber until death or pupation. As controls, 24 larvae were allowed to drink the inoculation solution without OBs. Individual larvae were weighed daily and moribund insects showing signs of the final stages of lethal polyhedrosis disease were individually transferred to microtubes and incubated at 26ºC without food for up to 8 hours until death. Virus-killed insects were reweighed and stored at -20ºC. The entire process

was performed three times. To determine OB yields, larvae were individually homogenized in 1 ml of distilled water and the OBs were counted in triplicate using a Neubauer improved hemocytometer. As the prevalence of mortality varied with the inoculation time, the OB yield was also estimated for groups of 100 inoculated larvae, in order to determine the treatment that achieved the OB yield for each cohort of 100 inoculated larvae. Average OB production values (OBs/larva, OBs/mg larval weight and OBs in each cohort of 100 inoculated larvae) were normalized by log transformation whereas initial larval weight, cadaver weight and mortality percentage values were normally distributed. All results were subjected to ANOVA and Tukey test using the SPSS 15.0 program. The correlations between larval weight at inoculation and cadaver weight, and also between log OB yield and cadaver weight were determined by Pearson coefficient as both variables were normally distributed.

2.4 Effect of inoculum concentration

To determine the influence of inoculum concentration on OB production, groups of 24 recently molted (1-8 h post-molting) L_5 larvae were inoculated with one of the concentrations corresponding to the LC_{95}, LC_{90} and LC_{80} i.e., 1.5×10^8, 2.5×10^7 and 5.5×10^6 OBs/ml respectively (Arrizubieta et al., 2014). As controls, 24 larvae were treated in exactly the same way, except that the aqueous inoculation solution contained no OBs. After inoculation insects were individually transferred to two 12-well plates, containing artificial diet and incubated at 26±1°C, 70±5% relative humidity and darkness in a growth chamber until death or pupation. Larvae were weighed daily and individuals showing signs of the final stages of polyhedrosis disease were individually transferred to Eppendorf tubes and incubated for up to 8 hours, without food at 26ºC until death. Virus-killed larvae were individually weighed and stored at -20°C. For counting, larvae were thawed, individually homogenized in 1 ml water and counted in triplicate in a Neubauer chamber as described above. The entire procedure was performed three times. The average OB production values (OBs/larva, OBs/mg and OBs from each cohort of 100 inoculated larvae) were normalized by log transformation whereas initial larval weight, cadaver weight and mortality percentage were normally distributed. Results were analyzed as described in the previous section.

2.5 Effect of incubation temperature

To determine the effect of temperature on OB production, groups of 24 recently molted (1 - 8 h post-molting) L_5 larvae were inoculated with an OB suspension that contained 100 mg/ml sucrose, 0.05 mg/mL Fluorella Blue food dye and a LC_{95} (1.5×10^8 OBs/ml) concentration of OBs. After inoculation insects were individually transferred to 12-well plates containing artificial diet and were reared at 23±1ºC, 26±1ºC or 30±1ºC in different incubation chambers in darkness, until they died of virus disease or pupated. Virus-induced mortality was recorded at intervals of 8 h. Individuals showing the signs of lethal polyhedrosis disease were individually transferred to Eppendorf tubes and incubated without food at the corresponding temperature until death. Virus-killed insects were stored at -20°C. For OB counting, larvae were thawed, individually homogenized in 1 ml water and counted in triplicate in a Neubauer chamber as described above. The experiment was performed five times. Time-mortality results were subjected to Weibull analysis using the GLIM program (Crawley, 1993). The validity of the Weibull model was determined using the Kaplan macro present in the GLIM program. OB counts were normalized by log transformation and subjected to ANOVA and Tukey test using the SPSS 15.0 program.

2.6 Effect of production temperature on OB pathogenicity

The effect of incubation temperature on the pathogenicity of OBs produced at each temperature was estimated by concentration-mortality bioassays in *H. armigera* using the droplet feeding method. Groups of 24 recently molted (1 - 8 h post-molting) L_2 larvae were inoculated with one of five different 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, which were previously found to result in between 95% and 5% mortality (Arrizubieta et al., 2014). As controls, 24 larvae were inoculated with sucrose and food color solution without OBs. Following inoculation, insects were individually transferred to 24-well plates containing artificial diet and incubated at 26±1ºC, 70±5% relative humidity in darkness in a growth chamber. Mortality was recorded at 24 h intervals during 10 days. The bioassay was performed three times. Concentration-mortality results were subjected to Probit analysis using the POLO-PC program (Le Ora Software, 1987).

3. Results

3.1 Cannibalism

The prevalence of larval cannibalism was similar among the different instars evaluated, and similar in healthy and infected larvae (Tukey, P>0.05) (Fig 1), except in L_5 in which cannibalism among infected larvae was significantly higher (77-87%) than among healthy larvae (20-55%) (Tukey, P<0.05) (Fig. 1C). Cannibalism increased significantly with increasing larval density ($F_{23,71}$=18.8, P<0.001). In L_3 and L_4 instars, the effect of larval density was similar in infected and healthy larvae, with cannibalism increasing from ~40% at the density of 5 larvae/tub to ~80% at a density of 20 larvae/tub (Fig. 1A, B). In contrast, in L_5 cannibalism varied from 20-55% in healthy larvae compared to 80-87% in infected larvae across all densities (Tukey, P>0.05) (Fig. 1C).

Cannibalism directly influenced both the average numbers of virus-killed larvae and the final OB yields per larva and per tub. The percentage of virus-induced mortality (80-93%) was significantly higher in individualized larvae compared to those incubated at higher densities (12-53%) ($F_{23,71}$=45.20, P<0.001) (Fig. 1). OB production was inversely correlated with density in each of the three instars tested (Pearson, r=-0.97 for L_3, r=-0.99 for L_4 and r=-0.99 for L_5) (Fig. 2A). This effect was most clearly observed in L_4 and L_5 in which the production of OBs decreased progressively from 1.8 x 10^9 and 2.8 x 10^9 OBs/larva in individualized larvae, respectively, to 1.2 x 10^9 and 5.7 x 10^8 OBs/larva in L_4 and L_5 larvae reared at the highest density, respectively (Tukey, P<0.05).

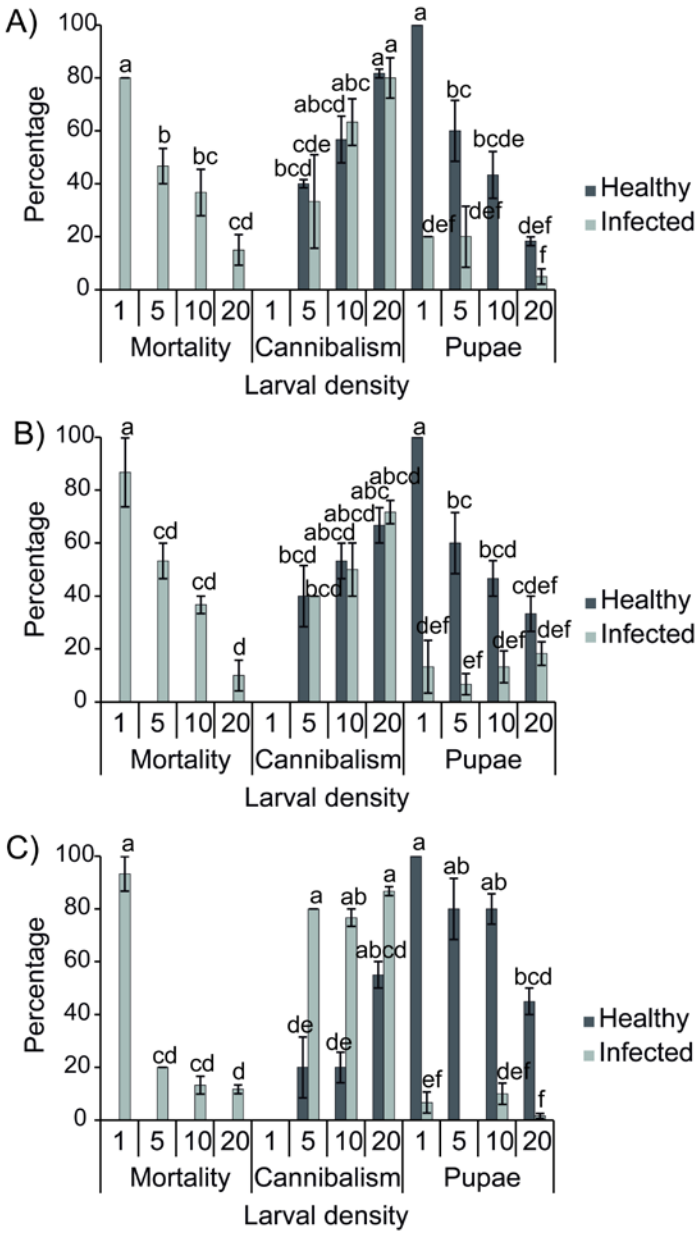

Figure 1: Percentage of pupae, mortality and cannibalism observed in *H. armigera* healthy and infected with LC_{90} of HearSP1B:LB6, at different densities: 1, 5, 10 and 20 larvae per tub. (A) L_3, (B) L_4, and (C) L_5.

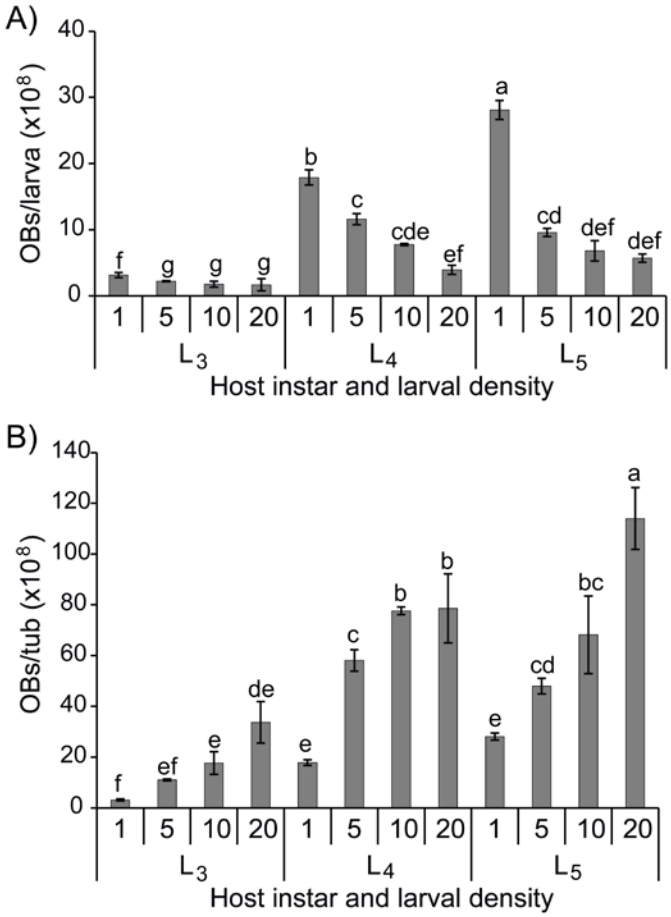

Figure 2: Productivity in L_3, L_4 and L_5 *H. armigera* infected with LC_{90} of HearSP1B:LB6 and reared at different densities: 1, 5, 10 and 20 larvae per tub. (A) Productivity expressed as OBs/larva, and (B) productivity expressed as OBs/tub. Values followed by identical letters did not differ significantly at P>0.05 by ANOVA and Tukey test.

The total production of OBs among all the virus-killed larvae from each tub, representing the product of the mean number of OBs per larva and the average number of virus killed larvae per tub (not including larvae that pupated or that died from cannibalism), varied significantly with instar ($F_{2,35}$=10.13, P<0.001) and density ($F_{2,35}$=6.80, P<0.001) (Fig. 2B). However, despite being seeded with 20-fold more larvae per tub than the individualized larvae, the highest density only produced 10.7-fold more OBs in L_3, 4.4-fold more OBs/tub in L_4 and 3.9-fold more OBs/tub in L_5 than observed in the individualized larvae (Fig. 2B). Overall, these

results led to the decision to perform the production of HearSP1B:LB6 OBs in individualized larvae.

3.2 Selection of inoculation time

The initial larval weight varied significantly among larvae inoculated at different ages and stages ($F_{5,17}$=1637.4, P<0.001) (Fig. 3A). All instars showed an 80% increase in body weight in the period between molting and when weighed at 1 day post-molting (Tukey, P>0.05). A positive correlation was observed between the initial larval weight and the cadaver weight (Pearson r=0.96). Larvae inoculated at later instars had significantly higher cadaver weights than larvae inoculated at earlier instars ($F_{5,17}$=75.1, P<0.001) (Fig. 3A). Cadaver weights were highest in L_5 larvae inoculated one day after molting that died with a mean body weight of 400.2 ± 25.0 mg, whereas newly molted L_3 larvae attained a body weight at death of just 16.2 ± 0.4 mg. Within the same instar, larvae inoculated one day after molting died at a consistently higher body weight than recently molted conspecifics (Tukey, P<0.05) (Fig. 3A).

Larval weight at the moment of inoculation had a marked effect on the percentage of larval mortality observed ($F_{5,17}$=15.1, P<0.001) (Fig. 3B). In newly molted larvae the expected prevalence of lethal polyhedrosis was observed across all instars tested (85-100%). However, larvae inoculated one day after molting presented significantly lower percentages of mortality (21-72%). The effect of the additional day in age at inoculation on the observed mortality tended to increase significantly with increasing instar (Tukey, P<0.05), so that the mortalities of L_3, L_4 and L_5 larvae inoculated one day after molting were 72, 36 and 21%, respectively.

OB production increased significantly with instar ($F_{5,17}$=15.1, P<0.001) so that a positive correlation was observed between OB production and cadaver weight (Pearson r=0.92). The greatest numbers of OBs were produced in larvae inoculated one day after molting to L_4, recently molted L_5, and one day after molting to L_5 (6.7-9.1 x 10^9 OBs/larva) (Tukey, P<0.05) (Fig. 3C).

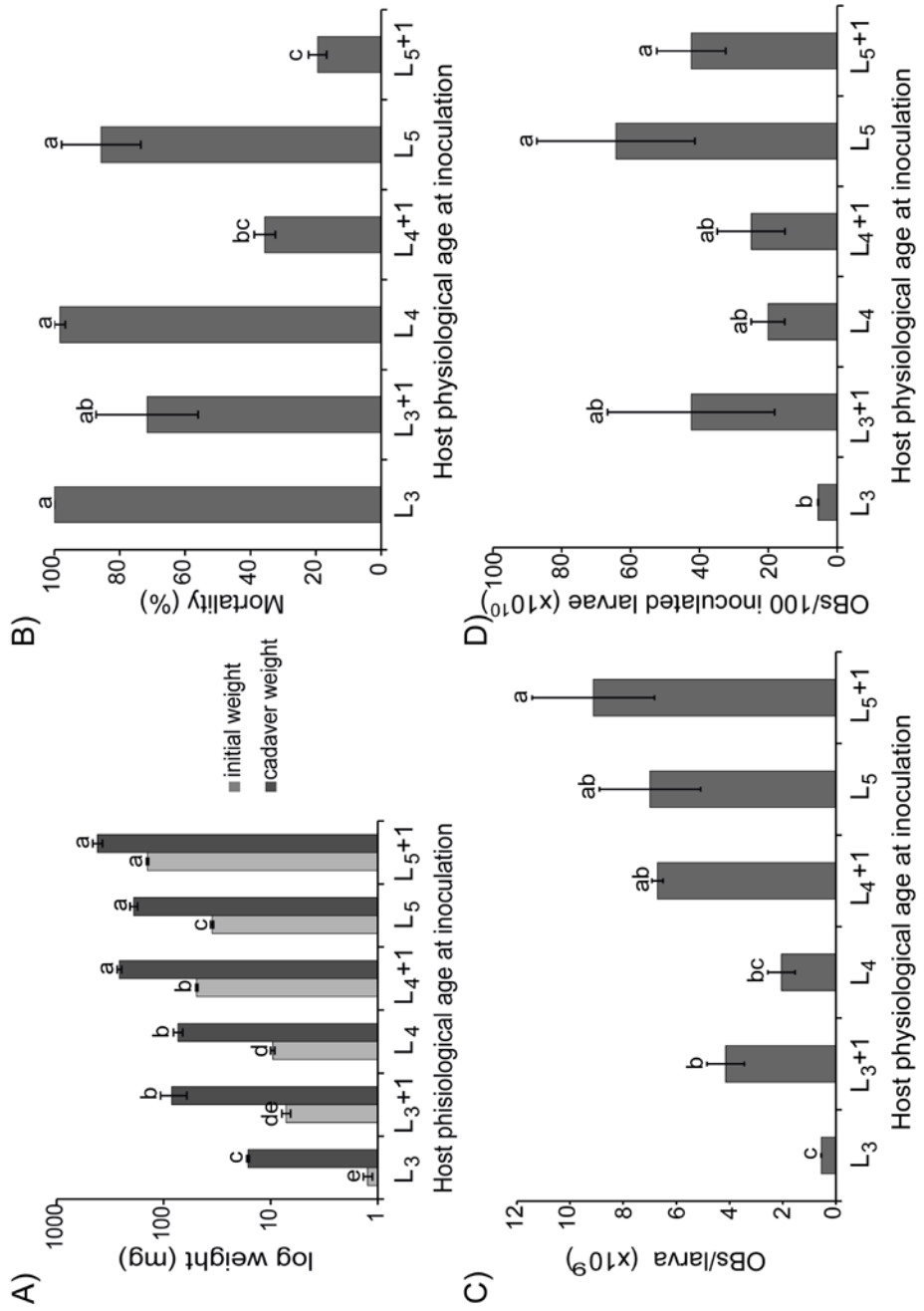

Figure 3: (A) Initial and viral cadaver weight, (B) Percentage of larval mortality, (C) Productivity expressed as OBs/larva, and (D) Productivity expressed as OBs/100 inoculated larvae in newly molted and one day after molting to L_3, L_4 and L_5 *H. armigera* infected with LC_{90} of HearSP1B:LB6. Values followed by identical letters did not differ significantly at P>0.05 by ANOVA and Tukey test.

