TESIS DOCTORAL

Development of a new bioinsecticide based on a *Chrysodeixis chalcites* nucleopolyhedrovirus from the Canary Islands

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Memoria presentada por

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Development of a new bioinsecticide based on a *Chrysodeixis chalcites* nucleopolyhedrovirus from the Canary Islands

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INFORMAN:

que la presente memoria de Tesis Doctoral titulada "**Development of a new bioinsecticide based on a *Chrysodeixis chalcites* nucleopolyhedrovirus from the Canary Islands**" elaborada por Dña. **ALEXANDRA BERNAL RODRÍGUEZ** 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 5 de noviembre de 2013,

Prof. Dr. Primitivo Caballero Murillo Dra. Oihane Simón de Goñi

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TABLE OF CONTENTS

RESUMEN

Chrysodeixis chalcites (Lepidoptera: Noctuidae) es una plaga importante que causa valiosos daños económicos en los cultivos de platanera de las Islas Canarias. El control efectivo de esta plaga con insecticidas químicos requiere muchas aplicaciones, aumentando los costes de producción, lo que puede derivarse en riesgos ambientales graves, y la acumulación de residuos químicos que dificultan la comercialización del plátano. En estos casos, una de las alternativas más realistas para el control seguro y eficaz de la plaga la constituyen los bioinsecticidas basados en microorganismos entomopatógenos incluidos los baculovirus. En condiciones naturales las poblaciones de *C. chalcites* se ven afectadas por un *Alphabaculovirus* (*Baculoviridae*) llamado *C. chalcites* nucleopoliedrovirus (ChchSNPV). El objetivo de esta tesis doctoral ha consistido en abordar algunos de los desarrollos biotecnológicos necesarios para la obtención de un nuevo bioinsecticida basado en un ChchSNPV autóctono de las Islas Canarias.

En primer lugar se ha evaluado la diversidad genética de ChchSNPV en las Islas Canarias. En un amplio muestreo larvario se encontró que, de las larvas recolectadas en campo, 97 murieron con los típicos síntomas de infección por NPV. Estos NPVs se agruparon en cinco tipos de aislados diferenciables en base al perfil de restricción del DNA genómico del virus. El aislado con mayor prevalencia y el más disperso en las Islas Canarias, ChchSNPV-TF1 (ChchTF1), a su vez fue el más patogénico y virulento para las larvas de una población autóctona de *C. chalcites*. La caracterización genotípica de este aislado puso de manifiesto que ChchTF1 está compuesto por al menos ocho genotipos diferentes, siendo ChchTF1-A el genotipo aislado en mayor proporción así como el más frecuente en la población silvestre (representando el 36%). Ninguno de los genotipos puros fue más patogénico que el aisaldo silvestre (ChchTF1) lo que sugiere la ocurrencia de interacciones entre genotipos que incrementan la patogenicidad del aislado silvestre. Sin embargo, ChchTF1 presentó una menor virulencia que los genotipos puros, que se reflejó en una mayor productividad viral. ChchTF1 está genotípicamente estructurado para maximizar su transmisibilidad en la naturaleza. Los ensayos con mezclas experimentales de genotipos pusieron de manifiesto que la mezcla co-ocluida compuesta por los tres genotipos más frecuentes (ChchTF1-

ABC), en una proporción similar a la encontrada en el aislado silvestre, era incluso más patogénica que el aislado silvestre. Inesperada y sorprendentemente, la virulencia de ChchTF1-ABC mejoró en 33 h la del aislado silvestre. Por ello, la mezcla co-ocluida ChchTF1-ABC fue seleccionada como materia activa de un bioinsecticida para el control de *C. chalcites* en las Islas Canarias. La secuenciación completa de los genomas de los tres genotipos más frecuentes, ChchTF1-A, -B y –C, y de dos de los genotipos menos frecuentes, ChchTF1-G y –H, puso de manifiesto una gran similitud (del orden del 99%) entre los mismos. Se diferencian dos regiones de variabilidad genómica, localizadas en los genes *hoar* y *bro-d*, que pueden estar involucrados en esas diferencias observadas a nivel fenotípico entre genotipos.

Una de las mayores limitaciones a la hora de desarrollar los baculovirus como agentes de control biológico es optimizar el sistema de producción masiva que, hoy por hoy, se hace *in vivo*. En este caso, se han evaluado variables tales como el estadio larvario, tiempo de inoculación, concentración de la suspensión viral y la densidad. Los CL_{90} produjeron mayores cantidades de OBs (media de $8.07x10^{13}$ OBs/container), que de densidades inferiores (1, 25, 50 y 100) y similares a las obtenidas con mayores (200) densidades. Esto representa un incremento de 59.645 veces sobre la concentración de inoculación. Tras ello, con el fin de determinar la eficacia probable de ChchTF1 como base de bioinsecticida se evaluó la susceptibilidad de los distintos estadio de *C. chalcites* a ChchTF1. La susceptibilidad de *C. chalcites* a ChcTF1 disminuye conforme incrementa el estadio larvario, larvas de segundo estadio fueron 10.000 veces más susceptibles que las de sexto estadio. El tiempo de mortalidad fue 42 h más rápido en larvas L_2 que en larvas L_6. Mientras que la producción de OBs incrementó en estadios más avanzados; larvas L_6 produjeron 23 veces más OBs que L_4. La formulación de ChchTF1 con abrillantadores ópticos mejoró sus propiedades insecticidas. La adición de 1% de Tinopal UNPA-GX aumenta la patogenicidad entre 4,43 y 397 veces dependiendo del estadio larvario tratado. La virulencia por su parte se vio también mejorada entre 14 y 26 horas; sin embargo, la producción de OB se redujo del orden de 8,5 veces. La actividad sinérgica de Leucophor UO fue significativamente menor. La formulación de ChchTF1 con abrillantadores ópticos puede mejorar la eficacia del producto consiguiendo niveles aceptables de control

a bajas dosis de virus. Estas formulaciones parecen especialmente útiles en situaciones reales de campo donde es necesario controlar diferentes estadios larvarios al mismo tiempo con un sol tratamiento.

Finalmente, se evaluó la eficacia de ChchTF1 en el control de infestaciones larvarias de *C. chalcites* en los cultivos de tomate y platanera, comparándolo con un insecticida químico (indoxicarb) y otro biológico (*Bacillus thuringiensis* ser kurstaki) usados habitualmente en este cultivo. Los tratamientos con el ChchSNPV ($1x10^9$ OBs/l) fueron entre 3 y 4 veces más efectivos que los insecticidas químicos y el Bt para el control de las infestaciones larvarias. Teniendo en cuenta que en los tratamientos plaguicidas, realizados en el cultivo de la platanera, se usan volúmenes de caldo de 1600-2000 l/ha, para aplicar una concentración de $1x10^9$ OBs/l la cantidad de inóculo viral para tratar una hectárea sería de $1.6\text{-}2.0x10^{12}$ OBs. Teniendo en cuenta que nuestro sistema de producción permite obtener hasta $8.07x10^{13}$ OBs, a partir de 150 larvas muertas por poliedrosis, este inóculo viral sería suficiente para proteger entre 40 y 50 ha de platanera. Toda esta información ha servido para hacer una solicitud de patente y constituye la base del desarrollo de un nuevo bioinsecticida. Este bioinsecticida, además de ser el agente de control biológico más efectivo para el control de esta plaga en la actualidad, es una herramienta muy útil para la implantación de programas de Protección Integrada de Cultivos y el establecimiento de una agricultura sostenible.

SUMMARY

Chrysodeixis chalcites (Lepidoptera: Noctuidae) causes valuable economic damage in banana crops in the Canary Islands. Effective control of this pest with chemical insecticides requires many applications that increase production costs and render serious environmental hazards and chemical residues that hamper banana commercialization. In these situations, microorganism-based bioinsecticides, particularly baculoviruses, constitute one of the most realistic alternatives for efficient pest control programs. Under natural conditions, *C. chalcites* populations are affected by the *C. chalcites* nucleopolyhedrovirus (ChchSNPV) (Fam. Baculoviridae, *Alphabaculovirus*). The aim of this thesis has been to address some of the biotechnological developments necessary to obtain a new biopesticide based on a ChchSNPV strain indigenous of the Canary Islands.

Firstly, the genetic diversity of ChchSNPV in the Canary Islands was evaluated. During the course of a large field sampling, 97 larvae died with the typical signs and symptoms of NPV disease. These NPV isolates were grouped into five different strains as indicated by restriction endonuclease analysis of their viral DNA genome. The most widespread and prevalent isolate, ChchSNPV-TF1 (ChchTF1), was also the most pathogenic and virulent against a local insect population. Genotypic characterization of this isolate revealed that ChchTF1 is composed of at least eight different genotypes, being ChchTF1-A the most isolated one and also the most prevalent in the wild-type population (36%). None of the pure genotypes was as pathogenic as the wild-type population, suggesting the occurrence of interactions among genotypes that increase the pathogenicity of the wild-type mixture. However, ChchTF1 was less virulent than single genotypes, which was correlated with a higher occlusion body (OB) yield. ChchTF1 is genotypically structured to maximize its transmissibility in nature. Experimental testing mixtures of genotypes revealed that the co-occluded mixture composed by the three most prevalent genotypes, ChchTF1-ABC, in a proportion similar to that found in the wild-type population, was even more pathogenic than the wild-type population. Unexpectedly, the virulence of the co-occluded mixture was improved by 33 h in relation to the wild-type isolate. Therefore, the ChchTF1-ABC co-occluded mixture was selected

as the active ingredient for a bioinsecticide to control *C. chalcites* in the Canary Islands.

Sequencing analysis of the most frequent genotypes, ChchTF1-A, -B and –C, and of the least frequent ones, ChchTF1-G and –H, revealed a high similarity (around 99%) between them. Two regions of genome variability, located in the *hoar* and *bro-d* genes, were identified that could account for differences in the phenotype of these genotypes.

One of the greatest limitations in developing baculoviruses as biocontrol agents is the mass production system, which, to date, is performed *in vivo*. To optimize ChchTF1 OB production, variables such as larval stage, inoculation time, viral suspension concentration and larval density were evaluated. Virus concentrations that killed 90% of inoculated larvae (LC_{90}) yielded the greatest OB productions ($8.07x10^{13}$ OBs/container). This represented a 59,645-fold increase over the inoculated concentration. Thereafter, the susceptibility of different *C. chalcites* instars to ChchTF1 was evaluated. Susceptibility to viral infection decreased as larvae aged and second instars (L_2) were 10^4-fold more susceptible than sixth instars (L_6). The virus speed of kill was 42 h faster in L_2 than in L_6 and OB production increased in late instars and L_6 larvae were 23-fold more productive than L_4.

Formulation of ChchTF1 with optical brighteners increased the virus insecticidal properties. For instance addition of 1% of Tinopal UNPA-GX increased the pathogenicity between 4.43 to 397-fold depending on the larval instar. The virulence was also improved by 14 to 26 h. However, OB yield was reduced by 8.5-fold. The sinergistic activity of Leucophor UO was significantly lower. In sum, formulation of ChchTF1 with optical brighteners may improve the efficiency of the product as they allow to achieve control levels at lower virus concentrations. These formulations seem particularly useful in field situations where it is necessary to control different larval stages simultaneously with a single dose.

Finally, the efficacy of the ChchTF1 in controlling larval infestations of *C. chalcites* in tomato and banana crops was compared with a chemical (indoxicarb) and a biological insecticide (*Bacillus thuringiensis* var kurstaki), commonly used in these crops. ChchTF1 treatments ($1x10^9$ OBs/l) were 3 to 4-fold more effective in reducing larval infestations than the chemical or Bt treatments. Considering that

pesticides are usually applied to banana crops at volumes of 1600-2000 l/ha, for an application rate of $1x10^9$ OBs/l, the amount of virus inoculum for an hectare would be $1.6\text{-}2.0x10^{12}$ OBs., Given that our system yields as much as $8.1x10^{13}$ OBs from 150 NPV-killed larvae, 40 to 50 ha could be protected efficiently with this inoculum. All this information has been used to make a patent application and is the basis for development of a new biopesticide. This new biopesticide, besides being the most effective biocontrol agent against *C. chalcites* to this day, is a very useful tool for the implementation of Integrated Pest Management programs and the establishment of a sustainable agriculture.

CHAPTER 1

Introduction

Content

1. GENERAL INTRODUCTION

The banana crop (Musa spp.), with an annual production of 95 million tons, ranks fourth in global importance, being only exceeded by rice, wheat and maize (Holmes, 2013). Currently it is the main crop in the Canary Islands, with a cultivation area of about 9,110 hectares, mainly in the islands of Tenerife (4,232 ha) and La Palma (3,032 ha). With an output of 352,397 tons, representing an annual value of € 165,768,000 (Gobierno de Canarias, 2013), it is the only cultivated product exported from the islands (ASPROCAN, 2012).

Phytosanitary problems associated with banana crops include an ample diversity of pests and diseases favoured by the optimal weather conditions on the Canary Islands. The tomato lopper *Chrysodeixis chalcites* (Esper) (Lepidoptera, Noctuidae), is one of the most important ones due to its economic impact and persistent presence (Bernal et al., 2013; Del Pino et al., 2011; Hernández-Santana, 2007). *C. chalcites* is a polyphagous polyvoltine species that feeds on the foliage and fruits of vegetable, fruit and ornamental crops. It is considered as one of the most serious lepidopteran pests in many countries (Shepard et al., 2009; van de Veire and Degheele, 1994; van Oers et al., 2004). In Peninsular Spain, it also causes damage in greenhouse crops in Almeria (Cabello and Belda, 1994) such as lucerne, maize and soybean crops (Amate et al., 1998). Infestations of this pest frequently result in up to 30% losses in bananas grown under greenhouse or mesh conditions in the Canary Islands (Del Pino et al., 2011). In the Canaries, *C. chalcites* populations have increased markedly during the past decade, and it is currently considered a re-emerging pest, possibly related to increased migration and range shifts in this and other noctuid pest species in response to global climate change (Sparks et al., 2007).

At present, *C. chalcites* control in banana crops is based only on the intensive application of synthetic chemical insecticides. However, the wide use of the few number of active ingredients authorized for this crop, has led to resistance and reduced effectiveness of these products (Perera and Molina, 2007) while generating pesticide residues that hamper the commercialization of bananas from the Canary Islands. These, together with the well recognized incompatibility of chemical control measures and natural enemies (Lara and Urbaneja, 2002;

Stansley et al., 2005) have raised public concern and fostered growing research efforts to develop secure, efficient, and sustainable pest control methods.

C. chalcites presents a diverse group of natural enemies that naturally regulate these populations in banana crops in the Canary Islands being *Trichogramma achaeae* (Nagaraja and Nagarkatti, 1973) (Hymenoptera: Trichogrammatidae) the most common parasitoid species is an egg-endoparasitoid, which may reach an 87% level of parasitism (Del Pino et al., 2011). However commercial availability of this insect is insufficient to keep *C. chalcites* populations low (Cabello, 2009). As for pathogens, experts observed the presence of *C. chalcites* larvae with clear signs of nucleopolyhedrovirus (NPV) disease due to infection by various local isolates of *C. chalcites* NPV (ChchNPV). This entomopathogen, which persists in natural *C. chalcites* populations frequently causing epizootics, has gained particular interest as a potential biopesticide to control this pest (Bernal et al., 2013; Del Pino et al., 2011; Hernández-Santana, 2007).

In the *Asociación de Organizaciones de Productores de Plátanos de Canarias* (ASPROCAN), there is a growing interest to reduce the phytosanitary problems associated with this crop by developing more rational techniques, especially those that implement biological and integrated control programs. However, the absence of effective biological products for the control of *C. chalcites* leaves growers with only chemical products (ASPROCAN, personal communication).

The present study arises from the existent need to control *C. chalcites* in banana crops in in the Canary Islands. With this aim, this thesis was devoted to develop an effective bioinsecticide product based on a local NPV to protect banana crops against *C. chalcites*.

2. Chrysodeixis chalcites

2.1. Taxonomy and morphology

C. chalcites (Esper) is a moth belonging to the order Lepidoptera, the Noctuidae family and the Plusiinae subfamily. Adults, with a 33-40 mm wingspan and 15-18 mm length, are golden brown and have two conspicuous silver to white drop-shaped spots in the forewings, while hindwings are paler. Two prominent crests are found on the thorax (Fig. 1A) (Bretherton, 1983; Goodey, 1991). Male and female adults are very similar but the female adults lack the yellowish tuft in the

lower part of the abdomen (Goodey, 1991). *C. chalcites* eggs are pearly white to pale green and shiny (Fig. 1B). They are dome-shaped (hemispherical) with 28 to 32 vertical ribs from the micropyle to the base (Bretherton, 1983; Goodey, 1991) (Fig. 1B-C). The eggs darken shortly before hatching (Harakly and Farag, 1975) (Fig. 1C). *C. chalcites* larvae are eruciform (Fig. 1D-E) and can reach six larval stages followed by a pre-pupal stage (Amate et al., 1998; Goodey, 1991). Larvae have only three pairs of prolegs, instead of the normal five, resulting in the looping gait giving rise to some of the common names as semi-looper, green garden looper, green looper, green semi-looper or tomato looper (Fig. 1D-E).