Due to the differences obtained in the percentage of virus-induced mortality in larvae inoculated at different times, which ranged from 19.5 to 100%, the total OB yield per cohort of 100 inoculated larvae differed significantly with inoculation age and stage ($F_{5,17}$=4.6, P=0.01) (Fig. 3D). The most productive treatment was newly molted L_5, with a total production of 6.0 x 10^{11} OBs per 100 inoculated larvae (Fig. 3D). According to these results, maximum HearSP1B:LB6 OB production should be achieved by inoculation of recently molted L_5 stage larvae.

3.3 Selection of inoculum concentration

Inoculum concentration did not significantly affect body weight at death (cadaver weight: 180-232 mg) ($F_{2,6}$=1.2, P=0.13) (Fig. 4A), or the percentage of mortality (80.1-96.4%) ($F_{2,6}$=0.6, P=0.58) (Fig. 4B). The different inoculum concentrations tested produced similar OB yields. For example, newly molted L_5 infected with the different concentrations produced between 6.3 x 10^9 and 7.2 x 10^9 OBs/larva ($F_{2,6}$=0.1, P=0.90) (Fig. 4C) and between 5.3 x 10^{11} and 6.9 x 10^{11} OBs for each cohort of 100 inoculated larvae ($F_{2,6}$=0.3, P=0.77) (Fig. 4D).

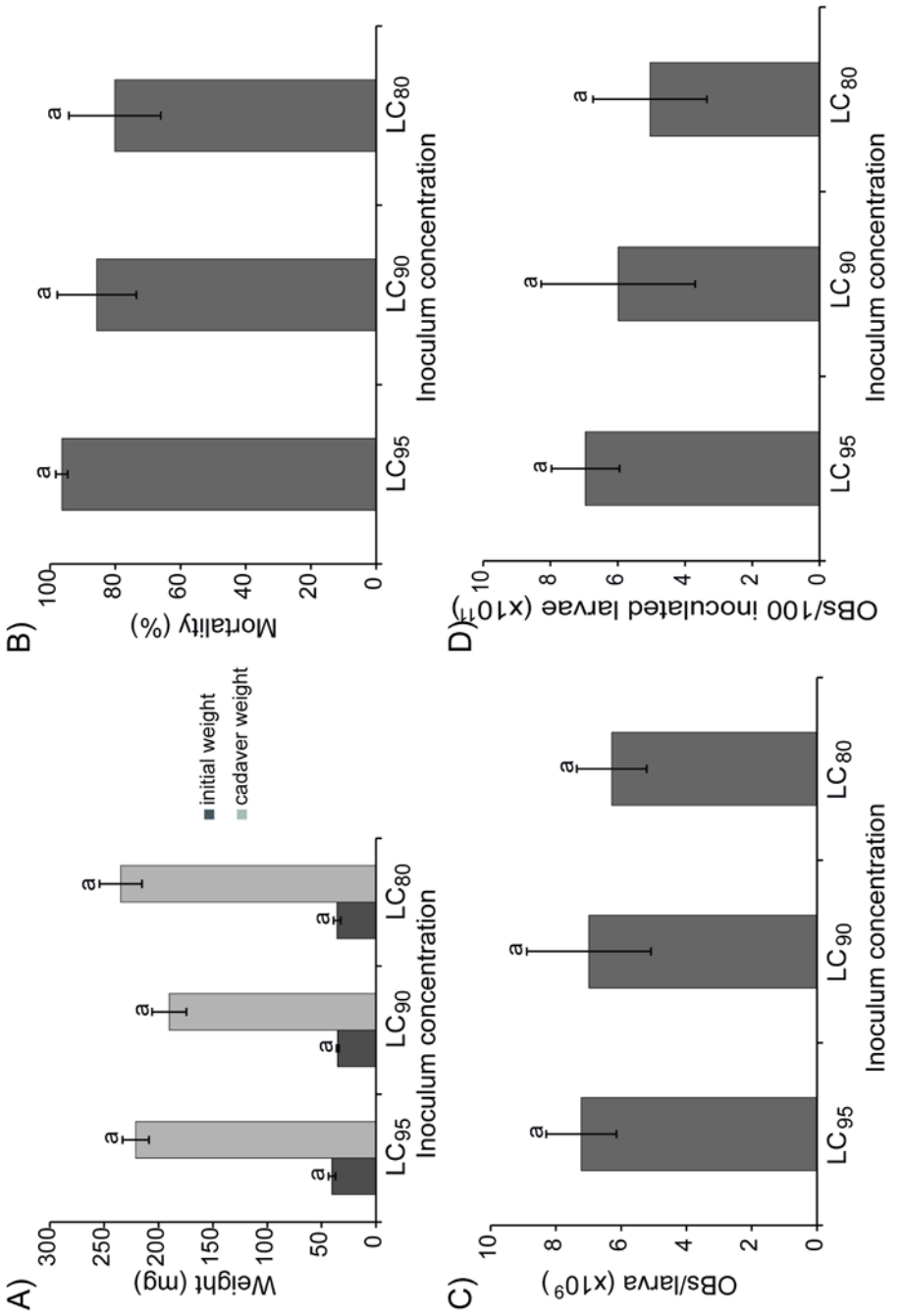

Figure 4: (A) Initial and viral cadaver weight, (B) Percentage of larval mortality, (C) productivity expressed as OBs/larva, and (D) OBs/100 inoculated larvae, in newly molted L_5 *H. armigera* infected with LC_{95}, LC_{90} and LC_{80} of HearSP1B:LB6. Values followed by identical letters did not differ significantly at P>0.05 by ANOVA and Tukey test.

3.4 Incubation temperature

Incubation temperature did not significantly affect the prevalence of larval mortality ($F_{2,12}$=1.5, P=0.52). The mortality observed in L_5 incubated at 23ºC, 26ºC and 30ºC was 88, 91 and 86%, respectively. Similarly, final OB yields (3.2-4.2 x 10^9 OBs/larva) did not differ significantly in cadavers that had been incubated at different temperatures ($F_{2,12}$=0.3, P=0.75) (Fig. 5A). However, insects incubated at 30ºC died significantly more rapidly; 13 and 34 hours before larvae incubated at 26ºC and 23ºC, respectively (Fig. 5B).

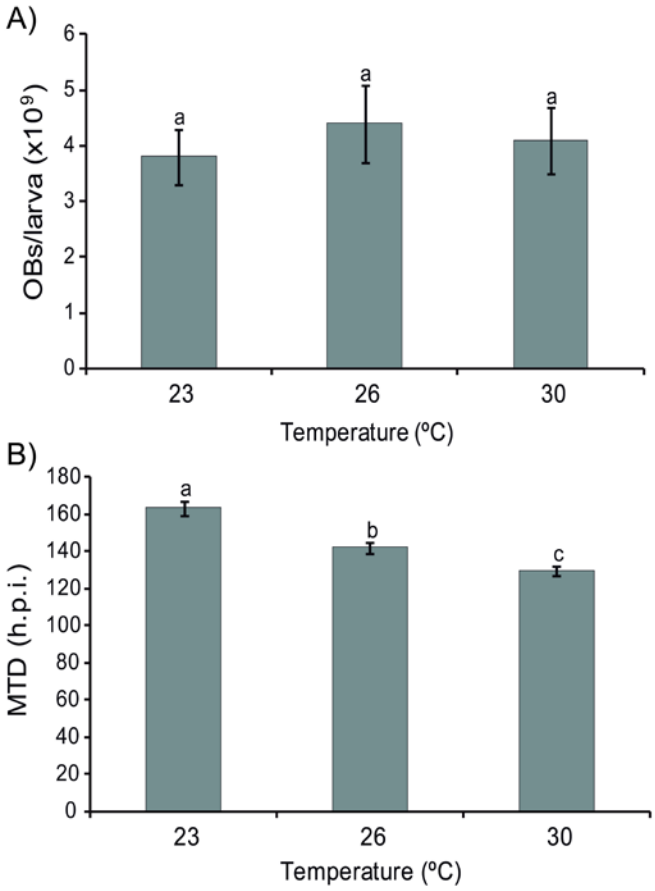

Figure 5: (A) Productivity (OBs/larva) obtained in L_5 *H. armigera* after inoculation with LC_{95} of HearSP1B:LB6 OBs and incubated at 23, 26 and 30ºC. Values followed by identical letters did not differ significantly at P>0.05 by ANOVA and Tukey test. (B) Median time to death (h.p.i.) of fifth instar *H. armigera* after inoculation with LC_{95} of HearSP1B:LB6 and incubated at 23, 26 and 30ºC. Values followed by different letters differed significantly at P<0.05 by Weibull analysis.

Finally, incubation temperature did not significantly influence the insecticidal properties of the OBs (Table 1). The LC_{50} values of OBs recovered from larvae maintained at 23ºC, 26ºC and 30ºC were very similar (1.1×10^4 - 1.8×10^4 OBs/larva). Therefore, the 30ºC incubation temperature was selected as the optimal temperature for the efficient production of HearSP1B:LB6 OBs in *H. armigera*, as L_5 larvae produced similar quantities of OBs, and OBs of similar pathogenicity, but died faster than conspecifics incubated at lower temperatures.

Table 1: LC_{50} values and relative potencies of HearSP1B:LB6 produced in L_2 *Helicoverpa armigera* larvae incubated at different temperatures.

Temperature	LC_{50}	Relative	Fiducial limits 95%	
	(OBs/ml)	potency	Low	High
23ºC	1.5×10^4	-	-	-
26ºC	1.1×10^4	1.3	0.8	2.0
30ºC	1.8×10^4	0.8	0.5	1.3

Logit regressions were fitted in POLO-Plus. A test for non-parallelism was not significant (χ^2=5.7, df=2, P>0.05), such that regressions were fitted with a common slope of 1.13 ± 0.15 (mean ± S.E.). Relative potencies were calculated as the ratio of effective doses relative to HearSP1B:LB6 produced at 23ºC.

4. Discussion

In previous studies, the co-occluded genotypic mixture HearSP1B:LB6 was selected as the basis for a biological insecticide (Arrizubieta et al., submitted). In the present study the most suitable conditions for the efficient production of HearSP1B:LB6 OBs were determined, in order to establish baseline conditions for *in vivo* production the co-occluded binary mixture of genotypes.

The HearSP1B variant was the most pathogenic of all the genotypes cloned from a field isolate (HearSNPV-SP1-wt) originating from Guadajira, Extremadura, Spain (Arrizubieta et al., 2014), whereas HearLB6 was the most virulent variant of those obtained from virus-killed insects that were the progeny of insects collected in the field in Lebrija, southern Spain (Arrizubieta et al., submitted).

Due to the high cost of *in vitro* production, in addition to mutations and deletions that occur during replication in cell culture (Chakraborty and Reid, 1999; Dai et al., 2000; Pijlman et al., 2004), large scale OB production has to be performed in *H. armigera* larvae. The cannibalism rate observed in this study was

very high in all three instars tested and increased markedly with larval density. Furthermore, in the fifth instar, cannibalism was significantly higher among infected larvae than among healthy larvae, whereas in L_3 and L_4 infection status had no significant effect on cannibalism. A high prevalence of cannibalism towards larvae infected by entomopathogenic viruses has been attributed in previous studies to lower mobility and sluggish responses of diseased larvae, making diseased insects more likely to be victims of conspecific predation than healthy insects (Dhandapani et al., 1993; Boots, 1998; Williams and Hernandez, 2006). Cannibalism rates in *H. armigera* were similar among the different larval stages, as observed in a previous study on *H. zea* (Chilcutt, 2006). In contrast, studies performed in other insect species have frequently observed that cannibalism is instar dependent, with a greater propensity for intraspecific predation in the later instars (Boots, 1998; Chapman et al., 1999; Dong and Polis, 1992; Polis, 1981). In contrast, published results provide evidence that insects in the final instar tend to present lower cannibalism rates than previous instars, and are less prone to aggressive behavior (Bernal et al., submitted; Champman et al., 1999; Elvira et al., 2010). Moreover, infected larvae are cannibalized in a higher rate than healthy larvae (Boots, 1998; Elvira et al., 2010). From an evolutionary perspective, cannibalism behavior may favor accelerated development, reduced mortality, increased pupal weight and higher fecundity in natural populations (Polis, 1981), and can also favor the survival of more robust and fecund individuals in laboratory colonies (Joyner and Gould, 1985). However, cannibalism is not desirable during OB production procedures as the total number of larvae is reduced, which reduces the overall yield of OB produced in each cohort of insects (Chapman et al., 1999; Shapiro et al., 1986). For these reasons, individualized rearing of *H. armigera* larvae post-inoculation appears to be necessary for efficient production of HearSP1B:LB6 OBs, despite the additional costs in handling that individualized rearing implies.

Productivity, measured in terms of OBs per larva, increased with increasing larval stage and age at inoculation time, which was directly correlated with the cadaver weight. Thus, larvae inoculated one day after molting to L_4, recently molted L_5, and L_5 at one day after molting, and were the most productive developmental states of the larva. Previous studies have reported a direct relationship between larval age and OB production in *H. armigera*. In the present

study, *H. armigera* larvae inoculated at later instars achieved an OB yield between 6.7 x 10^9 and 9.1 x 10^9 OBs/larva, which is comparable to the yields observed in *H. armigera* late instars reported previously (1.7 x 10^9-1.2 x 10^{10} OBs/larva) (Gupta et al., 2007; Kalia et al., 2001; Mehrvar et al., 2007).

The quantity of OB inoculum consumed by larvae can have an important effect on the production of OBs in each larva since high doses of OBs can hasten the death of the larva, resulting in reduced weight gain during infection and consequently fewer OBs produced in each insect (Grzywacz et al., 1998; Kalia et al., 2001). However, inoculum OB concentrations tested in the present study resulted in similar percentages of mortality and similar OB yields from experimental insects. OB production per mg of larval body weight did not vary significantly among the different inoculum concentrations, but was clearly affected by larval weight (Bernal et al., submitted; Sun et al., 2005). Sun et al. (2005) observed that HearSNPV OB production in third instar *H. armigera* was 6.0 x 10^9 OBs/larva, which increased to 1.0 x 10^{10} OBs/larva in the fifth instar. However, Sun et al. (2005) reported no differences in OB production/mg of larval weight, which was ~3 x 10^7 OBs/mg in all instars tested, clearly within the range of 2.0-5.0 x 10^7 OBs/mg of larval weight observed in the present study. Therefore, our observation that the three inoculum concentrations resulted in similar OB yields is likely due to the fact that inoculated larvae reached similar final weights.

OB production may also be influenced by incubation temperature, which directly affects larval development and the rate of virus replication (Subramanian et al., 2006). At high temperatures larvae feed and grow faster, and cell metabolism is accelerated resulting in faster virus replication, which can lead to premature host death and reduced OB yields compared to insects reared at lower temperatures. Mehrvar et al. (2007) obtained an almost 1.5-fold increase in OB yield by incubating *H. armigera* infected larvae at 25ºC rather than at 30ºC. Studies performed with other species, such as *S. litura* (Subramanian et al., 2006), *Lymantria dispar* (Shapiro et al., 1981) or *Mamestra brassicae* (Kelly and Entwistle, 1987), have consistently reported the highest OB production in larvae reared in the range 25-30°C following inoculation, which reflects the optimum temperature range of the host insects.

In line with these findings, the influence of incubation temperature on speed of kill of the virus was an issue of concern. In the present study rearing at 30°C

accelerated death by 13 or 34 h compared to larvae reared at 26 or 23°C, respectively. Similar effects have been reported in other species, including *Anticarsia gemmatalis* (Johnson et al., 1982), *S. litura* (Subramanian et al., 2006), *Diatraea saccharalis* inoculated with heterologous NPVs (Ribeiro and Pavan, 1994), and *Trichoplusia ni* inoculated with *Autographa californica* MNPV (van Beek et al., 2000).

Furthermore, high incubation temperatures may affect the insecticidal properties of OBs, particularly by favoring the propagation of bacterial contaminants (Jenkins and Grzywacz, 2000), which contribute to OB degradation (Subramanian et al., 2006). However in the present study, incubation temperature did not affect the biological activity of HearSP1B:LB6 OBs in terms of concentration-mortality metrics, which agrees with previous studies performed on *S. litura* infected with its homologous NPV (Subramanian et al., 2006).

Considering the results obtained in this study, we conclude that for efficient production of the HearSP1B:LB6 co-occluded mixture of *H. armigera* nucleopolyhedrovirus OBs should be performed by inoculation of recently molted L_5 with an LC_{80} concentration (5.5 x 10^6 OBs/ml) of inoculum followed by incubation of individualized larvae at 30ºC. Using this system it is possible to produce large quantities of OBs which previous studies by us indicate can be as effective in crop protection as commercial insecticides such as chlorpyrifos, *B. thuringiensis* or spinosad.

5. Acknowledgements

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CHAPTER VIII

Insecticidal efficacy and persistence of a co-occluded binary mixture of *Helicoverpa armigera* nucleopolyhedrovirus (HearSNPV) variants in protected and field-grown tomato crops in the Iberian Peninsula

Abstract

A binary co-occluded mixture (HearSP1B:LB6) of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) variants was previously found to be highly pathogenic under laboratory conditions. The insecticidal efficacy and persistence of this mixture were determined in greenhouse and field-grown tomato crops in Spain and Portugal. Concentrations of 10^9 - 10^{11} OBs/l of HearSP1B:LB6 resulted in 89-100% mortality of larvae on treated tomato plants in growth chambers. In protected tomato crops, application of 10^{10} OBs/l of HearSP1B:LB6 was as effective as *Bacillus thuringiensis* (Bt) and spinosad in reducing the percentage of damaged fruits, and resulted in higher larval mortality than the Bt treatment. In open-field tomato crops virus-treatments were as effective at reducing the percentage of damaged fruit as spinosad, Bt and chlorpyrifos treatments. The persistence of the insecticides on tomato plants was negatively correlated with solar radiation in both field and greenhouse settings. Residual insecticidal activity of OBs on protected tomato crops at 6 days post-application was 55% and 35% higher than that of Bt and spinosad, respectively. On field-grown tomato, OB persistence was significantly lower than that of spinosad or chlorpyrifos. In conclusion, the efficacy and persistence of HearSP1B:LB6 OBs was comparable to that of commercial insecticides in both field and greenhouse tomato crops. Future studies should focus on reducing application rates to determine insecticidal efficacy at lower OB concentrations.

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1. Introduction

Spain is the world's fourth largest producer of tomatoes with an average of 4.3 million tonnes/yr, whereas Portugal produces an additional 1.1 million tonnes/yr (INE, 2013; MAGRAMA, 2014). The total area of production comprises approximately 60,000 ha and 17,000 ha in Spain and Portugal, respectively, with 25% (Spain) and 60% (Portugal) of the production in greenhouses and the remainder as field crops (INE, 2013; MAGRAMA, 2014).

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), also known as the tomato fruitworm, is one of the most important pests of tomato Torres-Vila et al., 2003a). Females mainly lay eggs in the flowering period, although larvae may attack any phenological stage of the plant and are especially likely to damage the fruit. Therefore, the pest's preference for flowers and fruits, in addition to its polyphagy, high mobility and fecundity make it a major pest (Fitt, 1989). Quality control in the tomato processing industry set the damage limit to 2-5% of harvested tomatoes, but if larvae are present, this limit is reduced to 0-2% (Cameron et al., 2001; Torres-Vila et al., 2003a).

Control of this pest is usually achieved by applying chemical insecticides, especially organophosphates, the carbamate methomyl, or pyrethroids (Amaro and Mexia, 2006; Torres-Vila et al., 2003a). However, excessive dependence on chemicals has lead to a variety of problems, such as increased production costs due to multiple insecticide applications, development of marked insecticide resistance in the pest population, elimination of beneficial insects, or the presence of pesticide residues that may restrict the commercialization of tomato crops from this region (Torres-Vila et al., 2000, 2002a, b). Frequent use of insecticides also leads to a need for continual monitoring and analysis of residues in tomato fruits and tomato-based products (Garrido et al., 2004). These constraints have motivated the search for alternative control methods, including the use of biological insecticides (King and Colleman, 1989; O´Callaghan and Brownbridge, 2009).

HearSNPV is an alphabaculovirus (Baculoviridae) that has been used as a biological insecticide for control of this pest on cotton, soybean, pigeon pea, maize and tomato in several parts of the world (Moscardi et al., 2011). The virus comprises a single nucleocapsid containing a single genome within each virion;

with dozens of virions occluded into each viral occlusion body (OB). The virus is specific to certain species of *Heliothis* and *Helicoverpa* (Gettig and McCarthy, 1982), but shows the highest pathogenicity, in terms of dose-mortality metrics, to larvae of *H. armigera* (Cherry et al., 2003). As in other Alphabaculoviruses (Cory et al., 2005; Erlandson, 2009; Hodgson et al., 2001; Simón et al., 2004), natural isolates of HearSNPV comprise heterogeneous mixtures of genotypic variants that contribute to the transmission and survival of the pathogen in the host population (Baillie and Bouwer, 2012; Rowley et al., 2011).

In a previous study, a novel binary mixture of HearSNPV variants, named HearSP1B:LB6, had high insecticidal activity against *H. armigera* larvae under laboratory conditions. This unique combination of variants was produced by isolating individual variants *in vitro*, mixing and co-occluding them in different proportions, and testing the pathogenicity of the resulting OBs, using insect bioassay concentration-mortality metrics (Arrizubieta et al., 2015a). The HearSP1B:LB6 mixture comprised equal proportions of the most pathogenic variant present in a field isolate of HearSNPV from Badajoz (Spain), named HearSP1, and the fastest killing variant, HearLB6, from an isolate collected from dead larvae near Sevilla (Spain). These variants were mixed and co-occluded into OBs using a procedure that had been successfully employed for co-occlusion of mixtures of other alphabaculovirus variants (Bernal et al., 2013). The presence of variant mixtures within individual OBs was confirmed using end-point dilution and qPCR techniques (Arrizubieta et al., 2015a). Additionally, a production system for HearSP1B:LB6 OBs was developed aimed at efficient production of this binary mixture (Arrizubieta et al., 2015b).

Registration of bioinsecticidal products requires field trails to be performed under typical crop production conditions to demonstrate the efficacy of the product for pest control. The efficacy of biological insecticides can vary in laboratory, greenhouse and open field conditions (Arrizubieta et al., 2014; Bianchi et al., 2000; Dwyer et al., 2005; Grant and Bower, 2009). Furthermore, insect populations often differ in their susceptibility to a particular virus strain present in a virus-based insecticide, as local virus genotypes tend to be more pathogenic to local pest populations than genotypes from geographically distant regions (Barrera et al., 2011; Cherry et al., 2003; Erlandson, 2009; Fuxa, 1993).

The efficacy of a virus-based insecticide is also strongly influenced by its persistence on the surface of the crop plant, as with greater persistence the probability of the pest consuming a lethal dose of OBs over time also increases (Jones et al., 1993). Solar ultraviolet (UV) radiation is the main factor affecting the persistence of OBs deposited on plant surfaces (Ignoffo, 1992). However, the incidence of UV radiation can vary greatly from region to region, with crop phenology, and with growing conditions. For example, exposure to UV radiation is greatly reduced in greenhouse-grown crops compared to those grown in an open field, as the greenhouse's plastic structure filters much of the incident UV (Lasa et al., 2007).

As a contribution to the registration of the binary variant mixture as the active ingredient of an insecticidal product, the present study aimed to determine the efficacy of HearSP1B:LB6 OBs for the control of *H. armigera* and the persistence of OBs on tomato crops under both protected and open-field conditions. The performance of OBs was compared with that of commercial products commonly used for the control of this pest on tomato crops.

2. Material and methods

2.1 Virus and insects

The co-occluded genotypic mixture used in the present study HearSNPV-SP1B + HearSNPV-LB6, abbreviated here to HearSP1B:LB6, was characterized previously in terms of concentration-mortality response, speed of kill and OB production (Arrizubieta et al., 2015a). All virus preparations were propagated in fifth-instar *H. armigera* larvae by the droplet feeding method (Hughes and Wood, 1981). The wild-type HearSNPV-SP1 isolate (HearSP1) (Arrizubieta et al., 2014; Figueiredo et al., 1999) was included as a reference treatment in open-field trials.

The *H. armigera* colony used for artificial infestations in greenhouse and persistence assays was established with pupae received from the Centre for Ecology and Hydrology (CEH) (Oxford, United Kingdom) and maintained in the Universidad Pública de Navarra (Pamplona, Spain) at 25±2ºC, 70-80% relative humidity and 16:8h L:D photoperiod on a semisynthetic diet (Greene et al., 1976).

2.2 Determining the optimal OB concentration

A preliminary assay was performed in a growth-chamber under laboratory conditions, with the aim of selecting the optimal HearSP1B:LB6 OB concentration to be used in greenhouse and open-field trials described below. Groups of three tomato plants of ~1.2 m height were sprayed with 10^9, 10^{10} and 10^{11} OBs/l supplemented with 0.2% (v/v) of a commercial wetter-sticker based on nonylphenoxy polyethoxy ethanol (Agral®, Syngenta Agro, Spain), using a total of 60 ml for each plant. Plants sprayed with just water and wetter-sticker were included as negative controls. When plants were completely dry (~1 h), three tomato leaves from each plant, each comprising five primary leaflets were cut at the base of the petiole and the cut end was placed in 50 ml glass cups containing Hoagland nutritive solution (Hoagland and Arnon, 1950). Leaves were then placed individually in a 2 l glass container. Each container was artificially infested with ~150 second instar *H. armigera* larvae from the laboratory colony, covered with muslin and maintained at 25±2ºC, 70-80% relative humidity and 16:8h L:D photoperiod during a week. Groups of 20 larvae were collected from each container at three different intervals, following 1, 3 and 5 days of exposure. Collected larvae were individualized in 30 ml plastic cups containing artificial diet and incubated under the same conditions until death or pupation. Larval mortality was recorded daily. The experiment was performed three times. Results were subjected to repeated-measures analysis of variance (ANOVA) and Tukey test ($P<0.05$) for homogeneous groups using SPSS v.21 (IBM SPSS Statistics).

2.3 Greenhouse trials

Greenhouse trials were performed in 2011 in an experimental greenhouse 18 m length x 16 m width, with a total area of 288 m^2, located at the Instituto Superior de Agronomia (Lisboa, Portugal). Cropping practices such as bed formation, drip irrigation, application of fertilizers, transplanting, maintenance and manual weeding were performed following established procedures in the studied area. Plug-seedlings were transplanted with a ball of peat at the 3-5 true leaf stage, in pairs of rows separated by 20 cm between each row, and 50 cm distance between row pairs. Plants were spaced at 40 cm intervals along the rows resulting in a density of ~38,000 plants/ha. Greenhouse plastic was treated with a chalk-

based shade product (Spraychalk, Mardenkro, water dilution 1:8), as usual in the region for summer protected crops.

The experiment involved four treatments: (i) HearSP1B:LB6 OBs applied at a concentration of 10^{10} OBs/l (following the results of the growth chamber study described in section 2.2), equivalent to 10^{13} OBs/ha, (ii) Turex® 50WP (50% Bacillus thuringiensis (w/w), 25,000 IU/mg, from Biosani, Portugal; applied at 1 kg/ha), (iii) Spintor® (SC, 48% spinosad (w/v) from Dow AgroSciences, Spain; applied at 250 ml/ha) and (iv) control treatment (water). Turex and Spintor were applied at the product label recommended rates. All treatments included 0.2% Agral® wetter-sticker and were applied using 18 l hand-operated knapsack sprayers fitted with a cone nozzle.

The entire greenhouse was divided in 16 plots, according to a 4 x 4 Latin square design with four replicates per treatment. Experimental plots comprised 7.5 m long sections of two double rows (7.5 m^2) comprising 28 plants (22 border plants and 6 central plants). Each plot was artificially infested with 112 larvae, by placing two *H. armigera* second-instars from the laboratory colony on each of the two youngest clusters of tomato fruits on each of the 28 plants. Larvae were allowed to feed on plants for 1 day. After that, each plot was sprayed with 750 ml of each insecticide treatment, equivalent to an application volume of 1,000 l/ha. All treatments were applied between 18.00 and 20.00 hrs. The entire trial was performed twice, on July 19 and September 13. The efficacy of HearSP1B:LB6 OBs was evaluated by estimating the number of surviving larvae and number of fruit feeding injuries present on the six central plants of each plot at 10 days after application. The number of living larvae and fruit feeding injury, involving direct feeding damage caused by *H. armigera*, was measured by direct counting. Results were subjected to repeated-measures ANOVA and Tukey test ($P<0.05$) for homogeneous groups using SPSS software. The correlation between fruit feeding damage and larval mortality was determined using Pearson coefficient as both variables were normally distributed.

2.4 Open-field trials

Open-field trials were conducted in 2012 at CICYTEX Research Centre (Finca La Orden, Badajoz, Spain). Soil was prepared according to usual cropping practices in the study area: ploughing, harrowing, bed formation, drip irrigation and

transplanting. Plug-seedlings were transplanted with a ball of peat at the 3-5 true leaf stage in single rows spaced at intervals of 1.5 m on 1.0 m wide beds. Plants were spaced 25-26 cm apart along the rows, resulting in a density of ~26,000 plants/ha. Cultural practices, including the use of herbicide, fungicide, fertilizer, irrigation, crop maintenance and manual weeding were performed according to usual procedures. Plots were inspected daily for secondary pests; liquid sulfur was applied on four occasions to control mites. Experimental plots were arranged in a randomized plot design with four replicate plots per treatment. The whole trial consisted of 48 experimental plots. Each plot was 4 m long by one-row wide (6 m^2), comprising 15-16 plants. Plots were separated from each other by a buffer row of untreated plants. Plots at the edges of the experimental area were surrounded by additional rows of untreated plants to reduce edge effects. Two pheromone traps were placed at the edge of the experimental area and surveyed twice weekly to acquire information on likely infestation by *H. armigera*.

The experiment involved six treatments: (i) HearSP1B:LB6 OBs applied at a concentration of 10^{10} OBs/l (equivalent to 10^{13} OBs/ha following the results of the growth chamber study described in section 2.2), , (ii) HearSP1 OBs applied at a concentration of 10^{10} OBs/l, equivalent to 10^{13} OBs/ha, (iii) Turex® (50% *B. thuringiensis* (w/w) from Certis, Spain, applied at 2 kg/ha), (iv) Spintor® (SC, 48% spinosad (w/v) from Dow AgroSciences, Spain; applied at 250 ml/ha), (v) Dursban® (75% chlorpyrifos (w/w) from Dow AgroSciences; applied at 1.25 kg/ha) and (vi) control treatment (water). All commercial insecticides were applied at product label recommended rates for tomato. All treatments included 0.2% Agral® wetter-sticker and were applied using 18 l hand-operated knapsack sprayers with a cone nozzle. All treatments were applied between 18.00 and 20.00 hrs. Treatments were applied in a volume of 600 ml in each plot, equivalent to an application volume of 1,000 l/ha.

Treatments were applied either three or five times, depending on economic threshold levels observed during the trial. The first treatment was applied when 3% of fruits showed characteristic *H. armigera* feeding damage, which is the action threshold in integrated pest management (IPM) programs against *H. armigera* in tomato crops in this region (Torres-Vila et al., 2003a, b). Thereafter applications were performed every 10-13 days, which represents the usual time interval between treatments against *H. armigera* on processing tomato in this region. The

first application was performed on 11 June, when 3% of fruits showed characteristic feeding damage and the second on 21 June; in both cases all fruits were green. The third, fourth and fifth treatments were applied on 3, 16 and 26 July, which coincided with the presence of ~5%, 50% and 65% of red fruit development, respectively.

Plots were inspected for *H. armigera* damage twice weekly from early fruit set to one week before harvesting. The percentage of larval damage was estimated in each plot every 3-4 days by examining 100 randomly chosen fruits, which included green fruits larger than 2.5 cm in diameter, as well as pink and red fruits of all sizes. Insect feeding damage was classified as recent or old (scarred injuries). Data were grouped by fortnights (first fortnight: 1-15 June; second fortnight: 16-30 June; third fortnight: 1-15 July; and fourth fortnight: 16-31 July). Percentages were normalized by arcsine transformation prior to analysis followed by repeated-measured analysis of variance (ANOVA) and Tukey test ($P<0.05$). Within-subject pairwise comparisons were used to determine the time effects on fruit damage among the estimated marginal means with Bonferroni correction ($P<0.05$).

Following established criteria, plots were harvested when the 80% red fruit stage was reached. All tomato fruits in the central 1.5 m^2 of each plot were manually picked on a single occasion to simulate mechanical harvesting. Fruits were individually inspected, weighed, and classified into one of five groups: unmarked green fruits, damaged green fruits, unmarked red fruits, scarred red fruits and rotten red fruits. Scarred red fruits were marketable fruits, according to quality standards required by the processing industry, in which larval damage was superficial and well healed, whereas rotten red fruits were unmarketable fruits in which larval perforations were recent, unhealed, deep, and usually rotting.

For analysis, fruit production was expressed as tonnes per hectare, estimated from the harvested weight of tomatoes (kg/m^2) in each plot. The total number of fruits, fruit weight, percentage of healthy fruits and total yield per hectare were subjected to two-way factorial ANOVAs to examine the effects of insecticide, number of sprays applied and their interaction. Percentage values were normalized by arcsine transformation prior to analysis. Mean separation was performed by Tukey test ($P<0.05$). All analyses were performed using Systat (2000) statistical software.

2.5 Comparison of OB and insecticide persistence

The estimated UV radiation during the greenhouse trials was provided by the Instituto Geofisico Dom Luis Meteorological Station (Instituto Português do Mar e da Atmosfera, IPMA), while the data in open-field trials in Badajoz was collected by the Badajoz Meterological Station (Agencia Estatal de Meteorología, AEMET).

During greenhouse trials, three terminal leaflets were randomly collected from 15 leaves located in the upper half of the central plants of each plot. These samples were taken at 1, 60, 132 and 204 h post-application. In field-grown tomato crops, three terminal leaflets were collected from 30 leaves from the upper half of 15 treated plants at 1, 60, 156 and 228 h post-application. The three leaflets from each leaf were pooled, placed in labeled polythene bags, immediately frozen and stored at -20°C until use. Therefore, greenhouse trials had 4 replicas (4 plots per treatment), for each collection time, while open-field trials involved 8 replicas per collection time (8 plots per treatment). The concentration of viable OBs, and each commercial insecticide on leaflet samples was estimated by bioassay. For this, frozen leaflets were triturated and a 2 g sample (wet weight) of each leaflet sample was thoroughly mixed with 8 g of artificial diet. The resulting mixture was divided equally among 5 wells of a 24-well plate. A single second-instar larva was placed in each well and incubated at 25±2ºC, 70-80% relative humidity and 16:8h L:D photoperiod. Therefore each replica in greenhouses was assayed using 75 larvae (5 larvae were used for each of the 15 pooled leaflet samples), whereas in open-field trial each replica was tested using 150 larvae (5 larvae for each of the 30 pooled leaflet samples). Larval mortality was recorded daily for 7 days.

The relationship between the prevalence of mortality observed in the bioassay and insecticide concentration on leaf surfaces was determined by prior calibration of the bioassay technique. For this, 10 g of homogenized leaves collected prior to the application of insecticide treatments and 40 g of artificial diet were mixed with one of the five different concentrations of insecticides; 10^5, 10^6, 10^7, 10^8 and 10^9 OBs of HearSP1B:LB6 or HearSP1/l of diet; 1.6, 3.2, 8.0, 20 and 40 mg of Turex/l of diet (equivalent to 0.8 - 20 mg a.i./l Bt); 0.016, 0.625, 0.25, 1.0 and 4.0 µl/l of Spintor (equivalent to 0.0077 - 1.9 mg a.i./l spinosad); and 0.32, 1.6, 8.0, 40 and 200 mg/l for Dursban (equivalent to 0.24 - 150 mg a.i./l chlorpyrifos).