Mature larvae are 34 to 38 mm long, pale yellow-green with a glassy green to grey head edged with a black streak (Fig. 1E). Above the black spiracles on each side of the body is a thin dark green or black line stretching from the head to the seventh abdominal segment, below this is a thicker white line from the head to the tip of the anal proleg (Fig. 1E). The ventral region is speckled with white dots (Bretherton, 1983; Passoa, 1995). Pupae, which have a fusiform-shaped form, are

Figure 1. Morphology of *C. chalcites*: A) adult; B) young egg; C) mature egg; D) early instar larva; E) sixth instar larva; F) young pupa; G) mature pupa.

found between leaves wrapped in a silken cocoon produced by the larva at its final stage (Fig. 1F). The pupa size and width vary from 20 to 25 mm and 4 to 4.5 mm, respectively (Bretherton, 1983; Goodey, 1991). Considerable colour variation occurrs in the pupae, from pale green at the beginning of pupal development (Fig. 1F), turning into a hazel-dark brown colour (Fig. 1G) when pupae reach full maturity (Bretherton, 1983; Goodey, 1991; Harakly and Farag, 1975).

2.2. Geographical distribution

C. chalcites is endemic to Southern Europe and Tropical Africa but can be found in great parts of Europe due to its migratory behaviour. It is also found in the Middle East, Australia and South East Asia according to the invasive species compendium (CABI, 2013; Engle et al., 2008) (Fig. 2).

In Europe, *C. chalcites* has been reported as a serious pest in Bulgaria and Turkey (Loginova, 1992; Uygun and Ozgur, 1980) affecting tomato, cucumber and peppers. In northern Italy, *C. chalcites* is one of the principal arthropod pests on soybean (Zandigiacomo, 1990), also attacks artichokes (Ippolito and Parenzan, 1985) and is one of the four main noctuid pests of glasshouse crops in Sicily (Inserra and Calabretta, 1985). *C. chalcites* is also considered as a continuous pest in glasshouses in the Netherlands (Vos and Rutten, 1995) and Belgium (van de Veire, 1993). There are also around 50 records of *C. chalcites* as a migrant insect in the UK between 1943 and 1990 (Bretherton, 1983), and a recent study has revealed its presence in the UK (Woods, 2011). Although *C. chalcites* has been recorded in northern Europe, winter mortality prevents its long-term establishment outdoors (Lempke, 1982; Napiorkowska-Kowalik and Gawlowska, 2006). However, it has established in northern Europe glasshouses, where it is considered as a type of invasive species. *C. chalcites* is also located in the Iberian Peninsula, showing a major economic importance in the Guadalquivir Valley, the fertile lowland of Granada crops and in greenhouses of Almería in the South of Spain, feeding on lucerne, maize and soybean (Amate et al., 1998). Finally, in the Canary Islands, *C. chalcites* was first reported in 1904 in the Tenerife Island (CABI, 2013), where it is nowadays a severe pest in banana crops. (Bernal et al., 2013; Del Pino et al., 2011; Hernández-Santana, 2007).

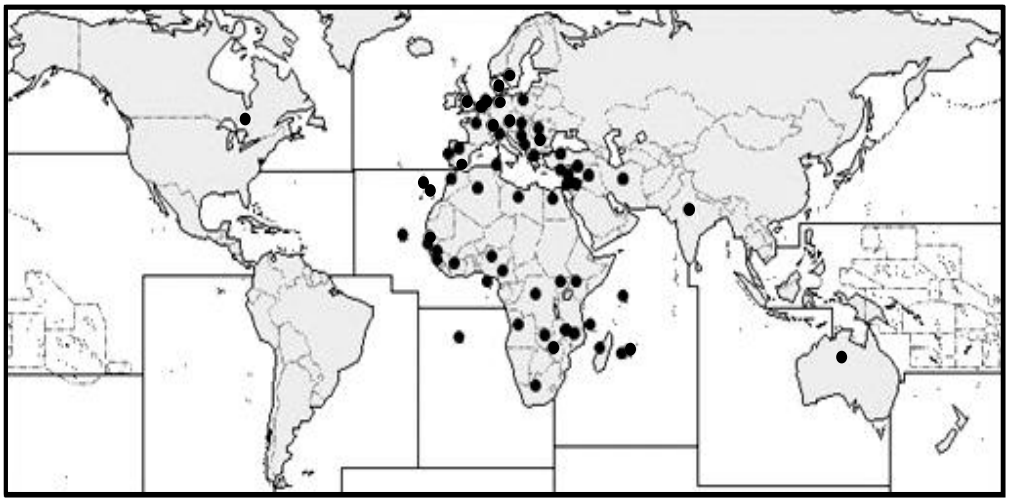

Figure 2. Worldwide geographical distribution of *Chrysodeixis chalcites* (black spots) (CABI, 2013).

In Africa, *C. chalcites* is broadly present and a quarantine pest in South Africa, (EPPO, 2013), while in Egypt it is considered as the most serious semi-looper pest attacking fruits and vegetables, and potato in Mauritius (Anon, 1984).

In Asia, this semilooper moth is the major pest of tomato in Israel during the growing season (Broza and Sneh, 1994; Harakly and Farag, 1975), and also one of themost important noctuid pests of fodder crops such as lucerne and clover (Avidov and Harpaz, 1969).

Specimens of *C. chalcites* have been also found in South Western Ontario, Canada since 2008 (Daricheva et al., 1983; Murillo et al., 2013), from where it may spread to protected cultivation elsewhere. Actually, *C. chalcites* has been found in *Pelargonium* crops in glasshouses in Ohio, USA (Passoa, 1995; USDA-APHIS, 2008).

Finally, *C. chalcites* larvae has been introduced internationally by trading, as described in Hugary, where it arrived from Germany on *Pelargonium* (Meszaros and Tusnadi, 1994), or in the UK, where it was introduced from the Canary Islands on *Chrysanthemum morifolium* and *Pelargonium* (Seymour and Kilby, 1978), and in Italy, which was hit by exported bananas from the Canary Islands (Jannone, 1966).

2.3. Biology and ecology

C. chalcites has a postembrionic holometabolistic development with four life stages or morphological phases: egg, larva, pupa and adult (Fig. 1). *C. chalcites*

populations may undergo up to 9 generations *per* year in certain climates, which vary depending on factors such as food, temperature and climatic conditions (Harakly and Farag, 1975). The life cycle from egg to adult is about 45 days at the optimal temperature of 25ºC (Gaumont and Moreau, 1961) but can last up to 50 days in cool climates (Harakly and Farag, 1975). Mating occurs predominantly at night and takes place shortly after adult emergence. Eggs are laid also at night, on upper and lower leaf surfaces, two or three days after adult emergence (Cayrol, 1972; Gasim and Younis, 1989). Females deposit one, two or a few eggs at each time so, eggs are very widely scattered in the crop (Harakly and Farag, 1975; van der Linden, 1996). Each female lays about 200 eggs in throughout her lifetime, but reports in the literature have shown considerable variation in the number of eggs oviposited (Harakly and Farag, 1975). Larval emergence occurs after 5-6 days at 25ºC (Gaumont and Moreau, 1961). Early instars are able to secrete silken threads to escape danger if disturbed (Goodey, 1991). The larval period consists of six instars and can last from 25 to 30 days at 25ºC. At the end of the last instar, they stop feeding, spin a cocoon to pupate and enter the prepupal stage. The cocoon is usually attached to the underside of a leaf but can be also found in the soil (Harakly and Farag, 1975). The pupal period at optimum temperature (25°C) averages 8.8 days (Goodey, 1991). Adults emerge and soon begin to fly and mate; they are semi-nocturnal and usually avoid strong sunlight.

C. chalcites generations follow one another throughout the year with no diapause. In protected cultivation, like that found in banana crops grown under mesh in Southern Tenerife, *C. chalcites* can occur at any time of the year (van der Linden, 1996) where it can reach high levels of infestation on vegetables and ornamental plants. However, there are two main peaks in *C. chalcites* populations per year: in May-June and in September-October (Izquierdo et al., 1996). Adults are able to migratet over great distances from Northern Europe to South Africa, mainly during spring and autumn (García et al., 1992).

2.4. Injury and damage

C. chalcites larvae are highly polyphagous, they consume the foliage and fruits of a wide range of crops grown both outdoors and in greenhouses, including fruit, vegetable, ornamental crops and wild plants from a wide range of plant families

(CABI, 2013). It is considered as the most important pest in tomato crops, as indicated by its common name, the tomato looper (Daricheva et al., 1983).

During the early stages, larvae are located on the underside of leaves feeding on parenchyma, where they, or the injuries they inflict, are quite difficult to detect (Fig. 3A). During the second and third instars, they begin to reach the leaves edges (Goodey, 1991) and perforate their surface, reducing the photosynthetic area (Goodey, 1991) (Fig. 3B). The last two instars are the most voracious feeders and eat the entire leaf but may avoid the midrib, or other large veins (Fig. 3B). The main injury caused by the pest is defoliation, especially in young plantations. However, *C. chalcites* larvae also attack the fruits of some crops, as in tomato or banana (Fig. 3C), reducing crop yields severely. On legumes, they may excavate deep into pods, sometimes cutting them in two.

During the past decade, *C. chalcites* populations have increased markedly in banana crops in the Canary Islands, not only because of increased migration and range shifts in this and other noctuid pest species in response to global climate change (Sparks et al., 2007) but also due to the restriction in the use of chemical pesticides (Del Pino et al., 2011). At present, *C. chalcites* can be found in all the islands in the Canaries both in open airfields and in those grown under plastic nylon mesh greenhouses (Del Pino et al., 2011). In banana crops, caterpillar food preferences focus on young leaves, the so-called leaf pipes (Cabello, 2009; Del Pino et al., 2011; Perera and Molina, 2007) (Fig. 4A). Formerly, small caterpillar

Figure 3. Injuries caused by *Chrysodeixis chalcites* in crops: A) early-stage larvae feeding on a leaf underside surface; B) defoliation caused by final instars; C) tomato fruit injury by *C. chalcites.*

Figure 4. Injuries caused by *Chrysodeixis chalcites* in banana crops: A) injured young plant leaves and leaf pipes; B) injured mature plant leaves; C) injured banana fruits caused by final instars.

damage was negligible in mature plants, but in young plants they could produce serious damage by cutting and even perforating leaves (Perera and Molina, 2007; Vilardebo and Guérout, 1964) (Fig. 4B). However, *C. chalcites* feeding habits have changed recently and larvae feed also on fruits, producing skin injuries and rendering important damages (Fig. 4C). This shift in larvae behaviour has made this insect an important emerging pest in banana crops in the Canary Islands (Perera and Molina, 2007).

2.5. Control methods

2.5.1. Cultural practices and monitoring

Cultural practices involve weed control, crop monitoring, leaf thinning, fruit packaging, the use of plants as reservoirs of natural enemies, and the use of trap crops, such as *Brassicae* plants (Del Pino et al., 2011). In protected crops, it is advisable to set up efficient barriers to avoid the entrance of adults (Perera and Molina, 2007).

Surveying methods such as light traps or pheromone traps (Fig. 5A) used by growers to evaluate pest density or time applications more precisely, are essential tools for the overall efficacy of IPM programs. Adult males can be monitored with pheromone traps (Del Pino et al., 2011: Perera and Molina, 2007), giving early warning of the infestation levels. The commonly used monitoring trap density is 3-4 traps *per* ha. The most effective lure was found to be a mixture of 1 mg (Z)-7-dodecenyl acetate and 0.2 mg (Z)-9-tetradecenyl acetate absorbed on rubber septa (Dunkelblum et al., 1981). At the time the first males are detected, an *in situ* monitoring of leaves, fruits, and other parts of the plant is recommended, and

followed by contact with the technical staff of the Pest Protection Service in the area, which will evaluate the situation on the ground and determine the measures to be taken in each case. Banana growers tend to treat banana plants when first adults appear or minimal larval injury is observed in the leaves (Fig. 5B), so as to prevent, or reduce to a minimum, the presence of later, more injurious, instars (Bernal, personal communication).

Figure 5. *Chrysodeixis chalcites* monitoring pheromone traps (A) and injuries that justify treatment for banana growers (B).

2.5.2. Chemical control.

In the last few decades, control of *C. chalcites* has relied on synthetic chemical pesticides, mainly carbamates, organochlorines, pyrethroids such as cypermethrin or deltamethrin, and organophosphates (Amate et al., 1998). More recently, insect growth regulators (such as flufenoxuron, lufenuron, methoxyfenozide, and tebufenozide) and biologically-derived insecticides (like spinosad 48%) have become increasingly popular. The insect growth regulator cyromazine gave good control of second and fourth-instar *C. chalcites* in glasshouses on tomatoes, lettuce and ornamentals when applied as a foliar spray (van de Veire and Degheele, 1994). Bassi et al. (2000) reported effective control of *C. chalcites* using indoxacarb (an oxadiazine) on vegetable crops in open fields and plastic houses in Italy.

Indoxacarb 30% is also commonly used in the Canary Islands, where *C. chalcites* control is currently based on the continued application of this and other autorized synthetic insecticides, like the limonoid azadirachtin 3.2%, initially extracted from the seeds of *Azadirachta indica* (Sapindales: Meliaceae), and the organophosphate chlorpyrifos 48% (Agrocabildo, 2013). Although effective, their use engages a number of risks. Intensive use generates pesticide residues on vegetables and fruits that hamper the commercialization of bananas from the

Canary Islands, which can result in batches of products being rejected for sale with important economic consequences. Furthermore, the presence of toxic pesticide residues has to be continuously monitored in banana products in order to comply with European Union established limits (http://europa.eu.int/comm/food/fvo/special Reports/pesticides_index_en.htm) for fruits and vegetables.

However, the wide use of a low number of active ingredients authorized for this crop, has led to *C. chalcites* developing resistance and to reduced effectiveness of these products (Horowitz et al., 1998; Perera and Molina, 2007). Resistance development is directly correlated with higher application frequencies, and therefore, with a dramatic increase in cost production. Finally, incompatibility between the use of chemical control measures and natural enemies is well recognized (Lara and Urbaneja, 2002; Stansley et al., 2005).

2.5.3. Natural enemies.

C. chalcites is a host for a diverse group of natural enemies that regulate naturally the pest populations in protected conditions. However, commercial availability cannot cover the grower's demands so far (Del Pino et al., 2011).

Alcippe brunnea (Passeriformes: Pellorneidae), a bird found in dense forest in India, is successfully used to control *C. chalcites* on sweet peppers grown in glasshouses in the Netherlands (van der Linden, 2000).

Many predators have been used extensively in different regions. In Italian glasshouses, the predatory *Podisus maculiventris* and *Podisus nigrispinus* (both Heteroptera: Pentatomidae), from North America, have been tested as good control agents (Vacante et al., 1996). Several parasitoids have also been described from different regions. In the UK, the endoparasitoid *Meteorus gyrator* (Hymenoptera: Braconidae) showed considerable potential as a biocontrol agent against *C. chalcites* under controlled conditions (Bell et al., 2000; Smethurst et al., 2004). In the Cape Verde Islands, the solitary endoparasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae) was introduced with some success for the control of *C. chalcites* in the field (Lima and van Harten, 1985).

Specifically, in the banana crops in the Canary Islands, *Chrysoperla carnea* (Neuroptera: Chrysopidae) has been recognized as an important predator. Larval parasitoids like *Cotesia* sp. have also been described in this region (Fig. 7A), but

the most common species is the egg-endoparasitoid *Trichogramma achaeae* (Nagaraja and Nagarkatti, 1973) (Hymenoptera: Trichogrammatidae) (Fig 7B), which can parasitize up to 87% *C. chalcites* populations (Del Pino et al., 2011; Nagaraja and Nagarkatti, 1973). However, the biology of the pest makes efficient control with this endoparasitoid dubious. In addition, commercial availability of *T. achaeae* is not sufficient to cover the needs for *C. chalcites* control (Del Pino, personal communication), hence the need to find alternative methods.

2.5.4. Microbiological control.

The microsporidian *Nosema manierae* (Protozoa: Microspora) is known to kill *C. chalcites* larvae in a few days (Toguebaye and Bouix, 1983). Products based on nematodes, in their vigorously infective juvenile stage, like *Steinernema carpocapsae* (Nematoda: Steinermatidae) also provide a feasible control of *C. chalcites* in a wide range of crops (Brødsgaard and Albajes, 1999). Several strains of *Bacillus thuringiensis* (*Bt*) are commonly used for the control of *C. chalcites* in different regions; like in Sicily, Italy, to efficiently protect tomato crops grown under net protection on in open-air greenhouses (Vacante et al., 2001) (Fig. 7C). In Israel, *B. thuringiensis* var. kurstaki is routinely used to protect horticultural and ornamental crops (Broza and Sneh, 1994). In the Canarian banana crops, *B. thuringiensis* var. kurstaki is the only microbial agent authorized to date (Agrocabildo, 2013).

As for viruses, high mortality rates have been found due to different local strains of the *Chrysodeixis chalcites* nucleopolyhedrovirus (ChchNPV) (Baculoviridae), during a period of high infestation of *C. chalcites* in banana crops in the Canary Islands (Fig. 7D). This virus has since gained particular interest as a potential biopesticide to control this pest (Bernal et al., 2013; Del Pino et al., 2011; Hernández-Santana, 2007). Insect-infecting baculoviruses are promising control agents for a number of lepidopteran pests due to their excellent insecticidal properties, host specificity and outstanding safety records (Caballero et al., 2009; Moscardi, 1999). A number of baculoviruses have been developed as the basis of effective biological insecticides against different agricultural and forest pests and applied to large field areas (Caballero et al., 2009; Cherry and Williams, 2001; Moscardi, 1999).