For each insecticide-diet combination, the resulting mixture was divided equally among the 48 wells of two 24-well plates. A single second-instar larva was placed in each well and incubated at 25±2ºC, 70-80% relative humidity and 16:8 h L:D photoperiod during a week. As a negative control, a 10 g sample of untreated homogenized leaves was mixed with 40 g of artificial diet and included in the bioassay. The entire calibration procedure was performed in triplicate. Logit regressions of larval mortality on the logarithm of insecticide concentration were computed (Table 1) and the quantities of the different insecticides per gram of leaf material were estimated by comparing the percentage mortality of larvae that consumed diet + leaf sample mixtures with the corresponding calibration curve for each insecticide. The logarithm of residual insecticide estimated concentrations were subjected to repeated-measures analysis of variance (ANOVA) using SPSS software. The significance of time effects on insecticidal persistence was determined by within-subject pairwise comparisons among the estimated marginal means with Bonferroni correction ($P<0.05$). In addition, the Pearson coefficient was calculated in order to determine the correlation between insecticidal persistence and incident UV radiation, as both variables were normally distributed.

Table 1: Logit regressions of larval mortality on the concentration of insecticides per gram of leaves used to calibrate the persistence bioassay.

	Treatment	Logit regression	log (units)
Greenhouse	HearSP1B:LB6 OBs	y = 28.30x – 53.85	OBs
	B. thuringiensis	y = 43.37x + 127.44	mg
	Spintor	y = 55.00x + 223.22	µl
Open-field	HearSP1B:LB6 OBs	y = 24.28x – 49.73	OBs
	HearSP1 OBs	y = 23.30x – 45.70	OBs
	B. thuringiensis	y = 41.32x + 122.38	mg
	Spintor	y = 55.72x + 224.40	µl
	Chlorpyrifos	y = 62.20x + 178.91	mg

3. Results

3.1 Determination of OB concentration

No virus mortality was registered in control larvae reared following exposure to control plants, indicating the absence of natural or accidental contamination of

experimental plants, and that the *H. armigera* colony insects used to infest the tomato plants were healthy. The percentages of larval mortality due to polyhedrosis disease in insects that fed on plants treated with 10^9 OBs/l were 88.9, 96.7 and 88.0% in larvae collected at 1, 3 and 5 days after virus application, respectively. These percentages increased to 100% at every collection time in plants treated with 10^{10} and 10^{11} OBs/l (Fig. 1). No significant differences were observed in the prevalence of virus-induced mortality among the different concentrations tested ($F_{2,6}$=4.96, P>0.05). As 10^{10} OBs/l was the lowest concentration that provided 100% mortality of experimental insects on tomato plants, this concentration was selected for use in greenhouse and open-field trails.

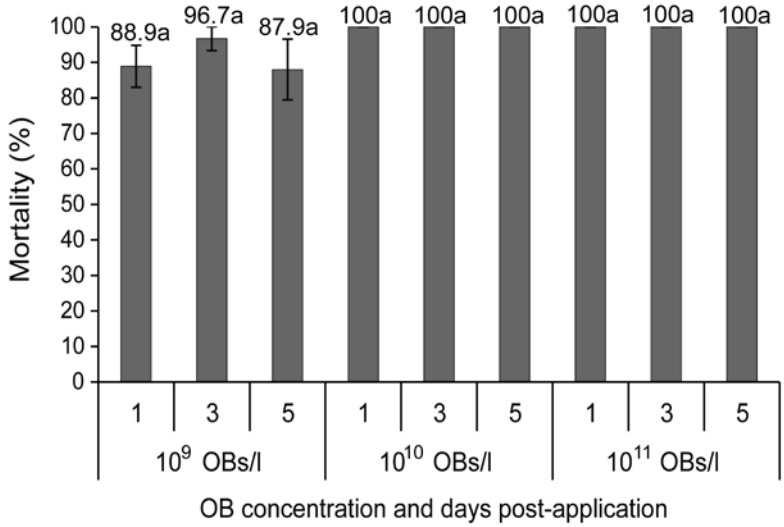


Figure 1: Percentage of virus-induced mortality in second-instar *H. armigera* larvae feed in tomato leaves treated under laboratory conditions 1, 3 and 5 days after the application of 10^9, 10^{10} and 10^{11} OBs/ml of HearSP1B:LB6. Bars labeled with the same letters did not differ significantly (repeated-measures ANOVA followed by Tukey at P<0.05). Vertical lines indicate the standard error.

3.2 Greenhouse trials

In the first trial, the numbers of released larvae that disappeared from plants, i.e. the reduction in the initial infestation (Moore et al., 2004), was taken as an indicator of larval mortality. At 10 days post-application significantly fewer larvae were present in plots treated with HearSP1B:LB6 OBs or commercial insecticides (93.2-97.9% mortality) than in control plots (75.0%) ($F_{3,12}$=48.6,

P<0.05) (Fig. 2A). HearSP1B:LB6 OBs (97.9%) and spinosad (97.9%) treatments resulted in significantly higher larval mortality than *B. thuringiensis* (93.2%) (Tukey, P<0.05) (Fig. 2A). Larval mortality was inversely correlated with fruit damage (Pearson r=-0.92); plants treated with HearSP1B:LB6 OBs or either of the commercial insecticides had significantly fewer damaged fruits (12.1-17.3%) than control plants (25.3%) ($F_{3,12}$=9.9, P<0.05) (Fig. 2A). However the different treatments resulted in similar percentages of damaged fruits, with 12.1% of damaged fruit in plots treated with HearSP1B:LB6 OBs, 16.3% in B. thuringiensis treated plots and 17.3% in plots treated with spinosad (Tukey, P>0.05) (Fig. 2A).

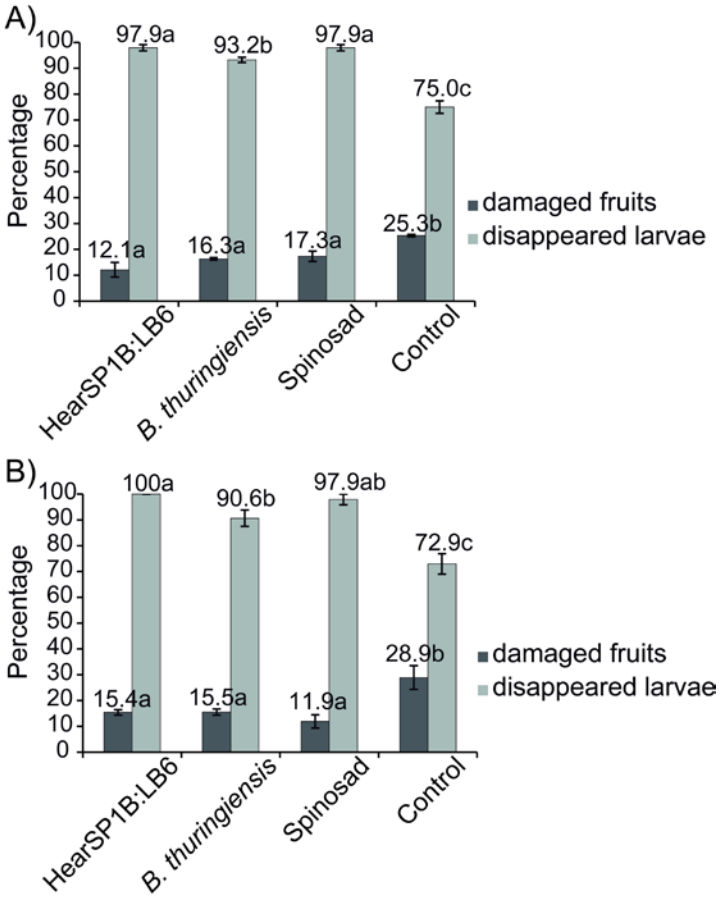


Figure 2: Percentage of reduction in infestation (dead and disappeared larvae) and percentage of damaged fruits on protected tomato plants at 10 days after the application of HearSP1B:LB6 OBs, *B. thuringiensis* and spinosad, A) first trial, and B) second trial. Bars labeled with the same letters did not differ significantly (repeated-measures ANOVA followed by Tukey at P<0.05). Vertical lines indicate the standard error.

The results of the second trial were very similar to those of the first trial. Larval mortality at 10 days post-application was significantly higher in plots treated with the different insecticides (90.6-100%) than in control plots (72.9%) ($F_{3,12}$=20.3, P<0.05) (Fig. 2B). Plots treated with HearSP1B:LB6 OBs (100%) resulted in significantly higher larval mortality than *B. thuringiensis* (90.6%) (Tukey, P<0.05), whereas spinosad resulted in an intermediate prevalence of mortality (97.9%) (Tukey, P>0.05) (Fig. 2B). Larval mortality was inversely correlated with fruit damage (Pearson r=-0.94); plots treated with viral OBs or commercial insecticides had significantly fewer damaged fruits (11.9-15.5%) than control plots (28.9%) ($F_{3,12}$=7.3, P<0.05) (Fig. 2B). No significant differences were observed in the percentages of damaged fruits among the insecticide-treated plots (15.4, 15.5 and 11.9% for HearSP1B:LB6, *B. thuringiensis* and spinosad, respectively) (Tukey, P>0.05) (Fig. 2B).

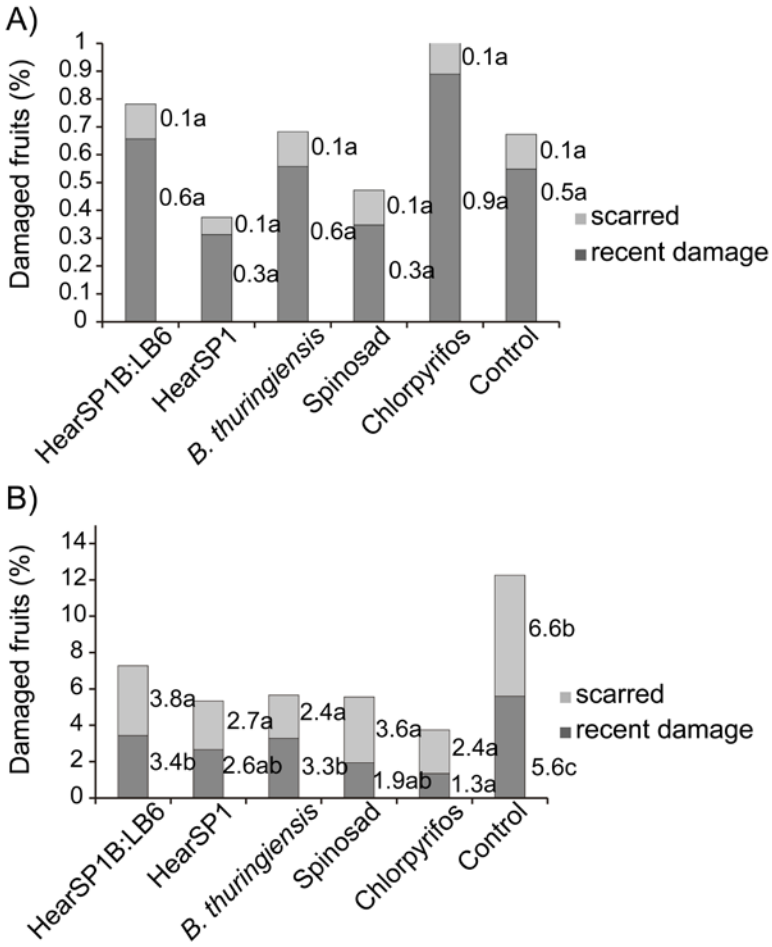
3.3 Open-field trail

No significant differences were detected between plots sprayed three or five times for any of the variables studied ($F_{1,33}$<3.12, P>0.09 in all cases), or in the interaction insecticide x number of applications ($F_{5,33}$<2.36, P>0.07 in all cases). Therefore the results for plots with different numbers of applications (3 or 5) were pooled for all subsequent analyses.

Fruit damage (recent and scarred) showed a clear seasonal pattern (Fig. 3). Significant differences between 14-day intervals (fortnights) ($F_{3,69}$>76.91, P<0.001 in all cases), insecticides ($F_{5,69}$>15.92, P<0.001 in all cases) and also in the fortnight x insecticide interaction ($F_{15,69}$=5.24, P<0.001), so that each 14-day period was considered separately in the following one-way ANOVAs.

In the first fortnight no differences were observed in the percentage of damaged fruits, which was always lower than 1% in all insecticide treatments ($F_{5,15}$=0.72; P=0.62) (Fig. 3A). However, in the second and third fortnights (Fig. 3B and 3C), control plots presented more damaged fruits, either scarred or recent (12.2-21.3%), than plots treated with the different insecticides (3.5-7.3%) (second fortnight: $F_{5,15}$=18.68; P<0.001; third fortnight: $F_{5,15}$=54.76; P<0.001). Finally, in the fourth fortnight, the percentages of scarred fruits differed significantly among insecticides ($F_{5,15}$=44.28; P<0.001), with significantly lower values in insecticide-

treated plots (2.1-4.5%) than in control plots (14.7%), whereas recent damage was very low (<0.4%) and similar among all treatments ($F_{5,15}$=1.00; P=0.45) (Fig. 3D).



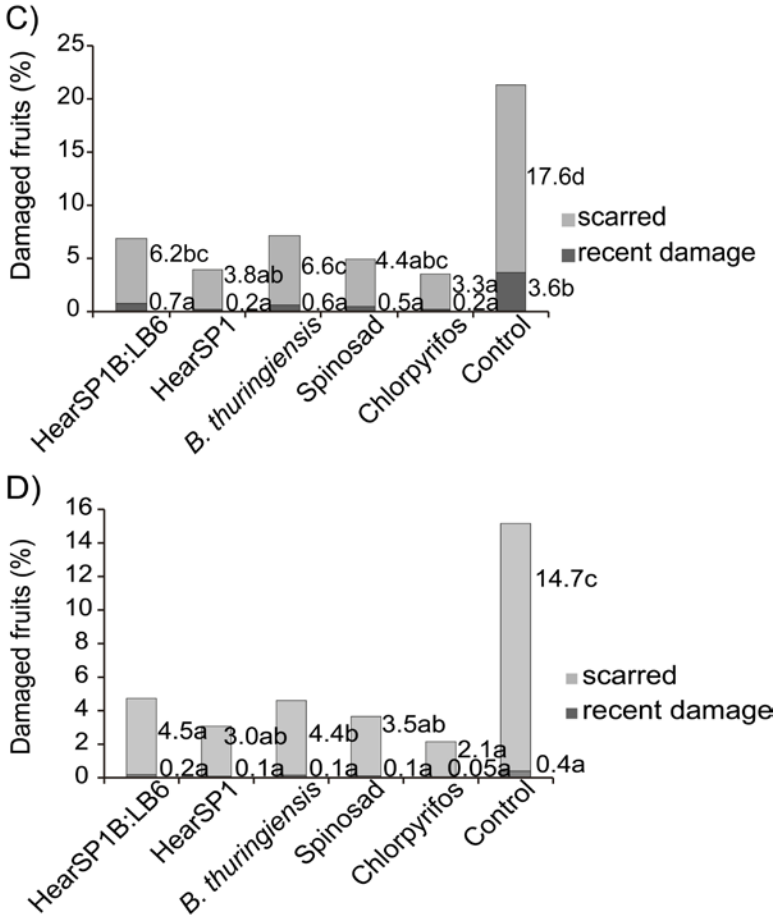


Figure 3: Percentages of damaged fruits, either scarred or recent, in field-grown tomato crops after the application of HearSP1B:LB6 OBs, HearSP1 OBs, *B. thuringiensis*, spinosad and chlorpyrifos, and their seasonal progression through the four fortnights of the months of June and July: (A) first fortnight, (B) second fortnight, (C) third fortnight, and (D) fourth fortnight. Bars labeled with the same letters did not differ significantly (ANOVA followed by Tukey test at $P<0.05$).

The percentages of fruits with recent damage was higher in the second fortnight (1.3-3.4%) compared to the first (0.3-0.9%), third (0.2-0.7%) and fourth fortnights (0.0-0.1%) in plots treated with the different insecticides ($F_{3,9}>41.2$, $P<0.001$, in all cases). In the control treatment, the percentage of recently damaged fruits was higher in the second (5.6%) and third (3.6%) fortnights than in the first (0.6%) or fourth (0.4%) fortnights ($F_{3,9}=12.7$, $P<0.002$). Lower percentages of scarred fruits were observed in the first fortnight (0.1% in all cases), compared to the second, third, and fourth fortnights, in plots treated with the different

insecticides (3.8-6.2%, 2.7-3.8%, 2.4-6.6%, 3.6-4.4% and 2.1-3.3% for HearSP1B:LB6, HearSP1, *B. thuringiensis*, spinosad and chlorpyrifos, respectively) ($F_{3,9}$>9.1, P<0.05, in all cases). In control plots the percentages of scarred fruits increased significantly over the time until the third fortnight (0.1%, 6.6% and 17.6% for the first, second and third fortnights, respectively) and was 14.7% in the fourth fortnight ($F_{3,9}$=69.7, P<0.001). As a result, similar quantities of green undamaged fruits were harvested in all insecticide treatments (8.5-12.3 tonnes/ha) ($F_{5,39}$=0.70; P=0.63) (Fig. 4A). In contrast, yields of red undamaged fruits differed significantly among insecticide treatments ($F_{5,39}$=2.78; P=0.03), with higher yields in insecticide-treated plots (143-166 tonnes/ha), than in control plots (121 tonnes/ha) (Fig. 4B). Similarly, lower quantities of either green damaged fruits (0.1-0.4 tonnes/ha, representing 1.1-3.2% of harvested fruits) ($F_{5,39}$=4.95, P<0.002), scarred red fruits (3.0-6.0 tonnes/ha, 2.0-4.2% of harvested fruits) ($F_{5,39}$=42.55, P<0.001) or rotten red fruits (5.0-8.9 tonnes/ha, 4.3-7.6% ($F_{5,39}$=10.15, P<0.001), were collected in insecticide-treated plots compared to control plots: 1.2, 18.1 and 14.9 tonnes/ha, for green, scarred and rotten fruits, respectively, equivalent to 9.7, 14.7 and 13.9% of harvested fruit, respectively (Fig. 4A, 4B).