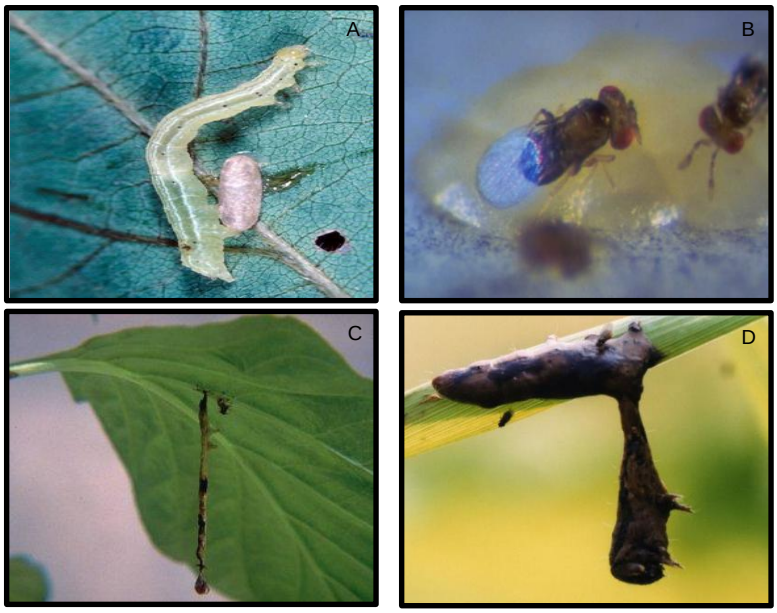

Figure 7. *Chrysodeixis chalcites* control with natural enemies: A) *C. chalcites* larva parasitized by *Cotesia sp.*; B) *C. chalcites* egg being parasitized by a *Trychogramma sp.* adult; C) larval cadaver resulting from a *Bacillus thuringiensis* infection; D) dead *C. chalcites* larva due to a baculovirus infection.

3. BACULOVIRUSES

Baculoviruses are infectious agents that cause fatal disease in arthropods. From an anthropocentric point of view, they are beneficial due to their potential for the control of insect pests. The spectacular symptoms induced by baculovirus infection were initially described two millennia ago in China in silkworm culture (Benz, 1986). Only much later, from 1950 to 1975, baculoviruses were observed to be effective biological control agents of insect pests (Ignoffo, 1981; Steinhaus, 1956). The first baculovirus registered as a pesticide in the United States was a commercial failure for a variety of reasons (Ignoffo, 1981). Nevertheless, the ever more obvious drawbacks of chemical pest control and the rapid build up of resistance to chemical pesticides has led to increased efforts to develop baculovirus insecticides and a concurrent increase in our understanding of the biology and ecology of these viruses.

3.1. Morphology and taxonomy

Baculoviruses (Baculoviridae) are a large family of arthropod-specific occluded viruses that have been isolated from more than 600 insect species in the orders Lepidoptera, Hymenoptera, Diptera, Orthoptera, Coleoptera, Neuroptera, Thysanoptera and Trichoptera (Herniou et al., 2003). They have a circular double-stranded DNA genome, of 90-160 kb, associated to a basic protein, and is included in a protein capsid constituting the nucleocapsid (Funk et al., 1997). Nucleocapsids are the elementary genetic units of baculoviruses and they are enveloped by a lipoprotein bilayer membrane, which they acquire from the host cell plasma membrane to form the budded virions (BVs), or which is newly synthetized to form occlusion derived virions (ODVs) (Blissard and Rohrmann, 1990; Caballero et al., 2001).

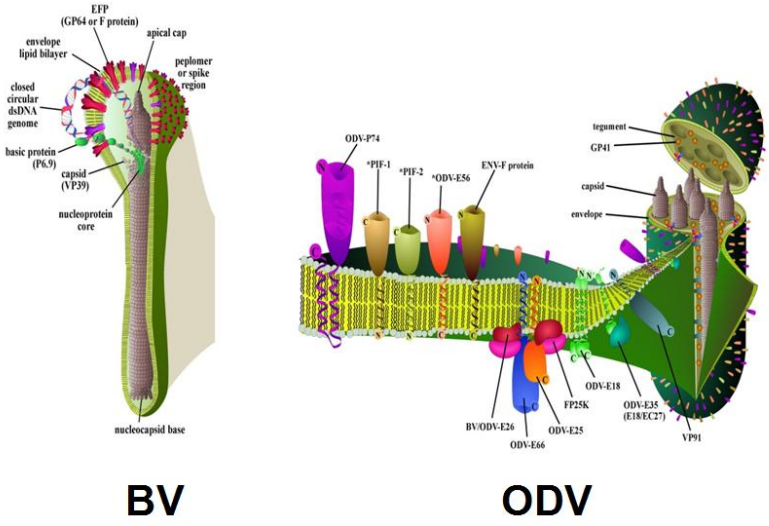

Figure 8. Composition and structure of two types of virions: budded virions (BV) and occluded virions (ODV). From Slack and Arif (2007).

All baculovirus virions share the same basic structure and constitute the morphological unit responsible for viral infection. The structure of the virion is critical to the ability of baculoviruses to replicate and spread efficiently within insects and then to transmit the infection throughout a population. Although these two types of virions are similar in their nucleocapsid structure, they differ in the origin and composition of their envelopes and their roles in the virus life cycle (Fig. 8). BVs

contain a single nucleocapsid and are formed when passing through the host cell plasma membrane from recognition sites including the glycoproteins, GP64 or F protein, which also form part of the membrane and allow recognition of new target cells. Meanwhile, ODVs may contain variable number of nucleocapsids and obtain their newly synthetized envelope in the nucleoplasm, associated with viral-encoding proteins. To date, several ODV proteins have been demonstrated to play an essential role in the ODV infectivity to the midgut cells. These proteins may form a heterotrimetric complex that fuses with receptors on the cell membrane (Peng et al., 2012). Deletion or inactivation of these proteins results in a lack of oral infectivity (Faulkner, 1997; Kikhno et al., 2002; Peng et al., 2012; Simón et al., 2012). ODVs are produced in the very late stage of the infection and become embedded in a protein matrix (polyhedron or granulin), which crystalizes around one or more ODVs forming a distinct structure known as occlusion body (OB) (Fig. 9), which is responsible for the horizontal transmission of the virus and is adapted to survival in the environment (Caballero et al., 2001).

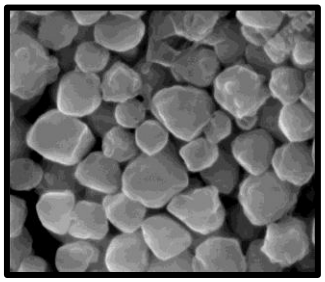

Figure 9. Scanning electron micrograph of occlusion bodies of *Chrysodeixis chalcites* NPV.

Formerly, baculoviruses were taxonomically divided into two genera based on their OB morphology (Blissard et al., 2000), namely: the granuloviruses (GVs) (Genus: *Granulovirus*) with smaller OBs called *granules* (150 nm in diameter and 400-600 nm in length), mainly composed by a protein matrix formed by granulin, and hosting a single virion; and the nucleopolyhedroviruses (NPVs) (Genus: *Nucleopolyhedrovirus*), large polyhedron-shaped structures called *polyhedra* (1-5 µm) composed of protein matrix formed by polyhedrin, and hosting multiple virions (Funk et al., 1997) (Fig. 9).

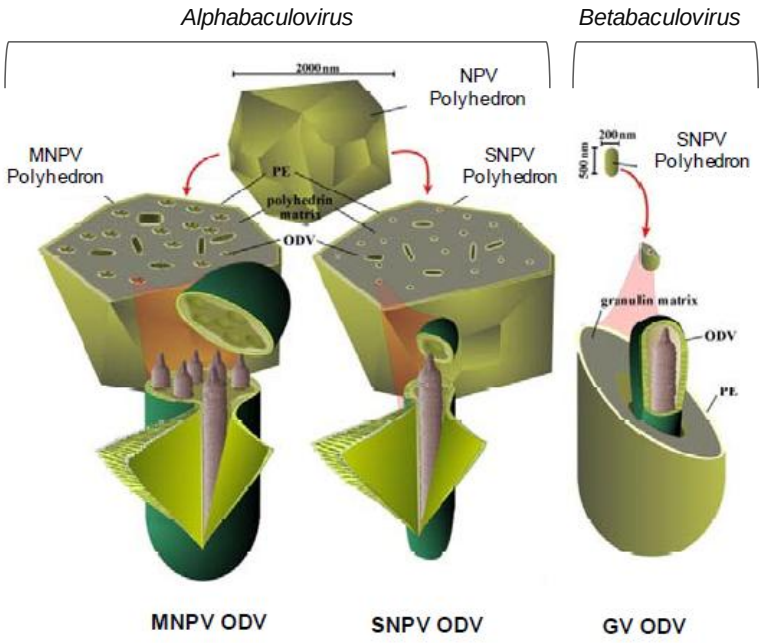

Figure 10. Structure of Alphabaculoviruses and Betabaculoviruses occlusion bodies (OBs) and of their respective occlusion-derived virions (ODVs). The two different ODV phenotypes within Alphabaculoviruses are also shown: multiple (MNPVs) and single (SNPVs) nucleopolyhedroviruses. From Slack and Arif (2007).

NPVs were further classified into two morphotypes, based on the number of nucleocapsids per virion: single NPVs (SNPVs), which contain a single nucleocapsid *per* virion; and multiple NPVs (MNPVs) with multiple nucleocapsids per virion (Fig. 10). However, the existence of both morphotypes did not have any taxonomic significance (Herniou et al., 2001; van Oers et al., 2005). Both, simple and multiple NPVs can contain numerous virions *per* OB. Co-occlusion is a usual feature in the biology of NPVs that directly influences genotypic composition. Multiple copies of the genome, monoclonal or polyclonal, can be found within the same OB (Bull et al., 2001), favouring NPV diversity within single OBs.

In the early days, it was believed that baculoviruses could only infect a single host species, and no cross-infection occurred. For this reason, baculovirus nomenclature is based on the host from which the virus was first isolated. However, although the cumulative host range of baculoviruses is very broad, each baculovirus can infect a single or a few related host species, with obvious implications for baculovirus classification and taxonomy. A clear alternative was the

use of molecular techniques in baculovirus taxonomy. In a first approach, restriction enzyme profile analysis were required to establish NPV identity (Blissard et al., 2000). Federici and Hice (1997) suggested defining a virus species by studying the relationship between common genes which are essential for baculoviruses. Herniou et al. (2003, 2001) proposed the use of multiple gene or complete genome arrangement to establish phylogenetic relationships between different baculoviruses. Since 2006, baculovirus classification has been based on genome sequence-based phylogeny in addition to the morphological and biological characteristics (such as the infected host), and the Baculoviridae family now comprises four genera: *Alphabaculovirus*, including NPVs infectious for lepidopteran, *Betabaculovirus*, with all GVs infectious for lepidopteran, *Gammabaculovirus*, encompassing NPVs infectious for phytophagous hymenopterans, and *Deltabaculovirus*, which includes dipteran-specific NPVs (Jehle et al., 2006). Additionally, phylogenetic studies based on the similarity of baculovirus common genes indicated that NPVs could be classified into two groups (I and II). Group I NPVs are composed only by MNPVs, while NPVs included in group II encompass both SNPVs and MNPVs (Herniou et al., 2001, 2003). Genome sequence comparisons further corroborated this finding and suggest that group II NPVs use an ancient furin-cleaved envelope fusion protein (or F protein) for cell-to-cell spread of the virus, whereas its function is replaced by GP64 in group I NPVs (Pearson and Rohrmann, 2002). ChchNPV is included within the *Alphabaculovirus* group II, phylogeneticaly close to *Trichoplusia ni* NPV (TnSNPV). Since ChchNPV is a member of the *Alphabaculovirus* genus, this genus will be described mostly in the rest of the chapter.

3.2. Infection cycle

3.2.1. In vivo *replication and pathogenesis*

A successful NPV infection starts when a larva of a susceptible species ingest the OBs present on leaf or soil material (Fig. 11A). Once they reach the insect midgut, the combined action of alkaline conditions (pH 10-11) (Terra and Ferreira, 1994) and of a non-essential alkaline protease present in the OB (Flipsen et al., 1995) causes OB dissolution and the release of ODVs. Virus encoded enzymes facilitate degradation of the peritrophic membrane and thus the contact of ODV

membranes with midgut epithelial cell membranes, which fuse (Fig. 11B), allowing virions to initiate a primary infection. ODVs may attach to midgut cells by interaction with midgut cell surface proteins (Horton and Burand, 1993; Peng et al., 2012). The nucleocapsids are liberated and cross the cytoplasm reaching the nucleus, where viral DNA is uncoated and unpacked. Then, viral DNA replication and new nucleocapsid formation takes place (Horton and Burand, 1993) (Fig. 11C).

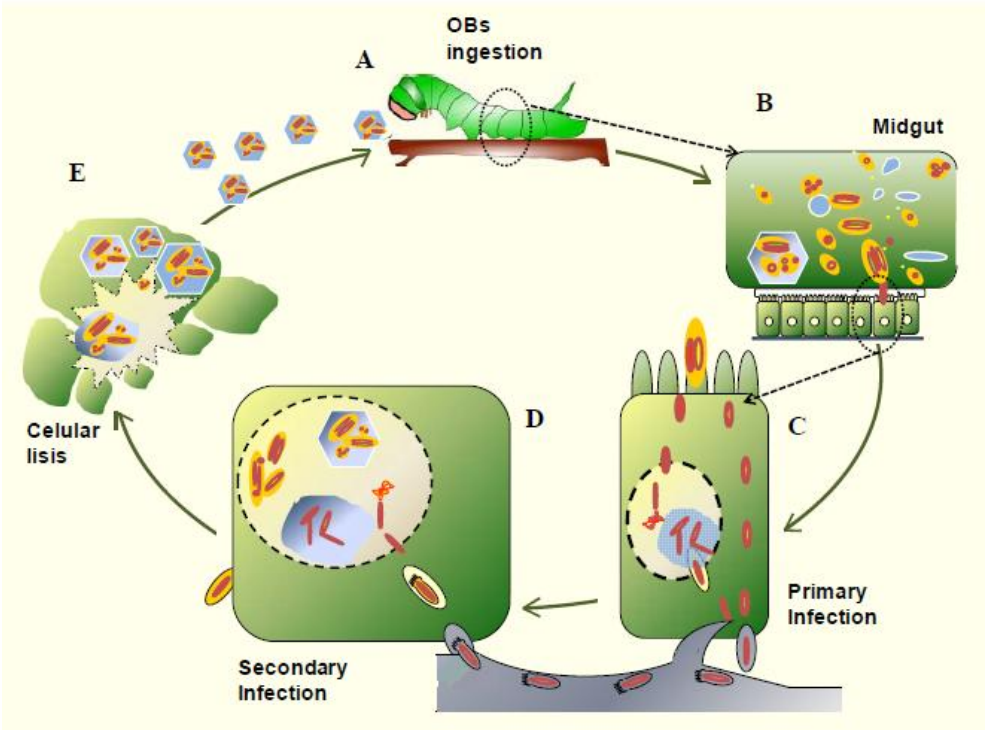

Figure 11. Life cycle of a NPV causing systemic infection. A) Polyhedra are ingested from surface-contaminated plant substrates along with feeding. B) Polyhedra are solubilised in the midgut and occlusion-derived virions (ODVs) are released. C) ODVs membranes fuse with the plasma membrane of the host midgut cell; nucleocapsids then they may follow one of two routes: i) cross the cytoplasm and reach the nucleus, where viral DNA replication and nucleocapsid formation take place; newly formed nucleocapsids are initially released by budding through the plasma membrane of the infected cell; and ii) bypass the nucleus and bud out throughout the basolateral membrane without entering and replicating in the nucleus. D) Virions from either route are spread throughout the insect tissues where they initiate secondary infections and produce more BVs, which are subsequently spread throughout the insect infecting furhter host tissues. E) ODVs produced in later stages of infection are occluded in occlusion bodies (OBs) and released in the environment.

Viral proteins of the BV envelope (GP64 or F protein), which confer tissue specificity for secondary infection, are transported to the cell surface and incorporated into the viral envelope, and newly formed nucleocapsids are initially

released by budding through the plasma membrane of the infected cell with which they form the budded virus (BV). BVs then pass into the hemocelic cavity through tracheoles, avoiding the basal membrane (Engelhard et al., 1994; Lepore et al., 1996; Slavicek and Popham, 2005; Wang and Granados, 1997) (Fig. 11C), and are able to enter cells of other tissues by endocytosis, in which they initiate a secondary infection process (Fig. 11D). Nucleocapsids from the ODVs may also bypass the nucleus of midgut epithelial cells and bud out through the basolateral membrane, allowing some ODVs to initiate secondary infections earlier than BVs (Fig. 11C). This strategy has been suggested as a mechanism to overcome the replacement of midgut cells, which can be faster than the rate of virus replication (Washburn et al., 1998, 1999). Early in the systemic infection, more BVs are produced, spreading the infection throughout the insect. The insect tracheal system and hemolymph are principally responsible for the successful transport of the BVs throughout susceptible tissues such as fat body, muscles and haemocytes (Fig. 11D) (Flipsen et al., 1995).

Once systemic infection is broadly spread, newly formed nucleocapsids remain in the host cell nucleus and assemble singly (SNPV) or in groups (MNPV) into ODVs. Later in infection, ODVs are wrapped in an envelope, followed by occlusion in polyhedra (OB) (Fig. 11D). This produces the hypertrophy of the nucleus, which occupies most of the cell volume. At the end of systemic infection, the nuclear and the plasma membranes breakdown, liberating the OBs and other cellular contents into the haemocoel. Virus encoded proteins, chitinase and cathepsin (O'Reilly, 1997), intervene in the subsequent degradation of the larval cuticle and hence in the releasing of OBs in the environment (Federici, 1997) (Fig. 11E).

NPV infections in lepidopteran larvae are characterized by alterations in the physiology and metabolism of the host. In general, the oxygen uptake increases markedly, indicating an acceleration of host cell metabolism (Granados and Williams, 1986), RNA synthesis increases to a maximum and total amino acid content, declines significantly. The hormonal titre in larvae is also affected by viral infection (Granados and Williams, 1986).

The external signs of a NPV-infected larva appear several days after virus intake, usually when infection is at the final stages (Granados and Williams, 1986). The first signs are gradual changes in colour and lustre of the integument with an

increase in opaqueness, milkiness and glossiness, mostly due to the infected hemocytes and the large amounts of OBs accumulated in the hemolymph. The larva becomes less active and loses appetite, but may continue to feed up to a few days before death. It is also possible to observe a retarded growth and an increased incidence of secondary infections, such as bacteria and fungi. The larva often secretes a dark-brown fluid from the anus or vomits a milky white fluid (Federici, 1997; Sciocco de Cap, 2001).

Figure 12. Characteristic signs of infection produced by alphabaculoviruses: A) typical position and flaccid tegument of a larva killed by NPV infection; B) dead larva with ruptured integument; C) healthy larva feeding on an NPV-infected cadaver.

Shortly before death, behavioural changes also occur. Larvae move away from the food and acquire a marked negative geotropism and an elevated location to hang from a branch or tree top by their abdominal and caudal prolegs (Federici, 1997). The death of the larva occurs within days, leaving the larvae typically hanging from their false legs (Sciocco de Cap, 2001) (Fig. 12A). A recent study revealed that *egt* gene (ecdysteroid uridine 5´-diphosphate (UDP)-glucosyltransferase) is responsible of this behaviour (Hoover et al., 2011). This gene inactivates the motilng hormone, and hence larval pupation or moulting, reducing the metabolic stress and extending the larval stage during infection, which favours viral progeny production. At this point, the larval corpse is flaccid with a fragile integument due to the action of viral encoded chitinases (Slack and Arif, 2007), which is easily ruptured to expel the liquid body contents, consisting almost entirely of OBs (Fig. 12B). These OBs contaminate plant surfaces and constitute the inocula for new infections (Federici, 1997) (Fig. 12C).

Several abiotic and biotic factors may contribute to NPV dispersion, and thus enhance horizontal transmission. The activity of natural enemies, including

predatory birds, arthropods and parasitoids, favours viral dissemination (Cossentine, 2009; Fuxa and Richter, 1994; Vasconcelos, 1996), as do agricultural practices (Moscardi, 1999). However, when horizontal opportunities are limited, especially during periods of low host densities, the NPVs may become persistent or latent (in a similar way as human virus), allowing NPVs to survive within apparently healthy host populations by vertical transmission (Burden et al., 2006). Persistent or latent infections may turn into overt infections when conditions are again suitable for horizontal transmission (Burden et al., 2006).

3.2.2. In vitro replication

Development of cell culture techniques, during the 1970s and 1980s, contributed to important advances in understanding the pathology and genetics of these viruses. In cell culture, the first step of baculovirus infection, which implies the infection of the midgut by dissolution of the OBs, is avoided. Instead, virus infection may be established most conveniently by harvesting BV-containing hemolymph from infected larvae and using it to inoculate susceptible cultured cells. It is relatively easy to establish continuous cultures of insect cells *in vitro* and many insect cells are susceptible to baculovirus infection. The most used system is that of *S. frugiperda* cell line (Sf21; developed from ovaries of the fall armyworm, *Spodoptera frugiperda*) with *Autographa californica* multicapsid nucleopolyhedrovirus (AcMNPV). Several other baculoviruses have been grown in cell culture using different cell lines; *Spodoptera exigua* MNPV in Se301 derived from a continuous Se3FH cell line of the beet army worm *S. exigua* (Hara et al., 1995) or *Trichoplusia ni* NPV in High Five cells (BTI-Tn5B1-4) derived from ovarian *T. ni* cells (Rhiel et al., 1997), in which ChchNPV also replicates. However, few attain the efficiency of the AcMNPV-Sf cell system.

The replication and morphogenesis of baculoviruses have been studied most intensely in cell culture systems (Erlandson, 2009; Hitchman et al., 2007). Notably, studies of baculovirus multiplication in insect cell cultures have provided fundamental knowledge of baculovirus DNA replication, gene function, gene expression, and gene regulation. Furthermore, baculovirus host specificity and other baculovirus characteristics, such as the diversity present within natural

populations, have also been elucidated in *in vitro* systems. Plaque purification techniques developed in cell culture have allowed the cloning of genotypic variants

Figure 13. Plaque assay technique. Monolayers of *Spodoptera frugiperda* cells are shown after staining a plaque assay with neutral red. Typical plaques are indicated by a blue circle. Each plaque, a clear area in a stained background, represents a group of cells killed by an infection originating from a single BV particle.

present in wild-type isolates, and contributed to scrutinize the heterogeneity present within baculovirus populations (Hitchman et al., 2007; Rowley et al., 2011; Simón et al., 2004; Wang et al., 2003; Wu et al., 2000) (Fig. 13). The cloning of individual genotypes is based on tissue culture infections with BVs.

However, continuous passage of viral progeny in cell culture has favoured the formation and accumulation of defective interfering particles (DIPs); 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). This is known as the passage effect.

From a practical point of view, cell-virus systems have proven useful for generating engineered baculoviruses for biological control (Moscardi, 1999) or for the expression of novel genes (Jarvis, 1997). The use of these viruses as expression vectors has arisen interest in the biomedical community to use baculoviruses for therapeutic purposes.

3.3. Genome organisation and evolution

During the last decades, several baculovirus genomes have been entirely sequenced, offering a wealth of information on the genetic diversity, gene

sequences, gene content, genome organization and phylogeny of baculovirus genomes (Fig. 14).

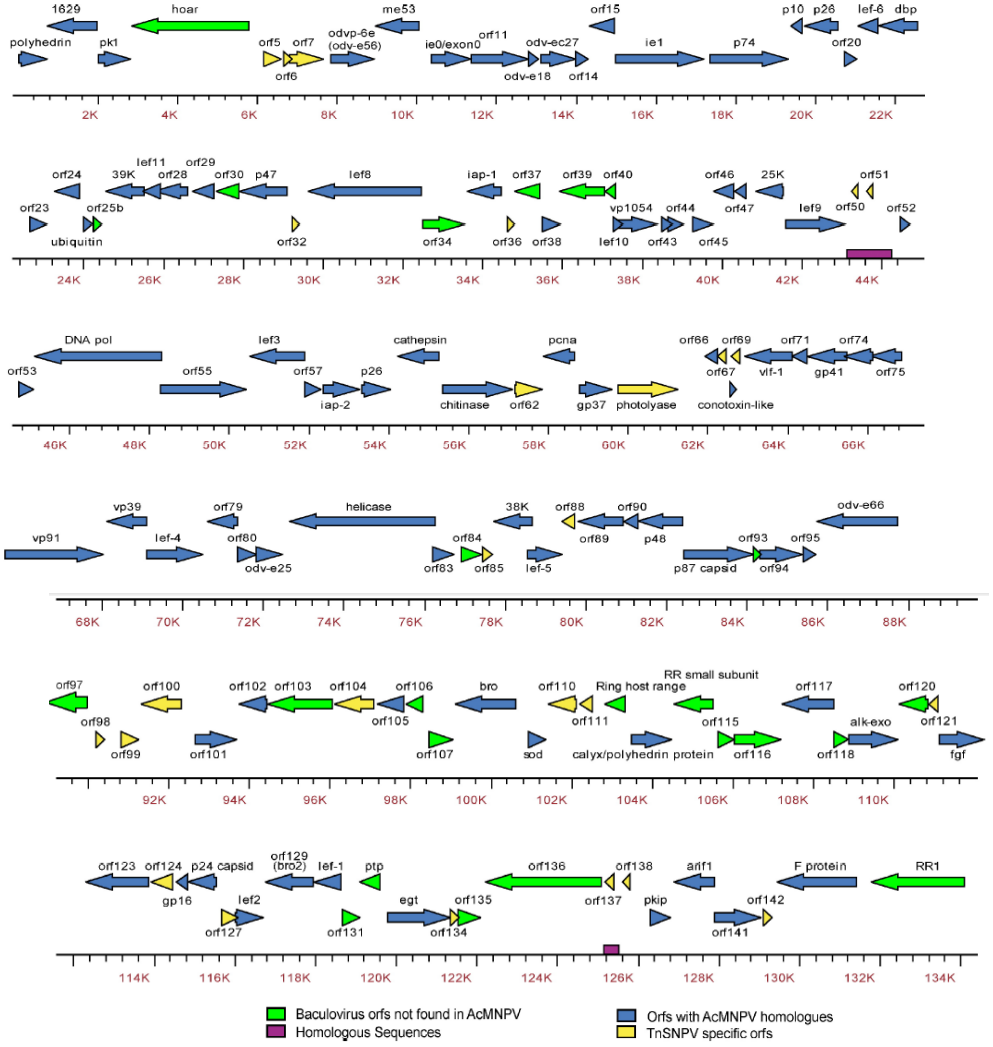

Figure 14. Linear representation of the genomic map of ChchNPV with annotated genes. Arrows indicate open reading frames and the direction of transcription. According to convention, the adenine residue of the start codon of the *polyhedrin* gene was designated as the zero point in the genome from Willis et al. (2005).

Currently, there are 57 complete baculovirus genomes deposited in GenBank. These include 41 *Alphabaculovirus* subdivided into Group I or II based on the type of fusogenic protein, 12 *Betabaculovirus*, 3 *Gammabaculovirus* and 1

Deltabaculovirus (Jehle et al., 2006). Baculovirus genomes consist of a single, circular, double-stranded DNA molecule. The genome size for different members of this virus family may vary from about 80 to over 180 kb that encode between 90 and 180 genes (van Oers and Vlak, 2007). Baculovirus open reading frames are tightly packed with minimal intergenic regions and the coding sequences are almost equally distributed over both strands and as non-overlapping contiguous sequences. Transcriptional analyses provided evidence of early, late and very late genes throughout the genome. All baculovirus genomes sequenced so far encode for a group of 37 genes, known as the core genes (Rohrmann et al., 2013). These genes represent a hallmark of the virus family and may play a role in essential biological functions (Lapointe et al., 2012). According to their function, baculovirus-encoded proteins can be grouped into the following functional classes: (i) transcription, (ii) replication, (iii) packaging, (iv) assembly, (v) cell cycle arrest/interaction with host proteins, (vi) oral infectivity, (vii) virion structure and (viii) proteins with auxiliary or unknown functions (Lapointe et al., 2012).

Comparisons of the overall gene content of the different baculovirus genomes provide an initial view of the genes present in all genomes that constitute the essence of a baculovirus. Sixty-two ORFs are common to all lepidopteran NPV and GV genomes sequenced to date (Jehle et al., 2006). These comparisons also allow identifying genes unique to each virus or characteristic of the different baculovirus genera. Unique genes are those that influence individual phenotypic traits of particular virus species. Comparison of the complete sequences of several baculoviruses has also revealed differences in gene content and genomic organization and hence led to establishing phylogenetic relationships among baculoviruses (Herniou et al., 2003).

In addition to genes, baculovirus genomes encode a number of small repeated sequences known as homologous regions (hrs). Hrs are interspersed in the genome (Erlandson, 2009; Possee and Rorhmann, 1997), have been shown to enhance early transcription and might also act as origins of replication (Lu et al., 1997). Moreover, sequence analysis has revealed that baculoviruses carry genes homologous to those of other organisms with eukaryotic or prokaryotic cells and to those of other viral families (Jehle et al., 1998). The origin of these baculovirus genes and the acquisition mechanisms by baculoviruses are now being

investigated. Recombination events and transposon insertions appear to play a role in the uptake of new genes from co-infecting viruses or from the insect host (Erlandson, 2009). Baculoviruses might be considered as a rich source of genes possibly acquired from many different organisms over a long period of evolutionary history. Phylogenetic analysis based on sequenced baculovirus genomes has revealed that they have evolved from non-occluded viruses infecting the midgut tissue, through occluded viruses infecting just the midgut (gamma- and deltabaculoviruses) to finally become occluded viruses with the ability to infect not only midgut cells but also to cause the systemic infections characteristic of NPV diseases (alpha- and betabaculoviruses) (Herniou and Jehle, 2007). It seems that baculoviruses have gained features to infect more cell types and become more independent from the host cell machinery.

3.4. Baculovirus diversity

Given the diversity and ubiquity of arthropod species, baculovirus diversity is probably enormous (Muñoz and Caballero, 2001). Baculoviruses have been identified in hundreds of insect species inhabiting forests, fields, rivers and households (Martignoni and Iwai, 1986). Likewise, the high baculovirus diversity can be explained by intrinsic factors, such as their high prevalence in the environment. Owing to the inclusion in OBs, baculoviruses are capable of surviving outside their host for long periods of time. Indeed, they can persist in the soil, plant crevices or other shelters for years (Miller, 1997).

3.4.1. Interspecific diversity

The *Alphanucleopolyhedrovirus* is the most common and widely distributed genus of baculoviruses. They have been reported from more than 400 insect species from seven different insect orders (Blissard et al., 2000; Jehle et al., 2006). They occur most commonly among lepidopterans, of which several hundred species have been reported as hosts. Since more than 200,000 species of Lepidoptera have been described to date, a great diversity of baculoviruses is estimated only within this genus. NPV isolates from hymenopterans, dipterans, coleopterans, thysanurans and trichopterans and even from shrimps (class Crustacea, order Decapoda) are much less common. GVs, however, have only

been reported from lepidopterans and, to date, slightly more than 100 lepidopteran species are known to be their hosts (Blissard et al., 2000; Jehle et al., 2006).

A better understanding of the natural diversity of baculoviruses will improve the taxonomy of this virus family, and more interestingly, will be of special importance for the development of highly insecticidal baculovirus strains (Muñoz and Caballero, 2001). The origin of this diversity lies in the different types of genes of each baculovirus species, the genomic organization, the degree of homology between shared genes, the structure of intergenic regions or even by small deletions or insertions (Serrano et al., 2013). The advent and widespread use of DNA-based techniques, initially restriction endonuclease analysis (REN) and lately genome sequencing, allowed these differences to be defined on a molecular level (Cory et al., 1997). Recently, phylogenetic analyses based on specific DNA sequences have favoured the naming and classification of the different viral species that are continuously emerging.

3.4.2. Intraspecific diversity: genotypes

Comparison of NPVs and GVs from the same species in different locations has shown considerable variation in genetic structure, demonstrated by the characterization of different geographical isolates of the same virus (Kamiya et al., 2004; Ogembo et al., 2007; Williams et al., 2011), and conversely, baculovirus collected from different species in the same area can be very similar (Cory et al., 1997). However, more interesting than the regional differences described above is the variation found within baculoviruses isolates (Cory et al., 2005; Redman et al., 2010; Rowley et al., 2011). Restriction endonuclease profiles of field-collected isolates frequently show submolar bands, indicating the presence of different genotypes. Cloning techniques, either in cell culture by plaque purification (Lynn et al., 1993; Simón et al., 2004) or in larvae by low dose *in vivo* cloning (Muñoz et al., 1998, 1999; Smith and Cook, 1988), has invariably demonstrated the presence of a wide array of genotypically distinct strains. The isolation of individual genotypic variants, therefore, has facilitated the genotypic characterization of wild-type populations and has allowed the evaluation of their relative biological activity contributing to our understanding of their diversity and evolution.

The presence of this variation raises some interesting questions, particularly with regard to the maintenance of baculovirus diversity and it effects on baculovirus-host interactions. Recent works have indicated that closely related baculoviruses undergo high levels of recombination (Cory et al., 1997; Herniou et al., 2003), which may produce the large numbers of variants observed. The genetic mechanisms most frequently involved in the generation of genotypic variants are small insertions or deletions, point mutations, and small genomic rearrangements (Cory et al., 2005; Muñoz et al., 1999). It seems that some genomic regions are more likely to generate variability. The *hrs*, are by nature regions with a high tendency to rearrangement and constitute "hot" areas in terms of variability (Erlandson, 2009; Pijlamn et al., 2002). Recombination between baculovirus genomes can also provide diversity and plasticity to baculoviruses (Cory et al., 2005). It is likely that this genetic interchange is occurring at a relative high frequency between baculoviruses which present homologous sequences in their genome or baculoviruses with overlapping host ranges (Cory et al., 2005; Erlandson, 2009). Finally, transposition is another mechanism that favours genetic interchange between the baculovirus and other related organisms (Li et al., 2013).

3.4.3. Intraspecific diversity: phenotypes

Differences found at the genome level between different isolates lead to distinct phenotypes in some instances. Closely related genotypic variants do not usually show large phenotypic differences, but in some cases minimal differences in their genomes affect important morphological and biological functions (Cory et al., 2005; Harrison et al., 2012; Rowley et al., 2011). For example, cloned variants of AcMNPV and TniSNPV (Harrison et al., 2012) show no significant phenotypic variation, whereas the speed of kill (virulence) of SfMNPV (Simón et al., 2004) or SeMNPV (Muñoz et al., 1999, 1998), cloned variants varies considerably. The phenotypic differences between these variants may alter baculovirus-host population dynamics.

Additionally, infections with experimental mixtures of different genotypes of a given viral species or different viral species have revealed antagonistic or synergistic effects in the phenotypic traits (pathogenicity, speed of kill, or virus yield) resulting from these interactions (Clavijo et al., 2010: Espinel-Correal et al., 2012;

Read and Taylor, 2001). In some instances, the mixture of viral genotypes enhances the efficiency of the virus as a biological insecticide (López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simón et al., 2005), but contrasting results have also been reported in other virus-host systems (Arends et al., 2005; Muñoz and Caballero, 2000).

The presence of naturally occurring deletion mutants in baculovirus populations is very common (Muñoz et al., 1998; Simón et al., 2004, 2005). In many instances deletions affect genes encoding for infectivity factors, essential for initiating primary infections (Dai et al., 2000; Kikhno et al., 2002; Simón et al., 2004). In these cases, maintenance of deletion mutants within natural populations is possible due to the co-occlusion and co-envelopment strategy, in which multiple genotypes are occluded in the same OB or co-enveloped in the same ODV (Clavijo et al., 2010). For instance, deletion mutants co-enveloped with a complete genotype are able to co-infect and co-replicate in the same cell by complementation (López-Ferber et al., 2003).