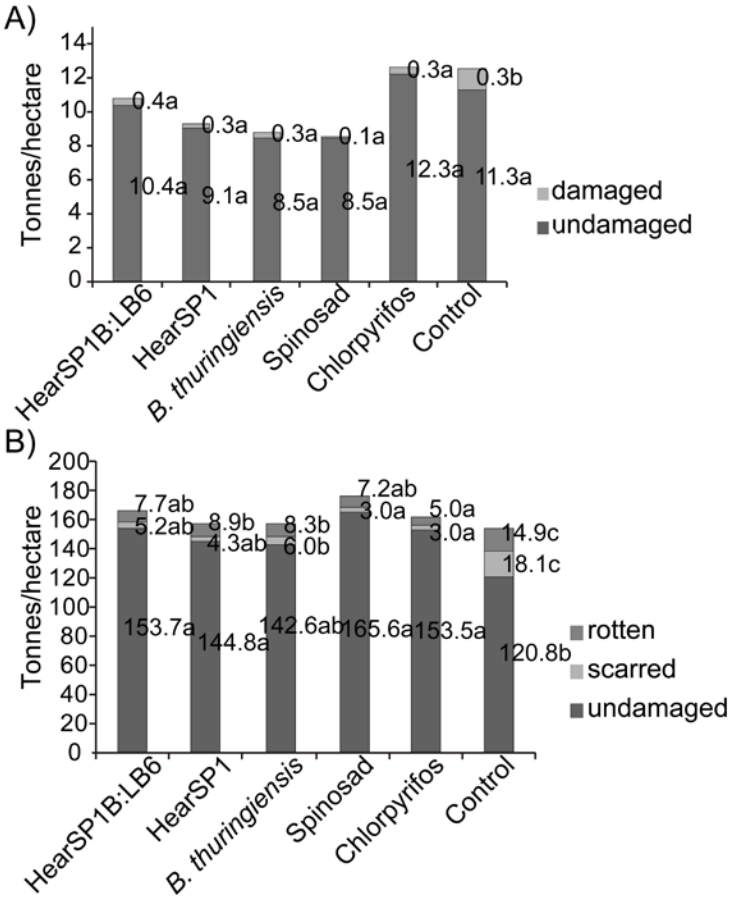


Figure 4: Yield (tonnes/ha) of (A) green fruits, either damaged or healthy, and (B) red fruits, either rotten, scarred or healthy, in open-field tomato crops after the application of HearSP1B:LB6, HearSP1, *B. thuringiensis*, spinosad and chlorpyrifos. Bars labeled with the same letters did not differ significantly (ANOVA followed by Tukey test at P<0.05).

The prevalence of scarred fruits was higher in the *B. thuringiensis*-treated plots (6.0 tonnes/ha, 4.2% of harvested fruits), compared with spinosad or chlorpyrifos treatments (3.0 tonnes/ha, 2-3% of harvested fruits), whereas HearSP1B:LB6 and HearSP1 OB applications resulted in intermediate values of scarred fruits (5.2 and 4.3 tonnes/ha, 3.8 and 3.5% of harvested fruits, respectively) (Tukey, P<0.05) (Fig. 4B). Application of chlorpyrifos resulted in significantly fewer rotten fruits (5.0 tonnes/ha, 4.3% of harvested fruits), compared to HearSP1 OBs (8.9 tonnes/ha, 7.6% of harvested fruits) or *B. thuringiensis* (8.3 tonnes/ha, 7.2% of harvested fruits) treatments, whereas HearSP1B:LB6 OBs (7.7

tonnes/ha, 6.6% of harvested fruits) and spinosad (7.2 tonnes/ha, 5.2% of harvested fruits) treatments resulted in intermediate values (Tukey, P<0.05) (Fig. 4B).

3.4 Insecticide persistence

The average accumulated dose of UV radiation inside the greenhouse during the sampling period was 4,134, 10,294 and 16,585 J/m^2 at 3, 6 and 9 days after treatment, respectively (Fig. 5), taking into account that the plastic structure and whitewash treatment intercepted ~55% of incident UV radiation which allowed pollinators to function within the greenhouse (Fig. 5).

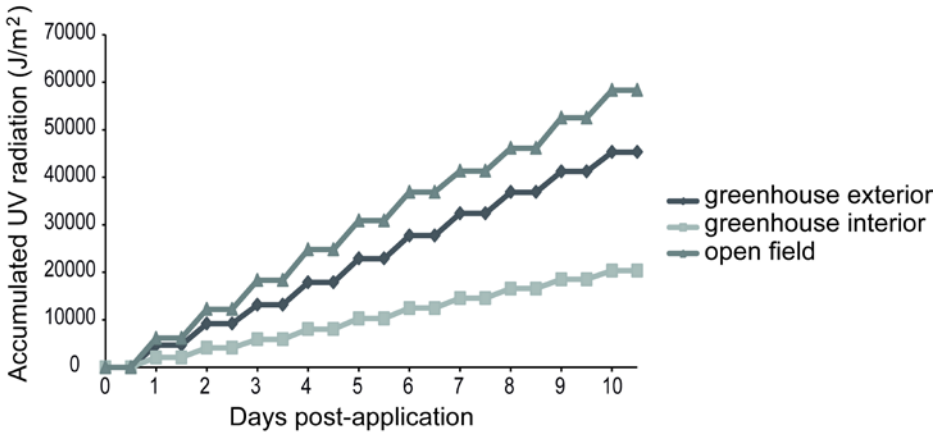
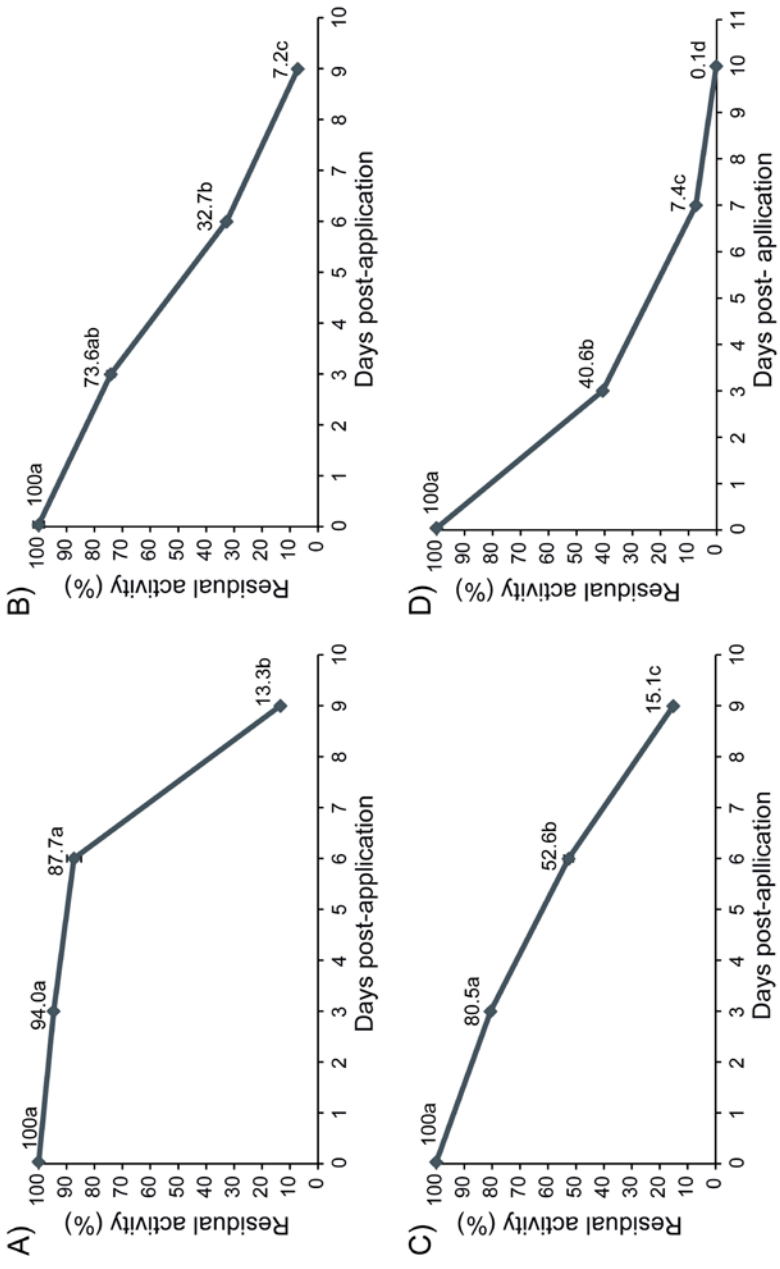


Figure 5: Accumulated ultraviolet (UV) radiation dose (J/m^2) during the field trials in protected crops (greenhouse exterior and greenhouse interior) and open-field tomato crops. UV radiation inside the greenhouse (greenhouse interior) was ~45% of the UV radiation outside the greenhouse (greenhouse exterior).

The activity of all insecticides on plants decreased significantly over time ($F_{3,9}$=18.9, P<0.05 for HearSP1B:LB6, $F_{3,9}$=170.5, P<0.05 for *B. thuringiensis* and $F_{3,9}$=8.3, P<0.05 for spinosad) and was negatively correlated with the accumulated dose of UV radiation (Pearson r=-0.89, -0.99 and -0.99 for HearSP1B:LB6 OBs, *B. thuringiensis* and spinosad, respectively). Insecticide concentrations on plant surfaces at 1 hour after spraying were estimated to be 2.73 x 10^5 ± 8.44 x 10^2 HearSP1B:LB6 OBs/g of leaf material (wet wt), 0.201 ± 0.016 mg/g of *B. thuringiensis*, and 0.0056 ± 0.0002 µl/g of Spìntor, which was taken to represent the initial (100%) value of insecticidal residues. At 3 and 6 days after application, the residual activity of HearSP1B:LB6 OBs on leaves was similar to the activity

observed at 1 hour after application (94.0-87.7% residual activity), but decreased significantly at 9 days post-application to 13.3% of initial activity (Fig. 6A). The *B. thuringiensis* and spinosad treatments showed similar patterns of persistence, with 73.6% and 80.5%, of initial activity remaining at 3 days post-application, respectively, falling to 7.2% and 15.1% at 9 days post-application, respectively (Fig. 6B and 6C).

In open-field crops, no rainfall occurred during the field trial and the accumulated doses of UV radiation at 3, 7 and 10 days post-treatment were 12,224, 36,885 and 52,517 J/m^2, respectively (Fig. 5). Residual insecticidal activities on plants decreased significantly over time ($F_{3,21}$=54.3, P<0.001 for HearSP1B:LB6, $F_{3,21}$=139.4, P<0.001 for HearSP1, $F_{3,21}$=38.5, P<0.001 for *B. thuringiensis*, $F_{3,21}$=12.3, P<0.001 for spinosad, and $F_{3,21}$=37.1, P<0.001 for chlorpyrifos). Residual insecticidal activity was negatively correlated with accumulated UV radiation (Pearson r=-0.92, -0.96, -0.88, -0.99 and -0.96 for HearSP1B:LB6, HearSP1, *B. thuringiensis*, spinosad and chlorpyrifos, respectively). Similarly to the results observed in protected crops the concentration of insecticide on leaves at 1 hour after treatment was 1.4 x 10^6 ± 1.1 x 10^3 OBs/g of HearSP1B:LB6 OBs, 1.7 x 10^6 ± 4.8 x 10^2 OBs/g of HearSP1, 0.26 ± 0.011 mg/g of *B. thuringiensis*, 0.0058 ± 0.0009 µl/g of Spintor and 0.054 ± 0.0021 mg/g of chlorpyrifos; these values were taken as the initial (100%) residual activities. Three days after application, the residual activity of HearSNPV OBs had decreased to 40.6 and 62.5% of initial activity for HearSP1B:LB6 and HearSP1 OBs, respectively (Fig. 6D and 6E). The activity of HearSNPV OBs continued to decrease to 0.1% of initial activity for HearSP1B:LB6 OBs and 0.05% for HearSP1 OBs at 10 days post-application (Fig. 6D and 6E). In the *B. thuringiensis* treatment, the residual activity was 33.3% at 3 days post-application, decreasing to 4.9% of initial activity at 10 days post-application (Fig. 6F). The residual activity of spinosad decreased gradually to 21.9% at 10 days post-application (Fig. 6G). Similarly, the activity of chlorpyrifos decreased gradually to 15.6% of initial activity at 10 days post-application (Fig. 6H).


A)
Residual activity (%)
Days post-application
100a
94.0a
87.7a
13.3b
B)
Residual activity (%)
Days post-application
100a
73.6ab
32.7b
7.2c
C)
Residual activity (%)
Days post-apllication
100a
80.5a
52.6b
15.1c
D)
Residual activity (%)
Days post- apllication
100a
40.6b
7.4c
0.1d

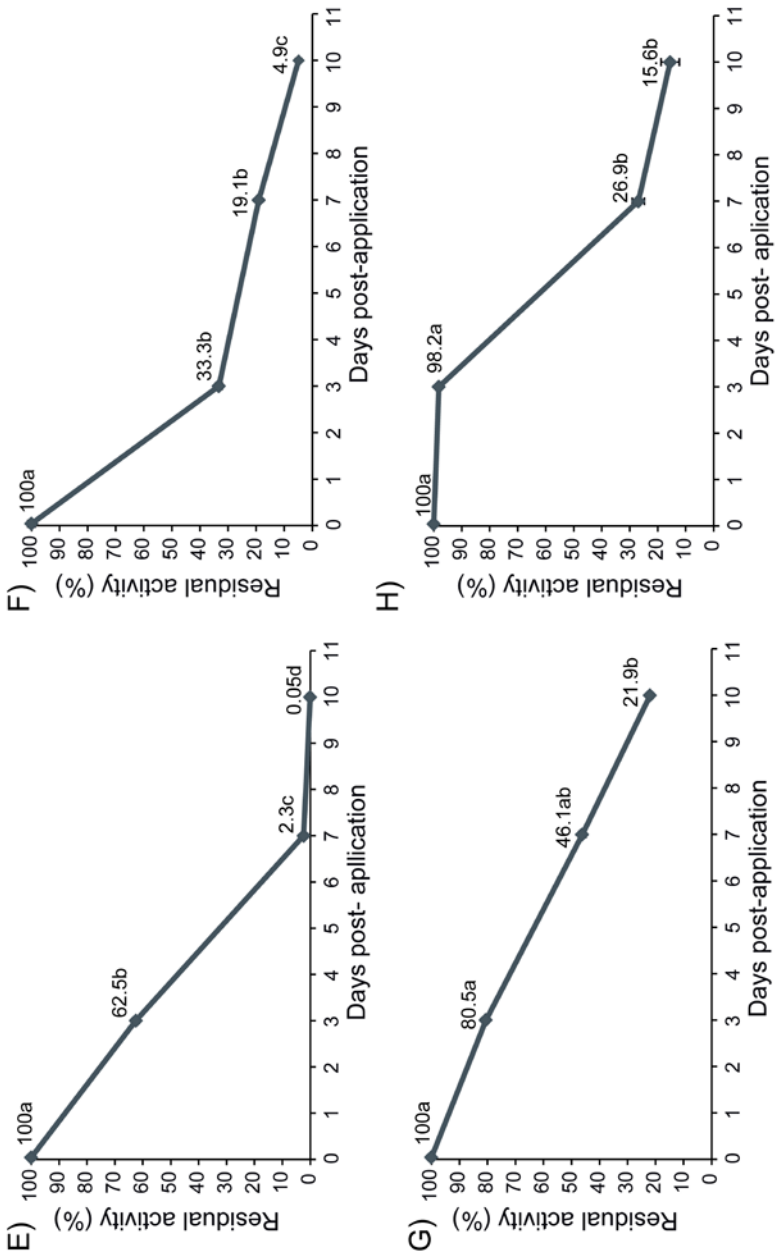


Figure 6: Percentage of insecticide residue on tomato leaves along the time with respect to the quantity of insecticide 1 hour after insecticide application: A) HearSP1B:LB6 OBs, B) *B. thuringiensis* and C) spinosad in protected tomato crops, and D) HearSP1B:LB6 OBs, E) HearSP1 OBs, F) *B. thuringiensis*, G) spinosad and H) chlorpyrifos in field-grown tomato crops. Bars labeled with the same letters did not differ significantly (repeated-measures ANOVA with intra-subject pairwise comparison of estimated marginal means, P>0.05 Bonferroni correction). Vertical lines indicate the standard error.

4. Discussion

In the present study the efficacy and persistence of a binary mixture of HearSP1B and HearLB6 variants co-occluded into OBs (Arrizubieta et al., 2015a), was evaluated as the basis for a biological insecticide for control of *H. armigera* on tomato crops grown under greenhouse and open-field conditions in southern Spain and Portugal. Initial growth chamber studies indicated that a concentration of 1 x 10^{10} OBs/l of HearSP1B:LB6 OBs resulted in 100% mortality of experimental larvae on treated tomato plants. This concentration was estimated to be equivalent to 1 x 10^{13} OBs/ha when applied in a spray volume of 1,000 l/ha, which is usual for fruiting tomato plants. Similarly, an unformulated strain of HearSNPV was tested for control of this pest at concentrations of 2.87 x 10^{8} - 1.35 x 10^{11} OBs/l in greenhouse and field-grown tomatoes and citrus in South Africa, although a rate of 1.15 x 10^{9} OBs/l (equivalent to 1.15 x 10^{12} OBs/ha in an application volume of 1,000 liters), was selected for further testing and provided excellent pest control (Moore et al., 2004). Studies elsewhere have indicated that applications of approximately 1 x 10^{12} OBs/ha provides control of this pest in Thailand, India and Botswana on a diversity of crops (Easwaramoorthy, 1998; Kunjeku et al., 1998; Roome, 1975). The high quantities of OBs that we applied, although highly effective as a pest control agent, likely require evaluation at lower concentrations (10^{11} - 10^{12} OBs/ha), in order to ensure that a virus-based product can be produced at an economically feasible cost (Daoust and Roome, 1974). We selected the higher concentration (10^{13} OBs/ha) rather than the lower one (10^{12} OBs/ha) given the fear that OBs would be rapidly inactivated due to the harsh conditions, with high temperatures and intense sunlight, that occur in southern Spain and Portugal during the summer months.

In greenhouses, the HearSP1B:LB6 OB treatment was as effective as spinosad at reducing larval infestation and slightly more effective than the *B. thuringiensis* treatment. However the degree of fruit damage was similar among virus, *B. thuringiensis* and spinosad treatments, which were consistently lower than that of the control. The reduction in infestation (close to 70%) observed in control plots at 10 days after insecticide application might have influenced the results. Previous studies have reported significant reductions in larval infestations after HearSNPV applications, comparable to those of *B. thuringiensis* or synthetic

insecticides, both in protected and field-grown tomato crops (Gupta et al., 2007; Moore et al., 2004; Ravi et al., 2008). Moore et al. (2004) also reported pest mortality exceeding 80% in control plots artificially infested with *H. armigera* eggs 10 days after treatment application, which was attributed to natural mortality and the inability to detect larvae once they had penetrated plants. Fortunately, the fact that both greenhouse trials produced near identical results provides strong additional support to the validity of these findings.