When developing baculoviruses as biopesticides, single genotypes or, more commonly, specific genotypic mixtures, with improved characteristics, such as increased pathogenicity or virulence, are selected. However, this may reduce the effectiveness of baculovirus-based bioinsecticides in the field, since traits promoting baculovirus survival, such as maximized yield, may act against selection of those enhancing their insecticidal properties. This underlines the importance understanding not only the diversity of isolates but also the interactions between them, so as to select those with well defined activity profiles for specific biocontrol programs. Genomic and biological characterization of the viral populations, either single or mixed genotypes, is thus strongly recommended before the release of new baculovirus insecticides.

3.5. C. chalcites simple nucleopolyhedrovirus

Natural populations of *C. chalcites* suffer NPV infections throughout the distribution area of this insect (Bernal et al., 2013; Murillo et al., 2000; van Oers et al., 2005). However, little is now about this NPV. To date only two ChchNPV isolates have been described, which can be clearly identified by restriction endonuclease analysis of their genomes (Murillo et al., 2000; van Oers et al., 2004).

One was obtained from *C. chalcites* larvae on greenhouse-grown tomato and sweet pepper crops in the Netherlands and is abbreviated as ChchSNPV-NL (van Oers et al., 2005, 2004); the other was isolated from *C. chalcites* larvae on greenhouse-grown in horticultural crops in El Ejido, Almería, Spain, and named ChchNPV-SP1 (Murillo et al., 2000). The first one has been completely sequenced (Gene Bank accession number: AY864330) (van Oers et al., 2005).

The geographical origin of both, the virus isolates and the host, can deeply impact control efficacy (Erlandson, 2009; Erlandson et al., 2007; Kouassi et al., 2009). Homologous viruses are usually more pathogenic than heterologous ones (viruses isolated from other host species) (Kouassi et al., 2009). Similarly, native isolates also tend to be more pathogenic to local insect populations than foreign ones (Erlandson et al., 2007). Clearly, there is an adaptive advantage for the virus to retain high infectivity toward the local host population, presumably as a result of continuous host-pathogen coevolution. Therefore, the characterization of indigenous NPVs in the regions where biological control programs are to be applied is specially recommended. Additionally, further selection of single genotypes or genotype mixtures is strongly advised, as these can be more efficient biopesticides than field isolates (Barrera et al., 2013; Behle and Popham, 2012; Muñoz et al., 1998).

4. BACULOVIRUSES BASED BIOINSECTICIDES

Of all viruses known to mankind, baculoviruses are the most beneficial from an anthropocentric viewpoint. While other viruses are studied because of their pathogenicity to humans, cattle or crops, the basis of modern baculovirology was stimulated by the potential utility of baculoviruses to control insect pests.

Humans have been aware of diseases caused by baculoviruses for over 2000 years. The earliest historical accounts originated in ancient Chinese literature describing silkworm culture (Benz, 1986). In addition, people in various cultures throughout history have witnessed, without knowing their cause, spectacular epizootics and subsequent population declines brought about by NPV and GV viruses in caterpillars and sawfly larvae that feed in the forests and on field and vegetable crops (Benz, 1986). However, it was not until the 19th century that polyhedral crystals could be observed in the “gore” (hemolymph) by microscopy and

correlated with the wilting disease of insects (Benz, 1986). In the 1930s and 1940s, Bergold discovered rodshaped virions within the crystalline polyhedral, initiating his studies on the biochemical properties of NPVs (Bergold, 1953). During the same period, baculoviruses were observed to be effective biological control agents of the European spruce sawfly (*Gilpinia hercyniae* (Hartig), Hymenoptera, Diprionidae), a pest that was accidentally introduced in North America, and which was found to be effectively controlled by the subsequent introduction of a baculovirus.

From 1950 to 1975, Steinhaus and his students defended the development of baculoviruses as biological control agents of insect pests (Steinhaus, 1956, 1963). In 1975, the first baculovirus was registered as a pesticide in the United States (Ignoffo, 1981), under the commercial name of Elcar®, however it was a commercial failure for a variety of reasons. In contrast, a notable success of a baculovirus pest control agent was the use of an NPV to control Douglas fir tussock (*Orgyia pseudotsugata* (McDunnough), Lepidoptera, Lymantriidae) by the US Forest Service (Martignoni, 1984). Significantly, the development of baculoviruses as biological pesticides stimulated efforts to understand the molecular biology of baculoviruses, and this, in turn, led to renewed industrial interest in baculovirus pesticide development on the 1990s.

The utility and effectiveness of these viruses to control lepidopteran pests has led to the commercial development of more than thirty baculovirus-based insecticides for use in both agricultural crops and forest ecosystems (Cherry and Williams, 2001; Moscardi, 1999), constituting a real alternative to chemical pesticides (Copping and Menn, 2000; Erlandson, 2008; Souza et al., 2007; Szewczyk et al., 2006, 2009; Zhang et al., 2007). Undoubtedly, the most prominent example is the successful Brazilian program for the control of *Anticarsia gemmatalis* in nearly two million soybean hectares. This program has demonstrated sustainability for more than two decades (Moscardi, 1999). In Spain, recently, a biopesticide based on a indigenous NPV of *S. exigua* (SeMNPV) for the control of this pest in vegetable crops in greenhouses of Almería, has been developed (Caballero et al., 2009). Another example is that based on *Helicoverpa armigera* SNPV to protect cotton against *H. armigera* in China, where the product has been used since its registration in 1993 (Zhang et al., 1995). After that several other products based on these viruses have been registered in this country. China is

currently the country with the highest number of baculovirus-based registered products (Ahmad et al., 2011; Yang et al., 2012). There are also well-known GV-based products (Vicent et al., 2007). Finaly, a number of baculovirus-based control programs are being presently developed in developing countries like Tanzania (Redman et al., 2010) or India (Bindu et al., 2011; Kumari and Singh, 2009; Srinavasa et al., 2008), where labour costs are cheaper than in developed countries.

4.1. Desirable characteristics

The ability of foliar-applied baculoviruses to protect crops from insect damage depends on the effective dose acquisition and the speed of action of the acquired dose (Black et al., 1997). Effective dose acquisition is critical to the success of any insecticide. In contrast with chemicals, which act by contact, baculoviruses need to be ingested in a sufficient dose to produce a systemic infection. The initiation of an infection is thus mainly reliant on the pathogenicity of the virus isolate or genotype forming the active compound. Consequently, the study of the natural diversity and the genotypic characterization of these viruses to select the genotype or mixture with improved phenotypic characteristics is a must.

Additionally, the success of baculovirus-based bioinsecticides relies on the intrinsic characteristics of baculoviruses as biocontrol agents. Among the desirable characteristics, bioinsecticides must have the following: (i) high virulence, (ii) high transmission capacity, (iii) high field persistence, (iv) feasibility for mass-production at practical and economic terms, (v) narrow host range, (vi) harmlessness to human, animals and environment, (vii) long shelf life, (viii) ability to be applied by conventional methods, and finally, (ix) susceptibility to be genetically modified (Ibarra and Del Rincón Castro, 2001).

4.2. Advantages and limitations

Baculoviruses have several inherent advantages that make them exceptional biological control agents, considering their safety, specificity and efficacy (Moscardi, 1999; Caballero et al., 2009; Rodgers, 1993). They are naturally occurring pathogens highly specific to insects and other closely related arthropods. Hence, they are safe in terms of pathogenicity against vertebrates, plants and other organisms (Gröner, 1986; Souza et al., 2007; Szewczyk et al., 2009). This fact

coupled with their narrow host range, sometimes limited to one or two species, make them harmless for non-target organisms, principally beneficial insects that naturally suppress insect pest populations and thus contribute to keep the biodiversity in agroecosystems (Ahmad et al., 2011; Ashour et al., 2007; Gröner, 1986). However, while single chemicals can cover a large portion of the crop pest complex or even all of it, baculovirus-based biopesticides usually target one pest species in a crop (Eberle et al., 2012). Baculoviruses are also highly persistent under natural conditions (specially in soil and litter), where they constitute an inoculum source for subsequent pest generations (Carinhas et al., 2010) and favour the establishment of the viruses as a mortality factor to regulate population densities, and extend the effect of an application. The capacity of these viruses to cause natural epizootics relies sometimes on their persistence but depends principally on the density of the pest population. Unfortunately, despite their persistence in the environment they are susceptible to UV degradation, resulting in a rapid degradation rate. For this reason, an appropriate formulation including UV protectans is especially recommended (Copping and Menn, 2000).

As occurred with most of the chemical insecticides commonly used in pest control (Georghion, 1991), long-term use of baculoviruses may derivate in insect resistance. In fact, field populations resistance to baculoviruses has been documented for one virus recently, *Cydia pomonella* granulovirus (CpGv) (Asser-Kaiser et al., 2007, 2011; Berling et al., 2009a, 2009b). Several populations of codling moth, *C. pomonella*, with a reduced susceptibility to its granulovirus have been detected in Germany and France (Asser-Kaiser et al., 2007; Eberle et al., 2008), and the mechanisms underlying such resistance are poorly understood. However, this resistance phenomenon could be overcame with the use of isolates from distant origins (Berling et al., 2009a, 2009b; Eberle et al., 2008), which recovered the formulates efficiency by selection against resistant hosts. The existence of a high diversity of genotypes within the same baculovirus species thus provides well-founded optimism that the emergence of baculovirus resistant populations can be pushed back.

As mentioned earlier, one of the biggest limitations of baculoriviruses as bioinsecticides is the slow speed of kill, compared with chemical pesticides. Chemical insecticides have a knockdown effect, whereas baculoviruses kill insects

after several days. The most rapid baculoviruses kill first instars 3-4 days after ingestion, e.g. SeMNPV (Caballero et al., 1992), but others like LdMNPV take longer, up to 20 days (Shapiro, 1986). Infected insects continue growing and feeding, causing substantial crop damage. Fortunately, this limitation and may be overcome in some instances by formulation. Incorporation of compounds such as optical brighteners (Morales et al., 2001; Zou and Young, 1994), free radical inhibitors, or certain enzymes has been demonstrated to prevent OB inactivation (Bonning et al., 2005; Zhou et al., 2004), enhance virus activity or even reduce the speed of kill (Zhou et al., 2004). Additionally, considerable research effort has been done toward developing faster killing agents through genetic modifications by the expression of insecticidal toxins, enzymes or hormones (Hammock et al., 1990; Maeda, 1989; Stewart et al., 1991; Tomalski and Miller, 1991), the deletion of life-stage manipulating virus genes (O'Reilly and Miller, 1991), or a combination of both (Bonning et al., 2005). Only a few genetically engineered baculoviruses with modified insecticidal properties have been tested in the field (Eberle et al., 2012), although none have been commercialized (Summers, 2006). Public aversion to release genetically modified organisms (GMOs) (Szewczyk et al., 2006) has rather placed the focus on the research o new isolates or genotypes with improved phenotypic characteristics.

The high costs associated with *in vivo* or *in vitro* production are also an important limitation for baculovirus-based production and commerciability. Additionally, *in vitro* production is not yet technically amenable for commercial production (Claus et al., 2012; Rezende et al., 2009; Tramper et al., 2006). Moreover, pesticide registration also carries associated costs linked to evaluation tests that are developed for broad-spectrum chemicals (Chandler et al., 2011). Fortunately, the entry into force, in January 2014, of a new European Union regulation for microbial control agents (Commission Regulation (EU) No 283/2013) will simplify the data requirements for these products. In developing countries, lower registration procedures and costs coupled with local production with little investment (Szewczyk et al., 2006) have made baculovirus-based formulates particularly suitable for use.

Due to their inherent insecticidal activity, and despite the limitations previously described, several natural baculoviruses have been registered, and successfully

used as safe bioinsecticides. The future of biopesticides relies on our ability to improve implementation and development of the products to make them competitive with chemicals.

4.3. Selection of the active material

Interest in baculoviruses as potential biopesticides naturally led to efforts to screen insect populations for new and potentially more effective isolates, single genotypes or mixtures of genotypes (Erlandson, 2009; Ogembo et al., 2007). Genotypic variation appears to be very common in natural baculovirus populations (Erlandson, 2009; Graham et al., 2004; Murillo et al., 2006). This genetic variability provides a large opportunity for natural selection, which maximizes the probability of successful infection of a potential host (Caballero and Williams, 2008; Hodgson et al., 2004; Simón et al., 2005) as well as survival in the environment (Murillo et al., 2006). It has been demonstrated that closely related isolates can differ in both pathogenicity and speed of kill, though it is not yet clear which genetic changes lead to these differences (Cory et al., 2005; Harrison et al., 2012; Rowley et al., 2011; Takatsuka et al., 2003). Geographical origin of both virus and host, can affect the characteristics of the dose-response curve and the survival period of infected hosts (Barrera et al., 2011; Erlandson, 2009; Erlandson et al., 2007; Kouassi et al., 2009). Homologous viruses are normally more pathogenic than viruses from other host species (Barrera et al., 2011; Kouassi et al., 2009) and native isolates also tend to be more pathogenic to the local insect population than homologous isolates from elsewhere (Erlandson et al., 2007). Therefore, determining the natural diversity present within the region in which the control program is going to be performed is a prerequisite to select the isolate with better insecticidal characteristics.

Additionally, genotypes that coexist in the same geographical isolate may differ greatly in pathogenicity (Simón et al., 2008), virulence (Hodgson et al., 2001; Simón et al., 2008), in the number of OBs produced *per* larva or *per* milligram of larva (Hodgson et al., 2001; Muñoz and Caballero, 2000; Murillo et al., 2006; Simón et al., 2005, 2008) or in their ability to disrupt the larval integument (Muñoz and Caballero, 2000).

Finally, infections with experimental mixtures of different genotypes of a given viral species or of different viral species have yielded antagonistic or synergistic

effects in phenotypic traits (pathogenicity, speed of kill, or virus yield) due to genotype interactions (Espinel-Correal et al., 2012; López-Ferber et al., 2003; Read and Taylor, 2001; Simón et al., 2005). In some instances, the mixture of viral genotypes enhances the efficiency of the virus as a biological insecticide (López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simón et al., 2005), but contrasting results have also been reported in other virus-host systems (Arends et al., 2005; Barrera et al., 2013; Muñoz et al., 1998; Muñoz and Caballero, 2000). Therefore, the genotypic characterization of field isolates is a key step in the process of active material selection during bioinsecticide development.

4.4. Massive production

The selection of a virus for pest management programs depends not only on its bioefficacy but also on the ease to mass produce it (Claus and Sciocco de Cap, 2001; Sherman, 1985) given the great amounts of bioinsecticides needed for field applications. Thus, the inocula used for virus production should be selected carefully to maximize both insecticidal activity (pathogenicity and virulence) and OB yield (Shapiro, 1986). The mass production system is one of the greatest limitations for the use of baculoviruses as biocontrol agents. As obligate pathogens, baculoviruses need active host cells for their replication and, for that reason, viral production must be performed either in host larvae (*in vivo*) or in cell culture (*in vitro*). Associated costs of either of the two systems make it difficult for viral products to compete in the marketplace (Grzywacz et al., 1998).

In vitro production has been demanded for decades by the bioinsecticide industry to commercialize baculoviruses. Although feasible for heterologous expression of protein products in recombinant baculoviruses (Claus and Sciocco de Cap, 2001), these techniques have not been sufficiently developed for large-scale viral production and devise serious technical constraints. Virus instability is one of them. In many cell line-virus systems, continuous passage in cultured cells rapidly renders defective virus genotypes with reduced biological activity (Dai et al., 2000; Giri et al., 2012; Pijlman et al., 2004; Sun et al., 2005; Szewczyk et al., 2006). Also, cell lines with relevant technological properties and with the ability to produce high OB yields still need to be developed (Claus et al., 2012). Finally, with a few exceptions, insect cell lines have been developed in insect tissue culture media that

rely on a costly substance, fetal bovine serum (FBS), to supply essential growth factors (Black et al., 1997). Without a substitute for FBS, profitability of *in vitro* produced insecticides is not possible. Adaptation to serum-free media or proprietary media may be a way out and successful adaptation typically requires several months to attain the desired growth kinetics and final cell densities required for production (Black et al., 1997).

In vivo production is, to date, the only feasible method for large scale propagation of baculoviruses (Gupta et al., 2007; Hunter-Fujita, 1998). Several authors have reviewed this system in the last 40 years (Hunter-Fujita, 1998; Jaques, 1977; Moscardi et al., 1997, Shapiro, 1986; Sherman, 1985; Shieh, 1989; van Beek and Davis, 2007). *In vivo* production involves the inoculation of massive numbers of larvae, the rearing of larvae while the virus replicates, and the recovery of OBs from infected larvae. To make it economically efficient, an ideal production system is one that yields the 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). In consequence, the techniques and methodology should be specifically developed and adapted for each host-virus system. *In vivo* production has several advantages over *in vitro* production. However, it involves a number of limitations and the efficiency depends on both biotic and abiotic factors that affect virus replication and viability in the insect host.

One of the factors directly influencing *in vivo* production is the establishment of a host insect population that is easily reared under laboratory conditions on artificial medium, with high fertility and no in-breeding problems (Claus and Sciocco de Cap, 2001). Insect rearing also demands a profound knowledge of the host biology, particularly nutritional and environmental requirements. For example, in species such as *A. gemmatalis* or *T. ni*, individuals can be reared in the same container, but in cannibalistic species as *S. frugiperda*, *H. zea* or *H. virescens*, insects need to be individualized, increasing production costs.