The timing of application is an important determinant of insecticide efficacy since phenological state plays a crucial role in the association of *H. armigera* with the tomato crop. For instance, before or after flowering, tomato plants are less attractive to ovipositing *H. armigera* females and mature tomatoes are similarly sub-optimal for larval development (Torres-Vila et al., 2003a). Therefore late insecticide applications, during the tomato maturation period, often have little or no effect in reducing larval damage, because larval densities in the crop are usually low during this phenological stage. This is most likely the reason why the fourth and fifth insecticide applications, which were applied when more than 50% of tomatoes were red, did not improve the degree of crop protection.

The percentage of damaged fruits at harvest was lower in HearSNPV OB treated plots (13.6-14.3% of damaged fruits) than in control plots (38.3%), and similar to that observed with the other insecticides tested (8.4-14.3%), which is in agreement with previous studies performed in Australia (Kay, 2007). As scarred red fruits are the only pest-damaged fruits accepted by processing plants, and given that most green and red rotten fruits are discarded at harvest, the similar yields of marketable tomato fruits (red undamaged fruits) obtained in plots treated with HearSNPV OBs, compared with plots treated with commercial insecticides, indicates that this virus can be a highly effective pest control agent. HearSNPV-based treatments resulted in less than 4% of scarred red fruits, comparable to those achieved with the commercial insecticides (2-4.2%), and markedly lower than that of control plots (14%). Field-grown tomato plots treated with HearSNPV OBs in India also proved as effective in crop protection as chemical insecticides (Gupta et al., 2007). Similarly, Cherry et al. (2000) also observed that chickpea crops treated with different formulations of HearSNPV OBs resulted in yields similar to those of plots treated with *B. thuringiensis* or chemical insecticides.

The persistence of HearSNPV OBs and *B. thuringiensis* on tomato leaves was markedly higher in the greenhouse than in the open field, whereas the residual persistence of spinosad was similar in both environments. The persistence of OBs on plant surfaces determines the period during which a lethal dose can be ingested by susceptible insects (Jones et al., 1993). Solar radiation is one of the most important factors affecting the persistence of OBs (Killick, 1990; Morris, 1971). Consequently, inactivation of HearSNPV OBs was slower in protected than in open-field crops, as the plastic structure and whitewash coating are able to filter a large part of incident UV radiation (Lasa et al., 2007; Smits et al., 1987). In protected crops 87% of HearSNPV OBs remained viable at 6 days after treatment, whereas in the open-field just 7% of OBs remained viable after 7 days. Similar levels of OB persistence were observed on greenhouse-grown sweet pepper, in which 61% of *Spodoptera exigua* MNPV (SeMNPV) OBs retained their insecticidal activity 6 days post-application (Lasa et al., 2007). Furthermore, the physico-chemical characteristics of the crop can also influence OB degradation as exudates of some plants can rapidly inactivate OBs. For example, OBs on cotton leaf surfaces were rapidly inactivated by alkaline leaf exudates (Young et al., 1977). Similarly, on chickpea HearSNPV OBs were degraded almost completely 7 days after treatment (Cherry et al., 2000), an effect attributed to the isoflavonoids present in leguminous plants (Stevenson et al., 2010). In contrast, tomato is reported to be more favorable for OB persistence on treated foliar surfaces (Farrar and Ridgway, 2000; Froschler et al., 1992; Young and Yearian, 1974). These observations underline the need for crop-specific persistence studies when developing a baculovirus-based bioinsecticide for use on different types of crops.

For the other insecticides, the persistence of spinosad and *B. thuringiensis* on protected tomato plants was lower than that of HearSNPV OBs. Like the virus, both these insecticides are of natural origin and have gained importance over the past decade as growers have adopted products to be used in integrated pest management programs that conserve natural enemy populations. The plastic structure of the greenhouse represents an important filter to the passage of UV light (Lasa et al., 2007; Smits et al., 1987), so that all the biological-based control measures that we tested likely benefited from partial protection from incident UV in the greenhouse setting. In contrast, in open-field crops, spinosad and chlorpyrifos persisted on tomato foliage for longer periods than HearSNPV OBs. The stability

of spinosad, although sensitive to sunlight (Saunders and Bret, 1997), might be related to its ability to penetrate leaf tissues by translaminar movement, increasing its persistence under field conditions (Tomlin, 2003).

The present study demonstrates that HearSP1B:LB6 OB applications, albeit at an elevated concentration, were as effective as commercial insecticides for controlling *H. armigera* damage in tomato crops. Moreover, the high persistence of OBs on tomato foliage favors the efficacy of this virus as an insecticide because pest larvae can consume OB-contaminated leaves over several days, increasing the likelihood of consuming a lethal dose. Additional advantages for baculovirus-based insecticides include a minimal safety period before harvest, the absence of xenobiotic residues in food produce, and virtually no impact on populations of natural enemies present in greenhouse or open-field crops. Moreover, as baculoviruses are compatible with other pesticides, they may be used in combination with other control agents in IPM programs in order to manage insecticide resistance (Eberle et al., 2012).

The results of this study are likely to provide useful efficacy information for the registration of the co-occluded HearSP1B:LB6 variant mixture as a biological insecticide for control of *H. armigera* in tomato crops in this region. Future studies should focus on determining the efficacy of lower OB concentrations and the use of formulations that improve the pest control characteristics of HearSP1B:LB6 OBs following their application to crops.

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CHAPTER IX

General discussion

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), causes important damage to field and greenhouse crops worldwide, exceeding annual losses of US$2 billion (Tay et al., 2013). In Spain, *H. armigera* is an important pest of peppers, cotton and maize, and is the key pest of tomato crops (Torres-Vila et al., 2003). Pest control based on chemical insecticides has led to the development of resistance to most of the active ingredients (Torres-Vila et al., 2000, 2002a, b). Therefore, research and development on more suitable and environmentally friendly control methods against *H. armigera* is a priority. Insect-infecting baculoviruses have been reported worldwide (Jehle et al., 2006; Moscardi et al., 1999), including those from *H. armigera*. In several countries, such as India, Australia and China, *H. armigera* is successfully controlled with applications of the *H. armigera* single nucleopolyhedrovirus, HearSNPV (Moscardi et al., 2011). Although several isolates of HearSNPV have been characterized in the Iberian Peninsula, none of them has been developed as a bioinsecticide product. Therefore the aim of the present thesis was to to design a new HearSNPV-based bioinsecticide to control *H. armigera* larvae in the Iberian Peninsula.

The first step in this process is the selection of the active ingredient that presents the most suitable insecticidal characteristics. Previous studies have shown a high intraspecific diversity of lepidopteran-infecting baculoviruses, which is reflected in high phenotypic diversity (Barrera et al., 2011; Bernal et al., 2013a; Erlandson, 2009). Moreover, local insect populations tend to be more susceptible to native virus isolates than to geographically distant strains, probably due to continuous host-pathogen co-evolution (Barrera et al., 2011; Erlandson, 2009). Additionally, from a practical point of view, indigenous isolates are easier to register (Muñoz et al., 1998). All this has prompted the need to find and evaluate the capacity of indigenous isolates from the Iberian Peninsula to control effectively *H. armigera* infestations in tomato crops, in order to select the most appropriate

one to be the active material of a baculovirus-based bioinsecticide. Therefore in **Chapter II**, seven isolates from different regions of the Iberian Peninsula (Figueiredo et al., 1999, 2009) were compared at the genomic and phenotypic level using a long-term laboratory colony of *H. armigera*. No major differences were found at genome level among the different Iberian isolates, and neither with the previously sequenced HearSNPV isolates (Chen et al., 2001; Ogembo et al., 2009; Zhang et al., 2005, 2014). However, the HearSP1 isolate presented the largest genome, whereas the other isolates lacked some restriction fragments present in HearSP1, suggesting smaller genomes. Phylogenetic analysis based on the conserved *polyhedrin*, *lef-8* and *lef-9* genes (Eberle et al., 2009) grouped Iberian strains together, suggesting a recent common ancestor. Iberian strains were more closely related to the isolate from Kenya, HearNNg1 (Ogembo et al., 2009), than to isolates from China or Australia (Chen et al., 2001; Zhang et al., 2005, 2014), possibly due to their geographical proximity. Those minimal differences found at the genome level were reflected in minimal differences at the biological level. The different HearSNPV isolates showed similar values of pathogenicity against the long-term insect population, being as pathogenic as isolates from Kenya, South Africa and China (Ogembo et al., 2007; Sun et al, 2002). However significant differences were observed in virulence. Speed of kill (MTD) values differed by 29 h with HearSP1 being the fastest-killing strain. Additionally, HearSP1 was one of the most productive isolates in spite of being the fastest killing. Prior to the development of a baculovirus-based insecticide the susceptibility of a local insect colony to native virus strains has to be evaluated, as in general, colony-dependent differences have been observed in susceptibility to NPVs; in general, local insect populations tend to be more susceptible to local virus isolates than to geographically distant strains (Barrera et al., 2011; Erlandson, 2009). Subsequently, a short term insect colony was started from a local field population and used to determine the host stage response to the most active isolate HearSP1, which was compared to that of a commercialized genotype from China, HearG4 (Chen et al., 2001; Zhang, 1994; Zhang et al., 1981). Although HearSP1 and HearG4 had similar pathogenicity against all instars tested, HearSP1 was faster killing in all instars than HearG4, indicating that local insect colonies are more susceptible to native virus isolates, as has been observed in other baculovirus host systems (Barrera et al., 2011; Erlandson et al.,

2007). These results proved that HearSP1 has suitable insecticidal characteristics to be the base of a biological insecticide to control *H. armigera* populations in Iberian Peninsula.

However, wild-type isolates that can be collected as natural isolates are not patentable, and additionally, specific genotypes or mixtures of genotypes could improve the insecticidal traits of the wild-type isolates (Bernal et al., 2013b; Clavijo et al., 2009; Espinel-Correal et al., 2012; Simón et al., 2004). Therefore, in **Chapter III** the genotypic interactions that modulate insecticidal characteristics were studied to develop novel products based on unique user-defined genotypic combinations in specific proportions that result in improved insecticidal properties of the final mixture. For this, the genotypic variants present within the most active Iberian strain selected in Chapter II, HearSP1, and those present within *H. armigera* larvae captured in Lebrija and died during an epizootic under laboratory conditions (HearLB) were isolated and characterized. Larvae that died during this epizootic belonged to the second laboratory generation of a colony established from larvae collected in cotton crops, so the virus might be maintained in the population as a covert infection, as has been described for other virus-host systems (Cabodevilla et al., 2011; Hughes et al., 1993; Simón et al., 2010; Vlak et al., 1981). In contrast, HearSP1 seems to be a horizontally transmitted isolate, as it was the most widespread and predominant isolate during a period of a high *H. armigera* infestation in tomato fields in the Iberian Peninsula (Figueiredo et al., 1999), which confirmed its high capacity of producing natural epizootics and to be transmitted horizontally. The fact that the baculovirus-based bioinsecticide contains both horizontal and vertical transmission genotypes appeared interesting, as it could adopt horizontal or vertical routes of transmission in certain moments in which environmental or ecological conditions are favourable for peroral infection (horizontal) or else when they are not favourable (vertical), such as low insect density (Burden et al., 2003).

Only two genotypic variants, HearSP1A and HearSP1B, were isolated from HearSP1-wt by plaque assay in cell culture. However, Sun et al. (1998) isolated seven HearSNPV genotypes by *in vivo* purification. Similarly, Wang et al. (2003) isolated ten HearSNPV genotypes from a single wild type strain using BAC cloning. Therefore, other genotypes may have also been present in the HearSP1-wt, but were not amenable to replicate in cell culture. The idea that some

HearSNPV genotypes replicate poorly in cell culture is supported by a previous study, in which only between one and five genotypes were isolated from different HearSNPV strains by plaque assay (Ogembo et al., 2007). Regarding the HearLB isolates, unexpectedly the seventeen HearLB-killed larvae analysed were each infected by a single genotypic variant, which were classified into six different genotypes by their *Eco*RI restriction profiles. Although the different genotypes differed minimally in their restriction profiles, they showed differences in biological activity, as occurs among other genotypes of many *Alphabaculoviruses* (Barrera et al., 2013; Bernal et al., 2013b) and HearSNPVs (Ogembo et al., 2007). HearSP1B was ~3 fold more pathogenic than HearSP1-wt. In contrast, among the HearLB variants, no differences were found in pathogenicity, although differences were observed in speed of kill, with HearLB1 and HearLB6 being among the fastest-killing variants, even more so than HearSP1-wt which was previously shown to be one of the fastest killing isolates (Arrizubieta et al., 2014). These results are in line with previous studies performed with different variants of HearSNPV, in which variants of similar pathogenicity presented marked differences in speed of kill (Arrizubieta et al., 2014; Guo et al., 2006). Generally, the fastest variants tend to produce lower OB yields, because insects die faster and the virus has less time to replicate (Barrera et al., 2011; Chen et al., 2000; Guo et al., 2006; Sun et al., 2005). However, HearLB1 was highly productive, despite being one of the fastest-killing variants. This fact has also been observed in other alphabaculovirus species, such as *Spodoptera exigua* MNPV, SeMNPV, in which a highly productive genotype was also among the fastest killing genotypes present in the population (Cabodevilla et al., 2011).

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 proved of great value for the development of several novel virus insecticides (Caballero et al., 2009, 2013, 2014). Therefore with the aim of selecting a genotypic mixture with improved insecticidal activities, eight co-occluded mixtures were constructed following *per os* inoculation (Bernal et al., 2013b), and using five genotypes selected based on their insecticidal properties (HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6). Surprisingly the genotypic mixture that included the most pathogenic genotype, HearSP1B, and the most virulent one, HearLB6, at 1:1 proportion (HearSP1B:LB6) was even

more pathogenic than the pure genotype HearSP1B. However this mixture, although showing a similar speed of kill, did not maintain the productivity of HearLB6. Nevertheless, in the field, in which large quantities of OBs are applied, the high pathogenicity and virulence might result in the rapid suppression of the pest using a low amount of product. According to these results, HearSP1B:LB6 was selected for development as a bioinsecticide for control *H. armigera* larvae in the Iberian Peninsula. The biological activity of this mixture was comparable to that of other baculoviruses previously commercialized as bioinsecticides, such as SeMNPV, which was successfully used to control *S. exigua* pest in greenhouses in Southern Spain (Caballero et al., 2009), *Chrysodeixis chalcites* SNPV, ChchSNPV, which has been recently developed as a bioinsecticide to control *C. chalcites* pest in the Canary Islands (Caballero et al., 2013), and also HearG4 genotype, that has been used extensively to control *H. armigera* pest on cotton crops in China (Zhang, 1994).

The co-occlusion of HearSP1B and HearLB6 in the same OB was confirmed by qPCR. However, prior sequencing of the complete genome of HearSP1B and HearLB6 was necessary to design the specific primers for each genotype (**Chapter IV and V**). For comparison, the genomes of HearSP1A, HearLB1 and HearLB3 were also included in the analysis in order to determine the genetic changes responsible for the differences observed in the biological activity of the viruses. Sequencing allowed the determination of genetic variation and also facilitated the detection of positively selected genes that could be responsible for the phenotypic differences (Cory et al., 2005; Harrison, 2009; Hitchman et al., 2007; Hughes et al., 1983; Simón et al., 2008). In **Chapter IV**, the accession numbers of the complete sequences are given with a short description of their genomes. The genomes sizes of HearSP1A (accession number KJ701032), HearSP1B (KJ701033), HearLB1 (KJ701029), HearLB3 (KJ701030) and HearLB6 (KJ701031) were 132,481, 132,265, 131,966, 130,949 and 130,992 bp in size, respectively, very close to the genome sizes of the *Helicoverpa* spp. SNPV genotypes sequenced previously (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). These differences in genome sizes were attributed predominately to the homologous regions (*hr*) and *bro* genes, and also to short deletions and insertions along the whole genome. In **Chapter V** the whole genome sequence alignment revealed that the Spanish genotypes showed a high degree

of homology with one another (97.9-99.7%), and with the previously sequenced HearSNPVs and HzSNPV (95.5%-98.8%). As mentioned in Chapter II the Kenyan genotypes genome, HearNNg1, was the most identical to the five Spanish genomes, probably due to their geographical proximity, suggesting that they probably evolved from the same ancestor. As described for other *Helicoverpa* spp. SNPVs, the major differences among these Spanish genotypes were attributed to the baculovirus repeat ORFs (*bro*) genes and the homologous regions (*hrs*). Therefore the phenotypic differences observed among the HearSNPVs genotypes might be explained by differences in *bro* genes and *hrs* (Chen et al., 2001, 2002; Ogembo et al., 2009; Zhang et al., 2005, 2014). However, point mutations observed in several ORFs could also be related to the phenotypic differences found among the Spanish HearSNPV isolates. As such, the increased productivity of HearLB genotypes compared to HearSP1 genotypes appeared to be related to point mutations found in genes involved in DNA replication (*ie-1*, *lef-3 DNA polymerase*) or viral transcription (*lef-8*, *lef-1*) as these mutations could influence late gene expression and virion production, and therefore OB production (Thézé et al., 2014). Moreover, these differences in OB yield might also be influenced by the mutations observed in structural genes (*p78/83, vp1054 desmop*, *calyx/pep*, *odv-e66*) involved in in primary infection and/or BV and ODV formation, affecting viral pathogenicity (Braunagel et al., 2003; Cohen et al., 2009). Additionally, those differences in OB production could also be attributed to differences in the *hrs*, as they clearly influence *ie-0*, *ie-1* and *polyhedrin* transcription, being affected BV and ODV production (Carson et al., 1991; Habib and Hasnain, 1996; Habib et al., 1996; Schultz et al., 2009; Stewart et al., 2005). The difference in the length of the *bro-a, bro-b* and *bro-c* genes of HearSP1B compared to those of the other genotypes could also be related to its increased pathogenicity, as previous results showed that HearC1 and HearNgg1 genotypes, with a shorter version of BRO-A protein in comparison with that of HearG4, were ~3 and ~300 fold more pathogenic than HearG4 against a Chinese and Japanese *H. armigera* colony, respectively (Ogembo et al., 2007; Zhang et al., 2005). As shown in previous studies, genes involved in the insect apoptosis inhibition (*iap-2*, *iap-3*) and *hoar* gene could be responsible of the transmission strategy or the ability to establish a covert infection in the insect host (Thézé et al., 2014), as different mutations were observed in these genes between the presumably horizontally transmitted

genotypes, HearSP1A and HearSP1B, and the vertically transmitted ones, HearLB1, HearLB3 and HearLB6. Furthermore, the presence of *bro-b* gene in HearSP1 genotypes and its absence in HearLB genotypes could have resulted from virus-host co-evolution (Zhang et al., 2014), as both groups were isolated from different geographical sources in the Iberian Peninsula and might be specialized in different routes of transmission. Experiments are in progress to determine the role of each of these genes in the phenotypic characteristics, with the aim of designing more effective viruses to be used in field.

Finally, in **Chapter V,** selection analysis (Yang, 1998) was performed that allowed identifying positively selected sites in proteins of high importance during viral infection, virus evolution or host adaptation (Furuse et al., 2009; Kuo et al., 2013; Rahm et al., 2013; Sérémé et al., 2014; Simón et al., 2011). Eight genes were identified undergoing diversifying selection; *p78/83*, *ie-0*, *odv-e56*, *p74*, *bro-d*, *egt*, *bv-ec31* and ORF130 of unknown function. All of these ORFs have homologs in other baculoviruses, which makes them candidates as being important in insect-virus relationships that have evolved to exploit the host machinery in the most effective manner (Dall et al., 2001). *ie-0* is essential for viral infection and replication (Stewart et al., 2005) and *p78/83*, *odv-e56* and *p74* genes encode for proteins of BVs and ODVs, influencing primary and secondary infection (Braunagel et al., 1996; Faulkner et al., 1997; Wang et al., 2008). As such, the positive selection of these genes may facilitate virus adaptation to a given host, and therefore, overcome the immune response of the insect. Furthermore, the *egt* gene is involved in the development of the insect host, resulting in a higher OB yield (Cory et al., 2004). Hence, diversifying selection of these genes may facilitate virus dispersion and transmission. Several experiments are in progress to evaluate the effect of these genes in HearSNPV phenotype, which might help to design more effective control methods based on this virus.

After sequence analysis, the specific primers for qPCR were designed for each genotype. qPCR analysis of OBs obtained from larvae that died after having consumed a single OB of HearSP1B:LB6 confirmed that HearSP1B and HearLB6 genotypes were co-occluded in the same OB, as both HearSP1B and HearLB6 were amplified from all OB samples obtained from each larva. Thereafter the genetic and biological stability throughout successive passages *in vivo* of the selected co-occluded mixture was performed in order to ensure that the product

has the same insecticidal characteristics as the original stock. Interestingly, the relative frequency of HearSP1B genotype increased along the passages, probably due to the increased pathogenicity of HearSP1B compared to HearLB6, which might increase its replication and transmission capacity. The accumulative frequency of HearSP1B was directly correlated with its biological activity. In fact, the OBs obtained after five passages, which comprised 85% of HearSP1B and 15% of HearLB6, were more pathogenic and virulent than the co-occluded mixture comprising both genotypes at a 1:1 proportion. These results suggest that the co-occluded 85%HearSP1B:15%HearLB6 mixture might be more suitable as the active material. This process represents a unique mechanism for selection of biological materials for use in biological insecticide, 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 *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) that were subjected to successive passages *in vivo* rapidly converged to the frequencies present in the wild type isolate (Clavijo et al., 2009; Simón et al., 2006). However, the genotypes HearSP1B and HearLB6 were isolated from different wild type isolates and this genotypic mixture reached genotypic frequencies that apparently favored improved transmission.

Although the selected mixture HearSP1B:LB6 showed desirable insecticidal characteristics against *H. armigera* larvae, its specificity might be a problem in the field, as it is only infective to *Helicoverpa* spp. insects (Gettig and McCarthy, 1982). However in some circumstances a crop is also attacked by other lepidopteran pest species, as occurs in tomato and cabbage crops in the Iberian Peninsula, which are attacked simultaneously by *H. armigera* and *M. brassicae* (CABI, 2014; Cartea et al., 2010; Rojas et al., 2000; Torres-Vila et al., 2003), or in soybean and cotton crops in South American, in which *S. frugiperda* and *H. armigera* can be present simultaneously (Casmuz et al., 2010; Czepak et al., 2013). Moreover *H. zea*, which is also susceptible to HearSNPV (Gettig and McCarthy, 1982), is also present in combination with other pest in several crops in the Americas (CABI, 2014). In such situations, HearSNPV specificity becomes a limiting factor for which the use of a baculovirus with a broader host range is desirable. Therefore, the **Chapter VI** aimed to obtain an OB sample that co-enveloped both HearMNPV and HearSNPV into the same ODV, in order to obtain

a mixture with the desirable insecticidal characteristics of HearSNPV (Sun and Zhang, 1994), and the broader host range of HearMNPV (Rovesti et al., 2000; Tompkins et al., 1988). Previous studies performed in our laboratory showed that a single host cell can be infected at least by two different genotypes of a multiple-nucleocapsid alphabaculovirus (Clavijo et al, 2010). Furthermore, it has been shown that a single insect cell can be co-infected with at least two genotypes from different multiple-nucleocapsid alphabaculovirus species, provided that co-infection occurs within a given time window that appears to vary depending on the insect and the viruses used in the co-occlusion (Beperet, 2014a). The co-infection of Sf9 cells by SfMNPV is only possible within 20 h following infection by *Autographa californica* MNPV, AcMNPV; after this time, an absolute block prevents SfMNPV co-infection (Beperet, 2014b). Co-infection of *S. frugiperda* larvae occurred in single cells with greater frequency in the said time window, however after this time no exclusion to the second virus infection was detected (Beperet, 2014b). These findings offer new possibilities in the custom-design of biological insecticides, with the aim of controlling specific complexes of pests in agricultural crops.

As HearMNPV was ~6 times less infective than HearSNPV, *H. armigera* larvae were firstly inoculated with HearMNPV and at different time intervals with HearSNPV, following the protocol developed by Beperet (2014b). Hence, the HearMNPV isolate had time to initiate its replication in *H. armigera* cells before HearSNPV replication occurred. As expected, larvae inoculated at the same time (T0 sample) with both viruses produced OB progeny composed mostly of HearSNPV (97%); as the most pathogenic virus, HearSNPV had an advantage over HearMNPV. In contrast, HearMNPV was the dominant virus in OB progeny obtained from larvae inoculated at 48 (T48) and 72 (T72) hours intervals (96-97%), as most of the tissues were already colonized at the time of the second infection, likely preventing co-infection by another virus, as shown in other host-pathogen systems (Beperet, 2014b; Ellenberg et al., 2007; Schaller et al., 2007; Tscherne et al., 2007; Xu et al., 2013). However, when HearSNPV was inoculated 12 (T12) or 24 (T24) hours later than HearMNPV, the proportions of both isolates in the OB progeny were similar, suggesting that this time interval could offset the replication disadvantage of HearMNPV in *H. armigera*. The ODVs released from these OBs resolved into several bands by ultracentrifugation, each

corresponding to ODVs with a specific number of nucleocapsids. HearSNPV was detected by PCR in multiple ODV bands, suggesting that HearSNPV nucleocapsids were enveloped within HearMNPV ODVs. Therefore in **Chapter VI**, we demonstrated for the first time the co-envelopment of a SNPV within a MNPV. This was confirmed by end point dilution assay performed with serial dilutions of ODVs released from the T12 and T24 OB samples. In cells infected by a single ODV both HearSNPV and HearMNPV were detected, demonstrating that nucleocapsids of HearSNPV and HearMNPV were enveloped in the same ODV. However, T12 and T24 samples comprised ~50% of HearSNPV genomes but just ~1% was co-enveloped, indicating that most of the HearSNPV genomes were present as single-nucleocapsid ODVs and most of the multiple-nucleocapsid ODVs (99%) comprised HearMNPV genomes. This lower co-envelopment proportion might explain the absence of wells infected by HearMNPV alone, as HearMNPV would not be able to initiate the infection in cells due to its lower replication capacity in the HzAM1 cell line compared with HearSNPV. However, when both viruses are co-enveloped in the same ODV, HearSNPV could act as a helper facilitating the entry and replication of HearMNPV into cells as previously observed with other NPV genotypes (Clavijo et al., 2010; López-Ferber et al., 2003), and also with viruses pathogenic to plants (Stenger, 1998) and mammals (Ecknar, 1973). This lower rate might also suggest a barrier for co-envelopment of single NPV within multiple NPVs, as previous results demonstrated that two genotypes of SfMNPV could be co-enveloped in one out of three ODVs (Clavijo et al., 2010). However, the reason(s) for this natural barrier is still unknown.

However, the co-envelopment of HearSNPV and HearMNPV showed no significant interaction in terms of pathogenicity between HearSNPV and HearMNPV, either in the homologous host, *H. armigera*, or heterologous hosts such as *S. frugiperda* and *M. brassicae* larvae. This might be due to the fact that although ~50% of ODVs were composed by both HearSNPV and HearMNPV, just 1% of HearSNPV genomes was present in the multiple ODVs. Therefore these mixtures were composed mostly by single HearSNPV and multiple HearMNPV, and just 1% by both genotypes. Therefore, both viruses might act independently, and be transmitted in a similar manner. Thus the OB mixture was as pathogenic as the co-enveloped mixture. This independent transmission between co-occluded genotypes has been observed in co-enveloped and OB mixtures of AcMNPV and

SfMNPV inoculated in *S. frugiperda* and *S. exigua* larvae (Beperet, 2014b). Interestingly the co-envelopment increased the host range of HearSNPV, as HearSNPV could be maintained in the OB progeny at least during 5 passages in non-susceptible hosts, *S. frugiperda* and *M. brassicae* larvae, although its proportion decreased rapidly. The co-envelopment of HearSNPV within the same ODVs as HearMNPV might favour the infection of non-susceptible midgut cells. In contrast, in OB mixtures, HearSNPV would be lost in the first passage, as only HearMNPV ODVs would penetrate into non-susceptible midgut cells. Therefore, the co-envelopment of HearSNPV and HearMNPV could overcome the problem of the narrow host range of HearSNPV, favouring the control of specific complexes of agricultural pests using a unique baculovirus based bioinsecticide. However, it would be necessary to produce and test different HearSNPV:HearMNPV mixtures in *H. armigera* larvae, or even in other susceptible species to achieve a higher frequency of HearSNPV in multiple ODVs and therefore, better insecticidal properties of the mixture.

According to the results presented so far the HearSP1B:LB6 mixture was selected as the active material for a bioinsecticide product aimed at reducing the crop damage produced by *H. armigera* in the Iberian Peninsula. Following this, the next step was to determine the feasibility of large scale production, as viruses that cannot be massively produced are not commercialially viable (Claus and Sciocco de Cap, 2001; Szewczyk et al., 2006). Therefore, **Chapter VII** aimed to optimize the production conditions in order to reduce the production costs and make the product commercially competitive. As baculoviruses are obligate pathogens, the virus production may be performed in host larvae (*in vivo*) or in cell culture (*in vitro*). However, due to the high cost of *in vitro* production, in addition to mutations and deletions that occur during serial passage (Chakraborty and Reid, 1999; Dai et al., 2000; Pijlman et al., 2004), *in vivo* production is the most suitable option for massive virus production. Furthermore, due to the high specificity of HearSNPV (Gettig and McCarthy, 1982; Gröner, 1986; Herz et al., 2003; Ignoffo and Couch, 1981), the production of HearSP1B:LB6 must be performed in *H. armigera* larvae. Several factors that influence OB production in *H. armigera* larvae, such as cannibalism rate, larval age, inoculum concentration and incubation temperature were studied. Those factors allowed us to select the optimal conditions for the maximum larval growth after viral inoculation, reaching the maximum viral

mortality, which in turn maximizes the OB production (Ignoffo and Couch, 1981). In the present study, the cannibalism rate was very high in all three instars tested (L_3, L_4 and L_5) and increased markedly with larval density. In contrast to previous studies that showed that cannibalism is instar dependent (Boots, 1998; Chapman et al., 1999; Dong and Polis, 1992; Polis, 1981), cannibalism rates in *H. armigera* were similar among the different larval stages, as observed in *H. zea* (Chilcutt, 2006). Additionally *H. armigera* larvae inoculated at later instars achieved a higher OB yield (6.7-9.1 x 10^9 OBs/larva), values comparable to those obtained by infecting later instars *H. armigera* (1.7 x 10^9 - 1.2 x 10^{10} OBs/larva) (Gupta et al. 2007a; Kalia et al. 2001; Mehrvar et al. 2007). However, although in L_3 and L_4 infection status had no significant effect on cannibalism, in L_5 cannibalism was significantly higher among infected larvae than among healthy larvae, which was attributed in previous studies to lower mobility and sluggish responses of diseased larvae, making diseased insects more likely to be victims of conspecific predation than healthy insects (Dhandapani et al., 1993; Boots, 1998; Williams and Hernandez, 2006). During OB production procedures, cannibalism is not desirable as the total number of larvae is reduced, which reduces the overall OB yield (Chapman et al. 1999; Shapiro et al. 1986). Therefore, the high cannibalism observed in late infected instar made it necessary to rear *H. armigera* larvae individually for efficient production of HearSP1B:LB6 OBs. The effect of L_5 larval age was also determined, as previous studies showed that *C. chalcites* larvae inoculated with its homologous virus 24 h after molting produced up to 6 times more OBs than those inoculated shortly following their molt (Bernal, 2014). However in the present study similar OB yields were obtained when L_5 larvae were inoculated 24 h after molting compared to newly molted larvae, however the mortality rate was much lower. Therefore, the total OB yield was higher using newly molted L_5 larvae as it resulted in higher mortality. Additionally, the OB inoculum consumed by larvae can have an important effect of the final OB production since high doses of OBs can hasten the death of the larva, resulting in reduced gain weight during infection and consequently fewer OBs produced in each insect (Grzywacz et al., 1998; Kalia et al., 2001). However, inoculum OB concentrations tested in the present study resulted in similar percentages of mortality and OB yields from experimental insects, as previously observed in other host-pathogen systems (Bernal, 2014), and OB production per mg of larval body

weight did not vary significantly among the different inoculum concentrations. Therefore the lower OB concentration, LC_{80} (5.5 x 10^6 OBs/ml), was selected as the optimum inoculum concentration for the production of HearSP1B:LB6 in L_5 *H. armigera* larvae as it resulted in the maximal OB yield using the least virus stock. Finally, OB production may also be influenced by incubation temperature, as it directly affects larval development and the rate of virus replication (Subramanian et al., 2006). At high temperatures larvae feed and grow faster, and cell metabolism is accelerated resulting in faster virus replication, which can lead to premature host death and reduced OB yields compared to insects reared at lower temperatures (Mehrvar et al., 2007; Shapiro et al., 1981; Subramanian et al., 2006). In the present study, larvae reared at 30°C died 13 or 34 h sooner than those reared at 26 or 23°C, respectively. Similar effects have been reported in other species, including *S. litura* (Subramanian et al., 2006), *Diatraea saccharalis* inoculated with heterologous NPVs (Ribeiro and Pavan, 1994), *Trichoplusia ni* inoculated with AcMNPV (van Beek et al., 2000) or *Anticasia gemmatalis* inoculated with the homologous virus (Johnson et al., 1982). High incubation temperatures might affect the insecticidal properties of the OBs, as they favor the propagation of bacterial contaminants, which contribute to OB degradation (Jenkins and Grzywacz, 2000). However in the present study incubation temperature did not affect OB quality, as the biological activity of the recovered HearSP1B:LB6 OBs was similar to that of the original stock virus, which is in agreement with previous studies performed on *S. litura* infected with its homologous NPV (Subramanian et al., 2006). According to these results the efficient production of HearSP1B:LB6 co-occluded mixture can be achieved by inoculating L_5 newly molted *H. armigera* with the LC_{80} (5.5 x 10^6 OBs/ml), followed by incubation of individualized larvae at 30ºC. Using this system 5 x 10^{11} OBs would be produced in approximately 5 days by infecting 100 larvae. Similar HearSNPV productions were obtained in previous studies in *H. armigera* larvae (Gupta et al., 2007a; Kalia et al., 2001; Mehrvar et al., 2007).

Finally, registration of bioinsecticidal products requires field trails to be performed under typical crop production conditions to demonstrate the efficacy of the product for pest control, as this might vary between laboratory, greenhouse and open field conditions (Arrizubieta et al., 2014; Bianchi et al., 2000; Dwyer et al., 2005; Grant and Bouwer, 2009). Whereas in laboratory all conditions are

controlled, several factors can affect virus efficacy under greenhouse or especially under open-field conditions; these factors include temperature, rainfall, plant exudates, etc. (Elleman and Entwistle, 1985; Hoover et al., 1998; Ignoffo, 1992; Moscardi, 1999; Young et al., 1977). However differences detected under laboratory conditions might not be appreciated in field (Barrera, 2013), as large quantities of OBs are applied in the field for the rapid suspension of the pest, additionally environmental conditions might obviate this difference. Additionally, the efficacy of a virus-based insecticide is also strongly influenced by its persistence on the surface of the crop plant, as with greater persistence the probability of the pest consuming a lethal dose of OBs over time also increases (Jones et al., 1993). Solar ultraviolet (UV) radiation is the main factor affecting the persistence of OBs deposited on plant surfaces (Ignoffo, 1992). However, the incidence of UV radiation can vary greatly from region to region, with crop phenology, and with growing conditions. For example, exposure to UV radiation is greatly reduced in greenhouse-grown crops compared to those grown in an open field, as the greenhouse's plastic structure filters much of the incident UV (Lasa et al., 2007).