Research efforts have focused on basic aspects such as mass rearing methods, viral infection, and production processing (Black et al., 1997; Shapiro, 1986; van Beek and Davis, 2007). The geographical origin of the colony may also affect final OB yield, as local viruses tend to be more productive in local colonies in order to maximize their transmission (Barrera et al., 2011; Erlandson, 2009). Many

other studies have determined that the number of OBs produced *in vivo* depends on other biotic factors such as virus strain and concentration, host nutritional status, host species, larval stage, larval cannibalism or insect gender among others, being the main criterion the occlusion body (OB) production that larvae lethally infected can produce (Elvira et al., 2010a; Evans and Entwistle 1987; Gupta et al., 2007; Ignoffo and Couch, 1981; Vargas-Osuna et al., 1995). If the later factors are synchronized, insect death at a fully-grown larval stage guarantee maximized virus production. Therefore, viral mass production programs aim to develop production systems efficient enough to produce NPVs at a competitive cost. Productivity improvements of *in vivo* systems could come from selection of an improved strain combined with the optimization of production procedures (Ravensberg, 2011).

Environmental conditions directly affect *in vivo* production. For example, temperature plays an important role in viral replication, as observed with decreased OB yields in larvae reared at high temperatures (Claus and Sciocco de Cap, 2001). Humidity has not such a direct effect on production, but is important for final product quality, as excess humidity favours proliferation of bacteria, fungi or other contaminant microorganisms (Moscardi and Sosa-Gómez, 1996). Larval rearing containers may also affect final yields. On this respect, larval behavior, size and optimum density is important (Claus and Sciocco de Cap, 2001). Finally, insect diets constitute a major cost of *in vivo* production systems. Although natural diets, based on host plants, can be used, semisynthetic diets of varying complexity are much preferred (Claus and Sciocco de Cap, 2001). The cost of *in vivo* production has been reduced in some instances with the development of low cost diets (Bell et al., 1981; Elvira et al., 2010b; Gupta et al., 2004), in which some pricy ingredients are substituted by inexpensive ones that maintain the diet nutrient value for effective and sustainable colony rearing (Cohen, 2001).

In conclusion, biotic and abiotic factors should be determined in detail in each host pathogen system to optimize OB yields.

4.5. Formulation

An efficient virus along with a viable production system cannot reach commercial success without a formulation that protects the virus from environmental degradation. The main objective of a formulation is thus to preserve

the virus biological activity so as to deliver the product to the target system in time for the virus to be consumed by the host pest. It is also important to use conventional delivery techniques familiar to the end user. Appropriate formulations thus require previous studies to determine field persistence and storage stability.

Ultraviolet light is one of the most detrimental environmental factors affecting OB persistence (Támez-Guerra et al., 2005). During the last decades, several natural and artificial compounds have been evaluated as UV protectants, among them optical brighteners are the best studied. These stilbene derivates, which absorb energy in the UV portion of the spectrum and re-emit it in the blue portion of the visible spectrum, are commonly used in industrial processes to enhance the color appearance of fabric or paper, causing a whitening effect. Shapiro (1995) described their usefulness as UV protectants of LdMNPV OBs for the first time. Meantime, Shapiro and Robertson (1992) demonstrated they enhanced viral activity in some virus-host systems by disrupting the peritrophic membrane (Okuno et al., 2003; Wang and Granados, 2000) or inhibiting midgut cellular sloughing (Washburn et al., 1998). Virus induced apoptosis in insect midgut cells may also be blocked in the presence of optical brighteners (Dougherty et al., 2006). Moreover, optical brighteners can alter the rate of feeding of larvae (Shapiro and Farrar, 2003) and also increase the susceptibility of later instars to NPV infection (Shapiro, 2000; Shapiro and Robertson, 1992) to such low levels that viral concentrations used for earlier instars can also control later ones. This is an interesting aspect of brighteners for field applications, as mixtures of larval stages are likely to be present simultaneously in the field due to overlapping of pest generations. However, field applications of optical brightener formulations have shown some negative effects: they may reduce the growth of monocot crops (Goulson et al., 2003) or induce modification in the behaviour of bee pollinators (Goulson et al., 2000).

Other successful enhancers of viral activity are titanium dioxide (Farrar et al., 2003), folic acid (Shapiro, 1986), calcofluor (Wang and Granados, 2000), congo red (Shapiro and Shepard, 2008), boric acid (Cisneros et al., 2002), chitinase (Shapiro, 1995), enhancins (proteins isolated from GV) (Lepore et al., 1996; Mukawa and Goto, 2010), azadirachtin (Mascarin and Delalivera, 2012; Nathan and Kalaivani, 2006) and plant extracts (Shapiro et al., 2010). However, the release of chemical products has limitations and natural products are more recommended. Some plant

extracts, for instance, protect microbial agents from UV radiation and one of them has been the subject of a US patent (Shapiro et al., 2010). Other additives, such as sunscreens, phagostimulants and synergists directly could also be added to improve the insecticidal effect (Burges and Jones, 1998; Farrar and Ridgway, 1994; Moscardi, 1999).

Finally, given that baculovirus-based bioinsecticide products are used for inundative applications in agricultural systems, the plant coverage with these products needs to be optimized. Some additives such as wetting agents, stickers, surfactant, thickeners or humectants affect the physic-chemical structure of the mix to improve the application and the surface deposition efficacy. Since OBs are degradated in alkaline conditions, formulations should avoid the use of basic water or pH correctors (Cherry and Williams, 2001).

4.6. Field efficacy

Once the efficacy of a NPV has proven useful under laboratory conditions, it is necessary to validate it under controlled field conditions. It is recommendable to perform these assays first under greenhouse conditions that aid determining any potential variables (eg. percent leaf coverage, persistence, application rates, etc.) in the open field (Ibarra and Del Rincón Castro, 2001).

The success of this kind of products depends on a profound knowledge of the crop and the target insect pest, as applications must be designed to provide optimum deposits at the pest feeding site. Special interest has to be deposited in controlling young instars to prevent their getting more voracious and damaging in later instars (Briese, 1986). The biology of the target host insect, particularly its feeding preferences and habits, may significantly influence the rate of acquisition of a lethal infection upon consumption of contaminated foliage.

Formulation and cultural crop techniques should also be adapted to favour virus efficacy in each situation. On this respect, selection of the isolate with increased insecticidal characteristics and an appropriate concentration of the target pest are important initial steps in the implementation of control programs involving baculoviruses. Timing applications properly and larval density are also key features for a successful use.

In inundative releases, performed mostly in agriculture, large OB quantities are applied for the rapid suppression of the existing damaging pest population. In contrast, inoculative (low dose) releases, applied to more stable ecological systems such as forests, NPVs are supposed to exert action over the course of various pest generations, where they replicate and build up increasing population levels. The number of OBs in each application has an important influence on pest mortality, but also directly affects the cost of each application so that recommendations are usually based on the lowest quantity of OBs that result in adequate control (Cherry and Williams, 2001). The frequency of virus treatments required depends on the persistence of OBs in the crops, the feeding habits of the insect, the dynamics of pest infestations, etc. For some pests, frequent applications of low OB rates result in better levels of crop protection than occasional applications of high OB concentrations (Hunter-Fujita et al., 1998).

4.7. Registration

Once the viral efficacy of a baculovirus-based agent or formulate is confirmed, registration of either one follows. Paradoxically, despite application of a considerable number of biological programs based on these viruses, their use is still scant compared to other biological insecticides like *B. thuringiensis*. Newly emerging trends favouring specific regulations for low-risk products like baculovirus-based pesticides, along with newly established departmental branches specialized in registration of these products will reduce registration processes and costs in the near future (Hauschild et al., 2011; Kabaluk et al., 2010; Lapointe et al., 2012). In the European Union, the date for entry into force of the new regulation setting out data requirements for active ingredients is January 2014 (Commission Regulation (EU) No. 283/2013). Effective public extension services and farmer training in the application of biopesticides are also needed to expand the use of these products worldwide.

5. SCOPE OF INVESTIGATION

This study arises from the need to control *C. chalcites* in banana crops from the Canary Islands (ASPROCAN, personal communication). The aim of this thesis is focused on the biotechnological development of a new biopesticide based on a Canarian ChchSNPV isolate. Firstly, the natural diversity of ChchSNPV in the

Canary Islands was evaluated in order to select a ChchSNPV isolate with high insecticidal properties. The most prevalent and widespread isolate, ChchSNPV-TF1 (ChchTF1), merited further evaluation as the basis for a biological insecticide (**Chapter 2**), because of its high pathogenicity and virulence. However, in the search for an even higher insecticidal activity, the genotypic composition of ChchTF1 was determined (**Chapter 3**) along with the interactions between several ChchTF1 genotypes. This analysis allowed selecting a genotype or mixture of genotypes with improved insecticidal characteristics compared with the wild-type ChchTF1. In a subsequent study, five pure genotypes were sequenced, including those of the mixture, with the aim of providing genetic data relevant to the genotypic and phenotypic diversity of this virus (**Chapter 4**). Further experimentation was devoted to maximize an OB production system for ChchTF1 in *C. chalcites* larvae (**Chapter 5**). Optimization of different parameters, like larval stage, inoculation time, viral suspension concentration and larval density, had a significant impact on NPV productivity and represent key factors in making the commercial production of this virus economically feasible. The larval susceptibility to ChchTF1 OBs was analysed across different instars as well as the effect of OB and optical brightener mixtures on the insecticide properties of this virus was also evaluated (**Chapter 6**), with clear applications for the use of ChchTF1 as a biological insecticide. Finally, In **Chapter 7** the efficacy of the ChchTF1 to control *C. chalcites* infestations on tomato and banana plants in growth chambers, greenhouses and field trials was assessed. All this information has favoured a patent application.

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CHAPTER 2

A native variant of *Chrysodeixis chalcites* nucleopolyhedrovirus: The basis for a promising bioinsecticide for control of *C. chalcites* on Canary Islands' banana crops

ABSTRACT

Chrysodeixis chalcites (Lepidoptera: Noctuidae) larvae cause up to 30% production loss in banana crops in the Canary Islands. Larvae of this species are susceptible to a nucleopolyhedrovirus (ChchNPV). This study aimed at evaluating the genetic diversity and bioinsecticidal activity of ChchNPV isolates collected from C. chalcites larvae in the Canary Islands. From a total 97 isolates collected in different banana greenhouses, restriction endonuclease analysis identified five genetic variants that differed slightly from ChchNPV isolates from Netherlands (ChchSNPV-NL) and Almería, Spain (ChchNPV-SP1). Physical maps revealed minimal differences at genome level mostly due to variation in the position/existence of restriction sites. ChchSNPV-TF1 was the most prevalent variant, representing 78% of isolates examined, and was isolated at all Canary Island sampling sites. This isolate was the most pathogenic isolate against C. chalcites second instars in terms of dose-response metrics, compared to homologous variants or two heterologous viruses *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Anagrapha falcifera* multiple nucleopolyhedrovirus (AnfaMNPV). ChchSNPV-TF1 was also one of the fastest killing variant although no differences were observed in occlusion body production among the different variants in second instars. We conclude that ChchSNPV-TF1 merits further evaluation as the basis for a biological insecticide for control of *C. chalcites* in banana crops in the Canary Islands.

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1. INTRODUCTION

Chrysodeixis chalcites (Esper) (Lepidoptera: Noctuidae) is a mayor polyphagous pest in many countries (Shepard et al., 2009). In the Canary Islands, 100 km off the coast of Morocco, north-western Africa, *C. chalcites* populations have increased markedly during the past decade, possibly related to increased migration and range shifts in this and other noctuid pest species in response to global climate change (Sparks et al., 2007). Infestations of this pest frequently result in up to 30% losses in bananas grown under greenhouse conditions in the Canary Islands (Del Pino et al., 2011). Chemical-based control measures require multiple applications that tend to increase production costs and can hamper the commercialization of produce that contain pesticide residues and generates resistance development (Horowitz et al., 1998; Perera and Molina, 2007). Insect-infecting baculoviruses have been reported worldwide in over 600 host species, mainly from the order Lepidoptera (Jehle et al., 2006). Several of these viruses have been developed as the basis for effective biological insecticides against different crops and forest pests (Caballero et al., 2009; Moscardi, 1999).

Populations of *C. chalcites* can succumb to infection by a nucleopolyhedrovirus (ChchNPV; genus Alphabaculovirus). To date two ChchNPV isolates have been described; one from *C. chalcites* larvae on greenhouse-grown tomato and sweet pepper crops in the Netherlands that we will refer to as ChchSNPV-NL (van Oers et al., 2005, 2004) and the other from *C. chalcites* larvae on greenhouse-grown in horticultural crops in El Ejido, Almería, Spain, named ChchNPV-SP1 (Murillo et al., 2000). ChchNPV infects members of an important group of Lepidoptera known as semiloopers (Noctuidae: Plusiinae) such as *Trichoplusia ni*, and has been considered as a potential candidate for biological control of semilooper pests. Other viruses with comparatively wide host ranges include *Autographa californica* NPV (AcMNPV) and its genotypic variant Anagrapha falcifera NPV (AnfaMNPV) (Harrison and Bonning, 1999), both of which can infect and kill *C. chalcites* larvae.

Considerable genetic heterogeneity is usually observed in natural baculovirus populations (Erlandson, 2009). This diversity has been demonstrated by the characterization of different geographical isolates of the same virus (Gordon et al., 2007; Williams et al., 2011) and also within single isolates, that frequently comprise a number of genotypic variants (Cory et al., 2005; Redman et al., 2010). Restriction

endonuclease treatment of viral DNA provides profiles that are characteristic for each isolate or genotype (Erlandson et al., 2007; Harrison and Bonning, 1999). Closely related isolates do not usually show large phenotypic differences in terms of their insecticidal characteristics, although occasionally minimal changes in the genome of these variants may affect important biological traits such as pathogenicity, speed of kill, occlusion body (OB) yield, or even host range, OB size, and larvae liquefaction has also been noted (Cory et al., 2005; Harrison et al., 2012; Rowley et al., 2011; Takatsuka et al., 2003). It has also been demonstrated that the geographical origin of both virus and host, can affect the characteristics of the dose-response curve and the period of survival of infected hosts (Erlandson, 2009; Erlandson et al., 2007; Kouassi et al., 2009).

The aim of the present study was to select a nucleopolyhedrovirus isolate that could be evaluated for use in a control program targeted at *C. chalcites* in the Canary Islands. For this purpose, the natural diversity of ChchNPV isolates was evaluated by molecular and biological characterization of different isolates collected in the Canary Islands and compared with ChchNPV isolates from other geographical origins and with AcMNPV and AnfaMNPV isolates that can also infect *C. chalcites* larvae.

2. MATERIAL AND METHODS

2.1. Insect source, rearing and viruses

C. chalcites larvae were obtained from a laboratory culture at the Universidad Pública de Navarra (Spain) established in 2007 using pupae received from the Instituto Canario de Investigaciones Agrarias, (ICIA), Tenerife, Spain, and refreshed periodically with pupae from the Canary Islands. Larvae were reared at 25ºC, 70±5% humidity, and a photoperiod of 16:8 (light:dark), on a wheat germ, yeast and soybean meal based semi-synthetic diet described by Greene et al. (1976). Adults were fed *ad libitum* with 30% w/v diluted honey.

Over a period of two months from October 2006 to November 2006 a total of 97 isolates were collected from *C. chalcites* larvae showing the typical signs of lethal polyhedrosis disease. This was confirmed by direct microscopic observation of OBs in giemsa-stained smears of infected cadavers. All infected larvae were collected from Canary Islands' banana crops during periods of high infestation by *C.*

chalcites (Table 1). These isolates were genetically and biologically compared with previously characterized isolates of ChchNPV from Almería (ChchNPV-SP1; Murillo et al. 2000) and the Netherlands (ChchSNPV-NL; van Oers et al., 2004), kindly provided by Dr. M. M. van Oers (University of Wageningen, The Netherlands). AcMNPV was kindly provided by Prof. R. D. Possee (CEH Oxford, UK) and AnfaMNPV by Dr. P. Támez-Guerra (Universidad Autónoma de Nuevo León, Mexico).

Table 1. Origin of the nucleopolyhedrovirus isolates collected from *C. chalcites* larvae in greenhouses of the Canary Islands.

Location of greenhouses sampled		Coordinates		Isolates (n)	Canarian ChchSNPV isolates				
Islands	Place (greenhouse)	Longitude (W)	Latitude (N)		TF1	TF2	TF3	TF4	TF5
Tenerife	Guargacho (Bueype)	16º37'45"	28º02'41"	39	23	8	6	1	1
	Los Silos-Garachico	16º48'15''	28º22'11''	9	9	0	0	0	0
	El Fraile (S. Lorenzo)	16º40'46''	28º00'15''	4	4	0	0	0	0
	Las Galletas (Valle Grande)	16º39'07''	28º01'47''	1	1	0	0	0	0
	Las Galletas (Laura)	16º39'32''	28º01'52''	4	4	0	0	0	0
	Puerto de la Cruz	16º33'31''	28º24'22''	7	7	0	0	0	0
	Las Galletas (R.F.)	16º39'60''	28º01'52'	3	3	0	0	0	0
	La Frontera (J.A.)	18º00'49''	27º46'17''	5	5	0	0	0	0
La Palma	Los Llanos de Aridane	17º55'53''	28º37'27''	1	1	0	0	0	0
Gran Canaria	Vecindario	15º25'44"	28º07'52"	8	8	0	0	0	0
El Hierro	Frontera	18º00'28''	27º46'51''	16	16	0	0	0	0
Total				97	81	8	6	1	1

2.2. Occlusion body amplification, DNA extraction and restriction endonuclease analysis

Occlusion bodies (OBs) of the different isolates collected in field were amplified by a single passage in *C. chalcites* fourth instar larvae. Twenty-five larvae from the laboratory colony were starved overnight and then allowed to drink an OB suspension (10^6 OBs/ml) obtained from infected field-collected insects. Larvae that drank the suspension were individually reared on semisynthetic diet until death.

OBs were extracted from dead larvae by homogenizing the cadavers in water and purified by filtration and differential centrifugation. OBs were then resuspended

in double-distilled water and their concentration was determined by counting triplicate samples using an improved Neubauer hemocytometer (Hawksley, Laucing, UK) under phase contrast microscopy at x400. Purified OBs were stored at 4ºC for up to 1 month prior to use in bioassays.

For DNA extraction, virions were released from OBs by mixing 100 µl of OB suspension containing 10^9 OBs/ml with 100 µl 0.5 M Na_2CO_3, 50 µl 10% (w/v) sodium dodecyl sulfate in a final volume of 500 µl and incubating for 10 min at 60°C. Undissolved OBs and other debris were removed by low-speed centrifugation (3,800 x g, 5 min). The supernatant containing the virions was treated with 25 µl proteinase K (20 mg/ml) for 1 hour at 50°C. Viral DNA was extracted twice with saturated phenol and once with chloroform and isolated from the aqueous phase by alcohol precipitation. The pellet was resuspended in 50 to 100 µl of 0.1xTE buffer (Tris-EDTA, pH 8) for 10 min at 60°C. DNA concentration was estimated by reading the optical absorption at 260 nm. For restriction endonuclease analysis, 2 µg of viral DNA were mixed with 10 U of one of the following enzymes *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II (Takara Bio Inc., Shiga, Japan) and incubated for 4 to 12 h at 37°C. Reactions were stopped by addition of 4 µl of loading buffer (0.25% w/v bromophenol blue, 40% w/v sucrose). Electrophoresis was performed using horizontal 1% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) at 20 V for 10 to 24 h. DNA fragments were stained with ethidium bromide and visualized on a UV transilluminator (Chemi-Doc, BioRad, California, USA).

2.3. Nucleocapsid packaging

To determine whether the ChchNPV isolates from the Canary Islands were single or multiple type NPVs, occlusion derived virions (ODVs) were released from samples of $5x10^8$ OBs by exposure to alkaline buffer (0.1 M Na_2CO_3) for 30 min at 28ºC. Polyhedrin and other debris were removed by low-speed centrifugation (2,500 x g, 2 min). The ODV-containing supernatant was banded by density equilibrium centrifugation (30,000 x g, 1 h) on 30-60% (w/v) continuous sucrose gradient. The banding pattern was visually inspected and photographed.

2.4. Construction of a ChchNPV genomic library

A partial *Bgl*II genomic library of the ChchNPV-TF1 isolate was constructed using the pSP70 plasmid (Promega, Fitchburg, Wisconsin, USA) as receptor in

conjunction with a DNA ligation kit (New England Biolabs, Ipswich, Massachusetts, USA). Viral DNA was purified as described above, digested with *Bgl*II and ligated into the pSP70 plasmid at 16ºC overnight following manufacturer's instructions. The ligation reaction was dialyzed for 30 min against TE buffer and used to transform GeneHogs electrocompetent cells (Invitrogen, Carlsbad, California, USA), that were then selected on LB agar containing 100 µg/ml ampicillin.

To study the genomic variability among the field-collected isolates, the polymorphic restriction fragments of each isolate were cloned and subjected to terminal sequencing.

Colonies were amplified and DNA plasmids were purified and screened for the presence of inserts by *Bgl*II digestion and electrophoresis in 1% agarose gel. Inserts were authenticated by comparing their migration in agarose gels with the fragments from field collected variants ChchNPV-TF1, ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4 and ChchNPV-TF5 generated by digestion with the same enzyme.

2.5. Physical mapping and sequencing

The construction of the physical map of ChchNPV-TF1 was obtained by ordering the restriction fragments on the viral genome according to the multiple digestions of cloned fragments. All cloned *Bgl*II fragments were digested with *Kpn*I, *Sac*II and *Bam*HI. The fragments resulting from multiple digestions were electrophoresed and the fragment sizes were then determined by analysis of the overlapping portions of cloned fragments. Mapping of the viral genome was confirmed by sequencing information obtained from the termini of cloned *Bgl*II fragments. Nucleotide sequences were determined in an ABI PRISM 377 automated DNA sequencer (Sistemas Genómicos S.A., Valencia, Spain), employing standard SP6 and T7 primers.

The physical maps of ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4, and ChchNPV-TF5 were assembled by comparing their restriction profiles with that of ChchNPV-TF1, and using the sequencing information from the termini of each variant's polymorphic restriction fragments. The physical map previously constructed for the ChchNPV-TF1 variant was used as a basis. The conventional

practice of defining the REN fragment which contains the polyhedrin gene as the start point for physical maps of NPV was followed (Vlak and Smith, 1982).

2.6. Insect bioassays

The insecticidal activities of Canary Island variants were compared with those of ChchNPV-SP1, ChchSNPV-NL, AcMNPV and AnfaMNPV. Concentration-mortality metrics, mean time to death (MTD) and productivity (OBs/larva) were determined by per os bioassays performed using the droplet-feeding method (Hughes and Wood, 1981). Second-instar *C. chalcites* from the laboratory colony were starved for 8 to 12 h at 26°C and then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs at one of five different concentrations for each variant. For AcMNPV and AnfaMNPV the OB concentrations used were $1x10^9$, $1x10^7$, $1x10^5$, $1x10^3$ and $1x10^1$ OBs/ml, whereas for ChchNPV variants the OB concentrations were $1x10^5$, $2x10^4$, $4x10^3$, $8x10^2$ and $1.6x10^2$ OBs/ml. These concentration ranges were previously determined to kill between 95 and 5% of the experimental insects. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with a cube of semisynthetic diet. Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed on three occasions. Larvae were reared at 25°C and mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality results were subjected to logit analysis using the POLO-PC program (LeOra Software, 1987).

Time mortality analysis, expressed as mean time to death (MTD), was performed only for insects infected by variants of ChchNPV. Groups of 25 second instars were allowed to drink OB suspensions during a 10 min period as described in the concentration-mortality bioassay. The OB concentration used for the time mortality analysis was $1x10^5$ OBs/ml for all ChchNPV variants tested, except for ChchSNPV-TF1 which was inoculated at a concentration of $2x10^4$ OBs/ml; these concentrations resulted in 92-98% mortality in all cases. The bioassay was carried out three times. Larvae were individually reared at 25ºC and mortality was recorded every 8 h. Time mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993). Survival models, such as the Weibull, are preferred for analysis of time to death data as the

variance in age at death tends to increase with the mean. The time mortality distribution of different isolates was analyzed graphically. Only individuals that died from NPV disease, confirmed by the microscopic observation of OBs, were included in these analyses.

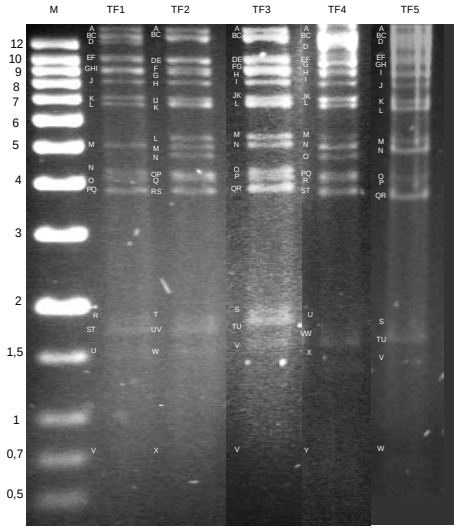
The OB production of ChchNPV isolates was determined in *C. chalcites* second instar larvae infected by treatment with the OB concentrations that were used in the time-mortality assay. Twenty-five larvae were inoculated for each treatment and the bioassay was performed three times. All the larvae that died of NPV disease (a minimum total of 50 larvae *per* virus treatment) were collected and stored at -20ºC until used for OB counting. For this, each larva was homogenized in 100 µl distilled water and the total yield of OBs per larva was estimated by counting in triplicate using a Neubauer hemocytometer. The promediums of each replicate were analyzed by ANOVA using the SPSS v12 program.

3. RESULTS

3.1. ChchNPV-TF1 was the most prevalent and widespread variant isolated from infected larvae

All of the 97 isolates collected from *C. chalcites* in the Canary Islands could be classified into one of five different variants differentiated by their restriction analysis profiles following treatment with *Bam*HI, *Bgl*II, *Kpn*I or *Sac*II, being *Bgl*II the restriction enzyme that differentiated all the isolates. All five variants were similar in restriction profile characteristic to the previously characterized ChchNPV-SP1 and ChchSNPV-NL isolates and therefore can be considered as geographically distinct variants of ChchNPV. The variants were named ChchNPV-TF1, ChchNPV-TF2, ChchNPV-TF3, ChchNPV-TF4, and ChchNPV-TF5 (Fig. 1A). The ChchNPV-TF1 isolate was selected as the reference variant because its *Bgl*II restriction profile was common to 78% of the isolates collected in field, indicating this to be the most prevalent variant. The prevalence of the remaining variants varied: 15% of isolates were classified as variant ChchNPV-TF2, 5% of isolates as variant ChchNPV-TF3, and two different single isolates as variant ChchNPV-TF4 and variant ChchNPV-TF5. The ChchNPV-TF1 variant was present in all the greenhouses sampled, making it the most widespread variant in the Canary Islands, whereas the other four

A)



B)

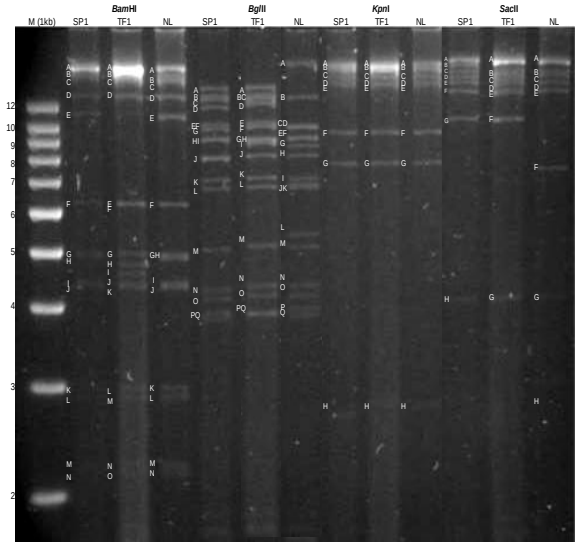


Figure 1. A) Restriction endonuclease profiles following digestion of ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) DNAs with *Bgl*II. B) ChchSNPV-TF1 (TF1) genomic DNA digested with *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II compared with the restriction patterns of ChchSNPV-NL (NL) and ChchNPV-SP1 (SP1). The DNA 1Kb Marker Ladder (Stratagene, California, USA) was used as a molecular size marker (kbp) (M). Fragments were designated alphabetically giving the letter A to the largest fragment for each endonuclease digest.

variants (ChchNPV-TF2 to ChchNPV-TF5) were only collected in Bueype, Tenerife (Table 1).

The *Bgl*II profiles of the five variants from the Canary Islands were quite similar among them. *Bgl*II treatment resulted in 23 visible fragments for ChchNPV-TF1, 25 fragments for ChchNPV-TF2, 24 fragments for ChchNPV-TF3, 23 fragments plus two submolar bands for ChchNPV-TF4 and 23 fragments plus one submolar band for the ChchNPV-TF5 variant (Table 2). Submolar bands were not visible in the

Table 2. Molecular size (kb) of *Bgl*II restriction endonuclease fragments of the genomic DNAs of ChchNPV isolates; ChchSNPV-NL (NL) from Netherlands, ChchNPV-SP1 (SP1) from Almería, Spain, and ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) from the Canary Islands, Spain. The DNA fragments are named alphabetically, starting with A for the largest fragment.

	Genotypic variants						
Fragments	NL	SP1	TF1	TF2	TF3	TF4	TF5
A	27.70	14.40	14.41	14.41	14.41	14.41	14.41
B	12.78	13.28	13.28	13.29	13.29	13.28	13.28
C	9.83	12.78	12.78	12.77	12.77	12.78	12.78
D	9.76	12.11	12.11*	9.77	10.19*	12.11	12.11
E	9.05	9.93	9.93*	9.57*	9.77	9.93*	9.93*
F	8.97	9.79	9.79*	9.05	9.05	9.79	9.79
G	8.52	9.77	8.97*	8.72	8.95*	8.97	8.97
H	7.99	8.97	8.92*	7.99	8.72	8.92	8.92
I	7.01	8.92	8.71*	7.01	7.99	8.71	8.71
J	6.80	7.50	7.99	6.81*	7.01	7.99	7.99
K	6.69	7.02	7.02*	6.68*	6.81*	7.01	7.01
L	5.33	6.69	6.69	5.33*	6.68*	6.69	6.68
M	5.06	5.06	5.06*	5.06*	5.33*	(5.33)	(5.33)
N	4.31	4.31	4.29*	4.76*	5.06*	5.06	5.06
O	4.14	4.14	4.13	4.29	4.29	(4.76)	4.29
P	3.94	3.94	3.86*	4.20*	4.13	4.29	4.13
Q	3.83	3.83	3.83*	4.15	3.88*	4.13	3.88
R	1.86	1.86	1.86*	3.88*	3.83*	3.86	3.83
S	1.75	1.75	1.75*	3.83	1.86	3.83	1.86
T	1.74	1.74	1.74*	1.86	1.75	1.86	1.75
U	1.52	1.52	1.52*	1.75	1.74	1.75	1.74
V	0.77	0.77	0.77	1.74	1.52	1.74	1.52
W	0.15	0.15	0.15	1.52	0.77	1.52	0.77
X				0.77	0.15	0.77	0.15
Y				0.15		0.15	
Total size (kb)	149.60	149.67	149.68	149.50	150.07	149.67	149.67

These fragments were obtained after digestion of the ChchNPV genomic DNAs with *Bgl*II restriction enzyme.
* Fragments cloned into pSP70 plasmid obtained by *Bgl*II digestion.
() Submolar bands not included in isolates total size.

restriction profiles of ChchNPV-TF1, ChchNPV-TF2 or ChchNPV-TF3. Whereas the submolar fragments were evident in ChchNPV-TF4 and ChchNPV-TF5 *Bgl*II profiles, suggesting the presence of a mixture of genotypes. It seems that ChchNPV-TF4 is a mixture of ChchNPV-TF1 and ChchNPV-TF2, while ChchNPV-TF5 is a mixture of ChchNPV-TF1 and ChchNPV-TF3 as RFLPs of ChchNPV-TF2 and ChchNPV-TF3 appeared as submolar bands. All the isolates presented restriction fragments length polymorphics (RFLP) that were characteristic for each one. The polymorphisms in restriction fragment lengths were fragments *Bgl*II-D for ChchNPV-TF1; *Bgl*II-L and *Bgl*II-N for ChchNPV-TF2; and *Bgl*II-M for ChchNPV-TF3. In contrast, the variants showing submolar fragments varied in *BglII*-D, *BglII*-M and *Bgl*II-O, in the case of ChchNPV-TF4, or *Bgl*II-D and *Bgl*II-M fragments in the case of ChchNPV-TF5 (Fig. 1A). The restriction profiles of the predominant variant ChchNPV-TF1 were compared with those of ChchSNPV-NL and ChchNPV-SP1 following treatment with *Bam*HI, *Bgl*II, *Kpn*I or *Sac*II (Fig. 1B). ChchNPV-SP1 and ChchNPV-TF1 appeared to be more similar to one another than to the ChchSNPV-NL variant. The genome size estimates derived from *Bgl*II fragments indicated that ChchNPV variants had genomes of 149.5 to 150.07 similar in length to that of the ChchSNPV-NL genome, previously reported as 149.6 kb (van Oers et al., 2005) (Table 2).

3.2. ChchNPV variants from the Canary Islands were single nucleocapsid NPVs

The banding pattern observed following ODV centrifugation revealed that all the ChchNPV ODVs contained single nucleocapsids. They are, therefore, single type NPVs (SNPV) as indicated by the unique band visible in the sucrose gradient (Fig. 2).

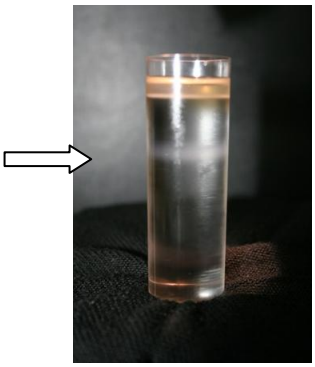


Figure 2. ODV banding pattern of ChchSNPV-TF1 after continuous sucrose gradient separation. The arrow points out the unique band.

3.3. Physical maps of ChchSNPV isolates showed minimal differences due to restriction site modifications

The similarity in variant genome sizes estimated from ChchSNPV-NL, ChchNPV-SP1 and ChchSNPV-TF1 restriction profiles suggested that differences in physical maps were likely due to modifications in restriction sites. To determine differences in physical maps among these variants an incomplete library of 15 of the 23 *Bgl*II fragments from ChchSNPV-TF1 was constructed (Table 2). Terminal sequence information from cloned *Bgl*II fragments was used to construct the physical map of ChchSNPV-TF1, using the ChchSNPV-NL genomic sequence as reference (van Oers et al., 2005). The homologous ORFs identified in the library are shown in Table 3. All the *Bgl*II restriction fragments analyzed fell within ORFs that showed similarity to 30 genes from ChchSNPV-NL. This allowed adjacent restriction fragments to be mapped together and provided further confirmation of the position of most of these ORFs. Following this approach and using the reference ChchSNPV-NL sequence information the physical maps of the ChchSNPV-TF1 variant were constructed for each enzyme used (Fig. 3A).