Therefore in **Chapter VIII** the efficacy of HearSP1B:LB6 OBs for the control of *H. armigera* and the persistence of OBs on tomato crops under both protected and open-field conditions were determined as a contribution to the registration of the binary genotypic mixture as the active ingredient of an insecticidal product. Initial growth chamber studies, performed to select the optimal HearSNPV concentration to be applied in field, showed that 1 x 10^{10} OBs/l (equivalent to 1 x 10^{13} OBs/ha) of HearSP1B:LB6 resulted in 100% mortality of experimental larvae on treated tomato plants. Previous studies have indicated that applications of 10^{12} OBs/ha provided effective control of this pest on a diversity of crops in different part of the world (Easwaramoorthy, 1998; Kunjeku et al., 1998; Roome, 1975). However, we selected a higher concentration for use in the field (10^{13} OBs/ha), as in the field OBs might be rapidly inactivated due to the harsh conditions, high temperatures and intense sunlight that occur in southern Spain and Portugal during the summer months. In the field, the co-occluded mixture, although being more effective under laboratory conditions, was as effective as HearSP1 (Barrera, 2013). Additionally, HearSP1B:LB6 applications provided comparable control to commercial insecticides, including spinosad, *B. thuringiensis* and chlorpyrifos, both

in protected and open-field tomato crops in the Iberian Peninsula. In greenhouses, the HearSP1B:LB6 treatment was as effective as spinosad at reducing larval infestation and slightly more effective than *B. thuringiensis*, as shown in previous studies (Gupta et al., 2007b; Moore et al., 2004; Ravi et al., 2008). However the degree of fruit damage was similar among HearSP1B:LB6, *B. thuringiensis* and spinosad treatments, which were consistently lower than that of the control. In open-field tomato crops, the percentage of damaged fruits at harvest was ~25% lower in HearSP1B:LB6 OB treated plots than in control plots, and similar to that observed with the other insecticides tested, which is in agreement with previous studies performed in Australia (Kay, 2007) and India (Gupta et al., 2007b). In addition, HearSNPV persisted on tomato leaves over several days, so larvae could acquire the virus infection during this period after virus treatment, increasing its efficacy (Jones et al., 1993). The persistence of HearSNPV OBs on tomato leaves was markedly higher in the greenhouse than in the open field, as the plastic structure and whitewash coating are able to filter a large part of incident UV radiation (Lasa et al., 2007; Smits et al., 1987). Additionally, the persistence of HearSNPV on protected tomato plants was higher than those of spinosad and *B. thuringiensis*. In contrast, in open-field crops, spinosad and chlorpyrifos persisted on tomato foliage for longer periods than HearSNPV OBs. Additional advantages for baculovirus-based insecticides include a minimal safety period before harvest, the absence of xenobiotic residues in food produce, and virtually no impact on populations of natural enemies present in greenhouse or open-field crops. The results of **Chapter VIII** demonstrated that HearSP1B:LB6 could be used to control effectively pest infestations caused by *H. armigera*. Moreover, using HearSNPV as a unique control method would reduce toxic residues in the environment and food and improved populations of natural enemies contributing to a better pest control.

That said, the use of a baculovirus-based bioinsecticide as a single pest control method might result in the development of resistance in insect populations, as shown previously. *Cydia pomonella* developed resistance to CpGV, which has been used massively as a unique control method during recent years (Asser-Kaiser et al., 2007; Shulze-Bopp and Jehle, 2012; Undorf-Spahn et al., 2012). However, insect resistance can be avoided using different genotypes or isolates from the same virus species (Berling et al., 2009). Therefore, determining the natural diversity of virus populations is of special interest in order to select new

genotypes to overcome resistance problems. In this thesis, several genotypes and genotypic mixtures have been studied. So, in case *H. armigera* develops resistance to the HearSP1B:LB6 mixture, other genotypes or new mixtures could be used to control this pest. Additionally, insecticide resistance could be prevented leaving untreated areas in the crop, which may act as refuge for susceptible insects, diluting population resistance by facilitating breeding with resistant individuals (Torres-Vila et al., 2002a, b; Jehle et al., 2010). Additionally, HearSNPV may be used in combination with other insecticides, both biological, such as *Bacillus thuringiensis*, and chemical, such as spinosad or synthetic insecticides, in order to manage insecticide resistance (Duraimurugan and Regupathy, 2005).

Biological control of *H. armigera* in tomato crops in the Iberian Peninsula had not been thoroughly studied until now. In the present thesis, intraspecific diversity and genotypic interactions of HearSNPV were evaluated, and the genotypic mixture HearSP1B:LB6 was shown to provide effective control of *H. armigera* in tomato crops, comparable to that of chemical insecticides. Moreover, an optimized OB production system was defined to reduce the costs of production. These results showed that HearSP1B:LB6 deserves to be registered as a biological insecticide to control *H. armigera* pest on tomato crops in Spain. For this reason, the HearSP1B:LB6 genotypic mixture has been the subject of a patent application (P201430956) (Caballero et al., 2014).

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CONCLUSIONES

1. El análisis molecular y biológico de siete aislados de HearSNPV originarios de la Península Ibérica puso de manifiesto que todos ellos son mezclas heterogéneas de genomas homólogos, con un elevado grado de identidad, y tienen propiedades insecticidas similares para las larvas de una población de laboratorio de *H. armigera*. Excepcionalmente, el aislado HearSP1 fue el más virulento y uno de los más productivos.

2. Los aislados HearSP1 y HearG4 fueron igualmente patogénicos para los diferentes estadios larvarios de *H. armigera*; sin embargo, el aislado HearSP1 fue significativamente más virulento que el resto de aislados en todos los estadios larvarios de *H. armigera*. El aislado HearSP1, que interesantemente fue tan productivo como el HearG4, fue seleccionado como material base para el desarrollo de un insecticida biológico.

3. El análisis de la estructura genética de la población silvestre HearSP1 reveló la presencia de al menos dos variantes genotípicas (HearSP1A y HearSP1B) mientras que en el aislado HearLB se identificaron seis distinguibles por su perfil REN (HearLB1 a HearLB6). La caracterización insecticida de dichos genotipos puros mostró que el valor de la CL_{50} de HearSP1B fue significativamente (~3 veces) menor que la del resto de genotipos y que la de los dos aislados silvestres (HearSP1 y HearLB). Los valores de la CL_{50} correspondientes a los genotipos puros clonados de HearLB no difirieron significativamente entre si; sin embargo, los genotipos HearLB1 y HearLB6 mataron las larvas más rápidamente y el genotipo HearLB1 fue el más productivo (OBs/larva) a pesar de ser uno con tiempo letal más corto.

4. Los genotipos HearSP1A, HearSP1B, HearLB1, HearLB3 y HearLB6 fueron seleccionados por sus propiedades insecticidas y utilizados para producir OBs compuestos por diferentes mezclas co-ocluidas de dos o más genotipos en una determinada proporción relativa. La mezcla HearSP1B:LB6, en la proporción 1:1, fue entre 1,7 y 2,8 veces más

patogénica que cualquiera de los genotipos individuales y que el resto de mezclas evaluadas. Además, la virulencia de dicha mezcla fue similar a la de los genotipos más virulentos y, consecuentemente, fue seleccionada como materia activa del nuevo bioinsecticida.

5. Las frecuencias genotípicas en la mezcla co-ocluida HearSP1B:LB6 varió, tras cinco pases sucesivos en larvas, del valor original 1:1 al de 5:1, lo cual supuso una mejora significativa de la patogenicidad y virulencia. Esto sugiere que un mayor número de pases sucesivos podría a otra proporción relativa de genotipo que mejore aún más la transmisibilidad de la mezcla.

6. La secuenciación completa del genoma de los cinco genotipos puros (HearSP1A, HearSP1B, HearLB1, HearLB3 y HearLB6) mostró un alto grado de identidad a nivel de nucleótido entre ellos (98-99%) y con los SNPV de *Helicoverpa* spp. previamente secuenciados (95-99%). Las mayores diferencias entre todos los genotipos se localizaron en las *hrs* y en los genes *bro*, que podrían ser responsables de las diferencias insecticidas.

7. Las mutaciones puntuales localizadas en genes implicados en la replicación del ADN (*ie-1*, *lef-3*, *DNA polimerasa*), la transcripción viral (*lef-8*, *lef-1*), o genes estructurales (*p78/83*, *desmop*, *vp1054*, *calix/pep*, *odv-e66*) podrían ser responsables de la menor producción de OBs de los genotipos de HearSP1 o mayor patogenicidad del genotipo HearSP1B.

8. El análisis de presión de selección mostró una fuerte selección en genes implicados en la infección primaria (*p78/83*, *odv-e56* y *p74*), la infección y replicación viral (*ie-0*) o la transmisión viral (*egt*). Otros genes seleccionados positivamente son de función desconocida (*bro-d*, *bv-ec31* y ORF130).

9. Las nucleocápsidas de HearSNPV se co-envolvieron con las del virus de espectro de huéspedes más amplio, HearMNPV. Por primera vez, un SNPV se co-envolvió con un MNPV, y esta estrategia permitió ampliar la especificidad de HearSNPV a especies no-susceptibles. La co-envoltura no mejoró las características insecticidas contra huéspedes homólogos o

heterólogos pero demostró ser una herramienta para la producción de baculovirus no recombinantes con un espectro de huéspedes más amplio, y por tanto suprimir la limitación de la especificidad de varios baculovirus.

10. La producción masiva más eficiente de la mezcla co-ocluida HearSP1B:LB6 se obtuvo mediante la inoculación de larvas de *H. armigera*, recién mudadas a $L_{5,}$ con la CL_{80} (5,5 x 10^6 OBs/ml), y manteniéndolas durante el proceso infeccioso individualizadas y a 30ºC.

11. Un bioinsecticida basado en HearSP1B:LB6 es un método de control eficaz frente a plagas causadas por *H. armigera* en tomate. En los cultivos de tomate protegidos, HearSP1B:LB6 redujo el porcentaje de frutos dañados con una eficacia similar a la de *Bacillus thuringiensis* (Bt) y spinosad y, además, produjo una mayor mortalidad larvaria que Bt. En cultivos en campo, el bioinsecticidas viral mostró una efectividad similar a la del spinosad, Bt y clorpirifos para reducir el porcentaje de frutos dañados.

12. La persistencia de los insecticidas en las plantas de tomate cultivadas en invernadero o campo abierto estuvo correlacionada con la radiación UV que incidió sobre el filoplano en ambas condiciones. La persistencia de los OBs en cultivos de tomate protegidos fue mayor que la de Bt y spinosad y, consecuentemente, las larvas pudieron ingerir concentraciones letales incluso varios días después de haberse realizado el tratamiento viral. Sin embargo, en tomates cultivados al aire libre la persistencia de los OBs fue significativamente menor que la de spinosad y clorpirifos.

13. Buena parte de los resultados de esta tesis forman parte del contenido de la solicitud de una patente española (P201430956).

CONCLUSIONS

1. The natural diversity of seven HearSNPV isolates from the Iberian Peninsula, as determined by identification of their genotypic composition and characterization of their insecticidal properties resulted in highly homologous genomes and, hence, similar phenotypes, as reflected by their pathogenicity against laboratory-reared *H. armigera.* However, HearSP1 was the most virulent and one of the most productive isolates.

2. Although HearSP1 and HearG4 showed similar pathogenicity against a range of instars from a Spanish *H. armigera* colony, HearSP1 was more virulent against all instars tested and, surprisingly, as productive as HearG4 in L_5 larvae. HearSP1 has, thus, suitable insecticidal characteristics as an active ingredient of a bioinsecticide.

3. The genotypic interactions that modulate the insecticidal characteristics of virus populations were analysed in two purified genotypic variants from the HearSP1 isolate, HearSP1A and HearSP1B, and in six genotypic variants from the HearLB isolate, HearLB1 to HearLB6. Phenotypic characterization revealed that HearSP1B was ~3 fold more pathogenic than HearSP1 and the other variants. HearLB genotypes showed no differences in pathogenicity, although HearLB1 and HearLB6 were among the fastest-killing variants and HearLB1 yielded most progeny OBs, despite its fast speed of kill. HearLB3 and HearLB6 showed intermediate OB production values.

4. Among the eight co-occluded mixtures that were constructed with genotypes HearSP1A, HearSP1B, HearLB1, HearLB3 and HearLB6, the co-occluded HearSP1B:LB6 mixture at a 1:1 proportion was 1.7-2.8 fold more pathogenic than any single genotype and other mixtures tested, and also killed larvae as fast as the most virulent genotypes, making this mixture the most suitable to develop it as a bioinsecticide.

5. After five serial passages in larvae, the proportion of genotypes within the HearSP1B:LB6 mixture varied from 1:1 to 5:1, while the pathogencity and

virulence of this mixture was higher than the original, suggesting that serial passage could further improve the transmissibility of the mixture.

6. Complete genomic sequencing of these five genotypes revealed high degree of identity at the nucleotide level between them (98-99%) and to previously sequenced *Helicoverpa* spp. SNPVs (95-99%). Major differences among all the genotypes were located in the *hrs* and *bro* genes, which could be responsible for insecticidal differences.

7. Point mutations occuring in genes involved in DNA replication (*ie-1*, *lef-3*, *DNA polymerase*), viral transcription (*lef-8*, *lef-1*) or structural genes (*p78/83*, *vp1054 desmop*, *calyx/pep*, *odv-e66*) could be responsible for the reduced OB production of HearSP1 genotypes or the increased pathogenicity of HearSP1B. Mutations in *iap-2*, *iap-3* and *hoar* genes could be involved in transmission or ability to establish covert infections.

8. Selection pressure analysis showed strong selection in genes involved in primary infection (*p78/83*, *odv-e56* and *p74*), virus infection and replication (*ie-0*), or viral transmission (*egt)*. Other positively selected genes have an unknown function (*bro-d*, *bv-ec31* and ORF130).

9. HearSNPV nucleocapsids were co-enveloped with those of the broader host-range HearMNPV. For the first time, a SNPV was co-enveloped within a MNPV, and this strategy allowed expansion of the absolutely specific HearSNPV host range to previously unsusceptible insect species. Co-envelopment did not improve the insecticidal properties against homologous or heterologous hosts but demonstrated to provide a means for producing non-recombinant baculoviruses with a wider host range and thus overcome the limitation of specificity of several baculoviruses.

10. The efficient production of HearSP1B:LB6 is optimum upon inoculation of newly molted L_5 *H. armigera* with the LC_{80} (5.5×10^6 OBs/ml), followed by individualized rearing of larvae at 30ºC.

11. HearSP1B:LB6 was a useful tool to effectively control pests caused by *H. armigera* in tomatoes. In greenhouses, HearSP1B:LB6 was as efficient in reducing the number of injured fruits as *Bacillus thuringiensis* (Bt) and

spinosad, and yielded higher larval mortality than Bt. In open-field crops, HearSP1B:LB6 treatment was as effective in reducing the percentage of damaged fruit as spinosad, Bt and chlorpyrifos.

12. Persistence of HearSP1B:LB6 and the other insecticides on tomato plants was correlated with solar radiation. In greenhouses, OB persistence was higher than that of Bt and spinosad, and larvae could effectively acquire the virus several days after virus treatment. However, on field-grown tomatoes OB persistence was significantly lower than that of spinosad or chlorpyrifos.

13. The results of this thesis have contributed to a patent application in Spain (P201430956).

LIST OF PUBLICATIONS

Arrizubieta, M., Williams, T., Caballero, P., Simón, O., 2014. Selection of a nucleopolyhedrovirus isolate from *Helicoverpa armigera* as the basis for a biological insecticide. Pest Management Science 70, 967-976.

Arrizubieta, M., Simón, O., Williams, T., Caballero, P. A novel binary mixture of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) genotypic variants provides improved insecticidal characteristics for control of the cotton bollworm. Applied and Entomological Microbiology. doi:10.1128/AEM.00339-15.

Arrizubieta, M., Simón, O., Williams, T., Caballero, P. Genomic sequence of five closely related *Helicoverpa armigera* single nucleopolyhedrovirus genotypes from Spain showing significant differences in their insecticidal properties. Genome Announcement. Submitted.

Arrizubieta, M., Palma, L., Williams, T., Caballero, P., Simón, O. Phenotypically diverse *Helicoverpa armigera* single nucleopolyhedrovirus genotypes differ minimally in *bro* genes and homologous regions (*hr*). Journal of Invertebrate Pathology. Submitted.

Arrizubieta, M., Simón, O., Williams, T., Caballero, P. Increased host range of *Helicoverpa armigera* single nucleopolyhedrovirus (HearSNPV) through co-envelopment with *H. armigera* multiple nucleopolyhedrovirus (HearMNPV). Applied and Environmental Microbiology. Submitted.

Arrizubieta, M., Simón, O., Williams, T., Caballero, P. Determinant factors in the production of a co-occluded binary mixture of *Helicoverpa armigera* alphabaculovirus (HearSNPV) genotypes with desirable insecticidal characteristics. Biocontrol. Submitted.

Arrizubieta, M., Simón, O., Caballero, P., Williams, T. Insecticidal efficacy and persistence of a co-occluded binary mixture of *Helicoverpa armigera* nucleopolyhedrovirus (HearSNPV) variants in protected and field-grown tomato crops in the Iberian Peninsula. Pest Management Science. Accepted.

Caballero, P., **Arrizubieta, M.**, Simón, O., Williams, T., 2014. Nuevos genotipos del nucleopoliedrovirus simple de *Helicoverpa armigera* (HearSNPV), procedimiento para su producción y uso como agente de control biológico. Patente de Invención P201430956.

Para la realización de esta tesis Maite Arrizubieta Celaya obtuvo una beca predoctoral JAE-predoc del Consejo Superior de Investigaciones Científicas (CSIC)

Helicoverpa armigera (Lepidoptera: Noctuidae) is an important insect pest of tomato crops in Spain. The extensive use of chemical insecticides has resulted in the development of insect resistance, in addition chemical residues hamper the tomato commercialization. In these situation, baculovirus-based bioinsecticides constitute one of the most realistic alternatives for efficient pest control programs. *H. armigera* populations are naturally affected by the single nucleopolyhedrovirus of *H. armigera* (HearSNPV). The aim of this thesis was to conduct the biotechnological developments necessaries to obtain a HearSNPV-based bioinsecticide to reduce the damage generated by *H. armigera*.

Tesis Doctoral - Maite Arrizubieta Celaya

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