The ChchSNPV-TF1 *Bgl*II physical map was used as reference for the construction of the physical maps of the rest four ChchSNPV isolates from the Canary Islands, since this enzyme allowed clear discrimination between the different isolates (Fig. 3B). The different isolates collected in the Canary Islands were quite similar among each other. The RFLP markers of ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF3 isolates were cloned into pSP70 (Table 2) and terminal sequencing was carried out to determine the identity of these

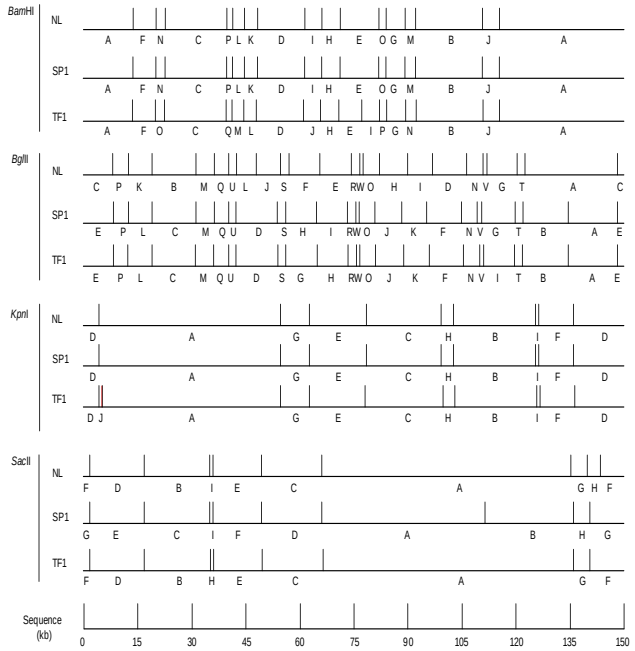
Table 3. The position and orientation of the 33 open reading frames (ORFs) in the ChchSNPV-TF1 genome.

ORF Nº	Gene family	Genomic fragment	Most homologous ORF	Size (nt)	Position in the ChchSNPV-NL genome	Dir*
1	*chch6*	*Bgl*II-P-E	*chch6*	503	7717-9165	>
2	*me-53*	*Bgl*II-P	*chch8*	41	11631-11755	<
3	*exon-0*	*Bgl*II-P	*chch10*	114	12038-12381	>
4	*p47*	*Bgl*II-M	*chch33*	51	31857-32011	<
5	*chch34*	*Bgl*II-M	*chch34*	158	32103-32557	>
6	*lef8*	*Bgl*II-M-Q	*chch37*	279	36221-37060	<
7	*bjdp*	*Bgl*II-Q	*chch38*	212	37084-37722	<
8	*chch41*	*Bgl*II-Q	*chch41*	142	40059-40485	>
9	*chch42*	*Bgl*II-Q-U	*chch42*	351	40505-41560	<
10	*lef-10*	*Bgl*II-U	*chch43*	12	41587-41623	<
11	*vp1054*	*Bgl*II-U-D	*chch45*	336	41932-42942	>
12	*DNA-polymerase*	*Bgl*II-D-S	*chc58*	292	53606-54487	<
13	*desmoplakin*	*Bgl*II-S-G	*chch59*	708	54487-56642	>
14	*lef3*	*Bgl*II-G	*chch60*	59	58159-58578	<
15	*gp37*	*Bgl*II-G	*chch67*	700	64428-65128	>
16	*DNA-photolyase I*	*Bgl*II-H	*chch68*	233	64329-65129	>
17	*chch75*	*Bgl*II-H-R	*chch74*	128	73255-73640	<
18	*vlf-1*	*Bgl*II-R	*chch76*	379	73715-74856	<
19	*gp41*	*Bgl*II-R	*chch78*	216	75272-75922	<
20	*chch88*	*Bgl*II-K	*chch88*	168	88902-88713	>
21	*p45*	*Bgl*II-K	*chch96*	59	94632-94453	<
22	*p87*	*Bgl*II-K-F	*chch97*	454	95233-96034	>
23	*chch106*	*Bgl*II-F-N	*chch106*	347	104455-105826	>
24	*chch107*	*Bgl*II-N	*chch107*	58	105651-105826	<
25	*chch108*	*Bgl*II-N	*chch109*	183	108518-109069	<
26	*chch109*	*Bgl*II-N	*chch110*	48	109174-109318	<
27	*chch110*	*Bgl*II-I	*chch111*	56	110097-110266	<
28	*chch111*	*Bgl*II-I	*chch112*	159	110420-110897	>
29	*chch119*	*Bgl*II-I	*chch119*	111	118015-118349	<
30	*dUTPase*	*Bgl*II-I	*chch120*	51	118504-118349	<
31	*calyx/pep*	*Bgl*II-I-T	*chch121*	331	118614-119609	>
32	*rr2*	*Bgl*II-T	*chch122*	266	119763-120563	<
33	*chch151*	*Bgl*II-E	*chch151*	266	148262-149062	<

*****Direction of transcription in the same (>) or opposite (<) sense of the *polyhedrin* gene; Chch: *C. chalcites* SNPV.

fragments (data not shown). The variability was located in two single regions of the genome, between nucleotides 42,267 and 54,411, and nt 56,161 and 65,133 of the ChchSNPV-NL genome (van Oers et al., 2005), involving the *Bgl*II-L, -J and -F fragments, or *Bgl*II-D and -G of ChchSNPV-TF1 profile (Fig. 3B). ChchSNPV-TF4 and ChchSNPV-TF5 present the same variability of the other strains as RFLPs of ChchSNPV-TF2 and ChchSNPV-TF3 appeared as submolar bands.

A)



B)

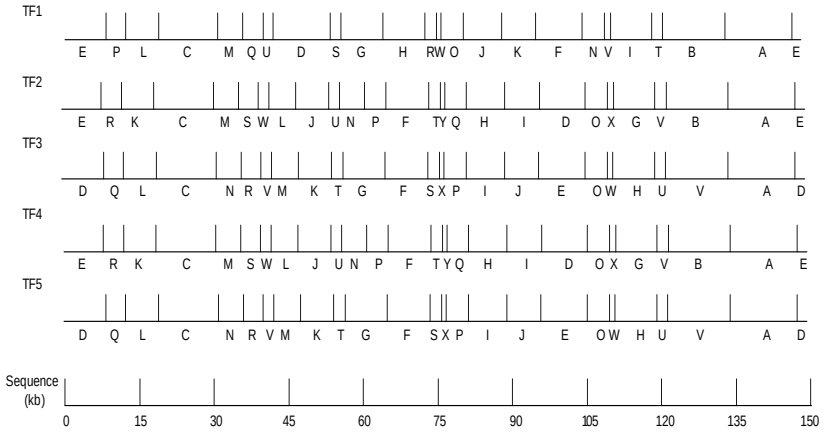


Figure 3. A) Physical maps of the ChchSNPV-NL (NL), ChchNPV-SP1 (SP1) and ChchSNPV-TF1 (TF1) genomes with *Bam*HI, *Bgl*II, *Kpn*I and *Sac*II. The genome size of ChchSNPV-TF1 was estimated to be 149.6 kbp. The first nucleotide in the different maps is the first nucleotide of the ChchSNPV-NL *Bgl*II-C fragment that carries the *polyhedrin* gene. The circular ChchNPV DNA is represented in linear form. Sequnce (kb) representing restriction sites are indicating below the maps. B) Physical maps of the genomes of the different isolates from the Canary Islands: ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) with *Bgl*II enzyme. The genome size was estimated to be around 149 kbp.

3.4. ChchSNPV-TF1 is the most pathogenic and one of the fastest killing variant

LC_{50} values of the ChchNPV variants ranged from $1.35x10^3$ OBs/ml for the most pathogenic variant ChchSNPV-TF1, to $2.94x10^4$ OBs/ml for the least one ChchSNPV-TF2 (Table 4). ChchSNPV-TF1 was significantly more pathogenic to insects from the Canary Islands' colony than any of the other ChchNPV variants tested being fifteen-fold more pathogenic in terms of concentration-mortality metrics than the ChchSNPV-NL variant and fourteen-fold than ChchNPV-SP1 or other isolates from the Canary Islands. The 95% fiducial limits of the relative potencies, representing the ratio of effective concentrations, overlapped in ChchSNPV-NL, ChchNPV-SP1, ChchSNPV-TF2, ChchSNPV-TF3, ChchSNPV-TF4 and ChchSNPV-TF5, indicating no significant differences in pathogenicity among these variants. Both AcMNPV and AnfaMNPV were markedly less pathogenic than any of the homologous viruses (Table 4). For this reason the heterologous viruses were not included in the speed of kill and OB productivity studies.

Table 4. LC_{50} values, relative potencies and MTD values of homologous ChchNPV variants, ChchSNPV-NL (NL) from Netherlands, ChchNPV-SP1 (SP1) from Almería, Spain, and ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) from the Canary Islands, Spain and heterologous viruses *Autographa californica* (AcMNPV) and *Anagrapha falcifera* (AnfaMNPV) in *C. chalcites* second instar larvae.

Virus	LC_{50} (OBs/ml)	Relative Potency	Fiducial limits (95%)		MTD (h)	Fiducial limits (95%)	
			Low	High		Low	High
NL	$2.03x10^4$	1	-	-	126abc	123	127
SP1	$1.89x10^4$	1.11	0.47	2.58	127bc	125	129
TF1	$1.35x10^3$	15.47	7.37	32.48	123a	121	124
TF2	$2.98x10^4$	0.71	0.28	1.78	128bc	126	130
TF3	$2.71x10^4$	0.76	0.32	1.82	125ab	123	126
TF4	$2.46x10^4$	0.85	0.36	2.04	129c	127	130
TF5	$2.41x10^4$	0.86	0.36	2.03	127bc	125	129
AcMNPV	$6.82x10^6$	$2.9x10^{-3}$	-	-	-	-	-
AnfaMNPV	$9.31x10^6$	$2.1x10^{-3}$	-	-	-	-	-

Logit regressions were fitted in POLO Plus (LeOra Software, 1987). A test for non-parallelism was not significant for ChchNPV viruses (χ^2=4.52; d.f=6; *P*>0.05). Relative potencies were calculated as the ratio of effective concentrations relative to the ChchSNPV-NL isolate. Mean time to death (MTD) values were estimated by Weibull survival analysis (Crawley, 1993).

Analysis of mean time to death (MTD) values revealed significant differences among the different ChchNPV variants being ChchSNPV-NL, ChchSNPV-TF1 and ChchSNPV-TF3 the fastest killing isolates for which the 95% confidence intervals of the MTD values overlapped in each variant (Table 3). The majority of the insects

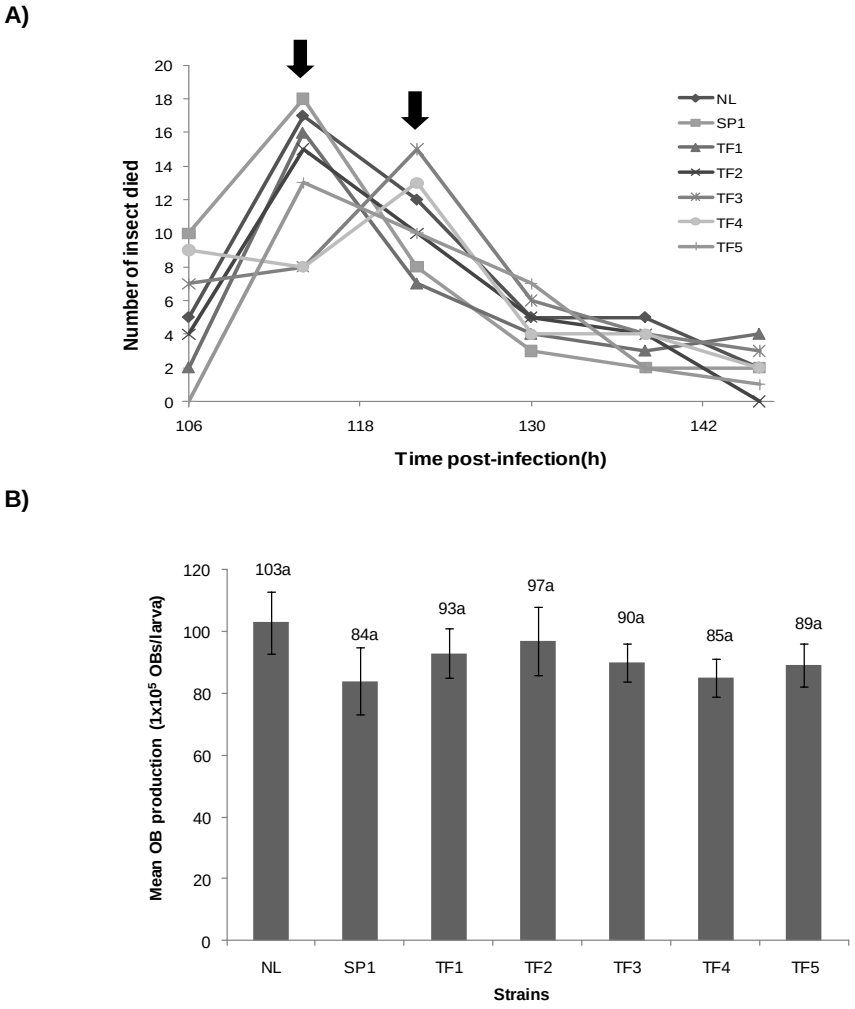


Figure 4. A) Mortality distribution over time of *C. chalcites* second instars killed by ChchSNPV-NL (NL), ChchNPV-SP1 (SP1), ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5) after inoculation with OB concentrations that resulted in ~90% mortality. B) Mean OB production per insect (x10^5 OBs/larva) for *C. chalcites* larvae that died from ChchSNPV-NL (NL), ChchNPV-SP1 (SP1), ChchSNPV-TF1 (TF1), ChchSNPV-TF2 (TF2), ChchSNPV-TF3 (TF3), ChchSNPV-TF4 (TF4) and ChchSNPV-TF5 (TF5). Values above the bars indicate means. Values followed by identical letters did not differ significantly for multiple post hoc comparisons (Tukey $P<0.05$).

infected with ChchNPV variants died between 106 and 120 h post-infection (Fig. 4A). Two mortality peaks were observed; one at ~115 h for ChchSNPV-NL, ChchNPV-SP1, ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF5 isolates and a later one at 120 h for insects infected with ChchSNPV-TF3 or ChchSNPV-TF4 (Fig. 4A).

No significant differences were detected in total OB production among the different variants ($F_{(6,14)}$=2.204; P= 0.105). The total yield values ranged from 8.40 $x10^6$ to 1.03 $x10^7$ OBs/larva for ChchNPV-SP1 and ChchSNPV-NL, respectively (Fig. 4B).

4. DISCUSSION

The aim of this study was to select a native ChchNPV isolate that could be developed as the basis for a bioinsecticide-based control program against *C. chalcites* in the Canary Islands. The occurrence of distinct isolates from different geographical origins has been demonstrated for several other NPVs (Rowley et al., 2011; Takatsuka et al., 2003) and granulosis viruses (Espinel-Correal et al., 2010; Léry et al., 1998). Given the great inter- and intra-specific diversity in lepidopteran baculoviruses, selection of isolates showing highly efficient insecticidal properties for biological control is an essential step in the development of a bioinsecticide product. Among the 97 field-collected ChchNPV isolates five genetic variants were identified by restriction endonuclease analysis, all of which were closely related to one another and showed clear similarities to the previously characterized ChchSNPV-NL from the Netherlands (van Oers et al., 2005) and ChchNPV-SP1 from Almería, Spain (Murillo et al., 2000). As *Bam*HI and *Kpn*I profiles of ChchSNPV-NL and ChchNPV-SP1 were identical, as were those of ChchNPV-SP1 and ChchSNPV-TF1 with *Bgl*II it is likely that these variants shared approximately the same sized genome. In addition, minimal differences were found in the restriction profiles among the ChchSNPV variants from the Canary Islands. Terminal sequencing indicated that genetic differences among the variants were mainly due to point mutations and small deletions or insertions that have modified restriction sites. These results find similarities with other studies that have reported that the genetic diversity of different isolates from distant geographical origins is often limited to minor differences in the presence and distribution of restriction sites

(Chen et al., 2002; Zhang et al., 2005). Differences were located mainly in two genomic regions, including *Bgl*II-L and -J and -F fragments of ChchSNPV-NL genome (*Bgl*II-D and –G in ChchSNPV-TF1 profile) that contain genes including *vp1054*, *fp25k*, *lef-9*, *bro-a*, *DNA polymerase*, *desmoplakin*, *lef-3*, *iap-2*, *p26b*, *v-cathepsin*, *chitinase*, *pcna* or *gp37* and other ORFs of unknown function (van Oers et al., 2005).

ChchSNPV-TF1, ChchSNPV-TF2 and ChchSNPV-TF3 appear to be composed of a highly abundant, genotype or possibly a mixture of genotypes that were not revealed by restriction enzyme analysis as previously observed for other isolates (Muñoz et al., 1999; Simón et al., 2004). In contrast, ChchSNPV-TF4 and ChchSNPV-TF5 appeared to be a mixture of the dominant genotypes present in ChchSNPV-TF1 and ChchSNPV-TF2, and the predominant genotypes present in ChchSNPV-TF1 and ChchSNPV-TF3, respectively. Although ChchSNPV-TF1 was demonstrated to be a single nucleocapsid NPV, multiple genomes are occluded within the same OB, favoring the presence of genotypic heterogeneity as previously found with other single nucleocapsid NPVs (Ogembo et al., 2007; Wang et al., 2003).

Differences in biological activity are common among virus isolates from distinct geographical regions (Alexandre et al., 2010; Rowley et al., 2011; Takatsuka et al., 2003) or even from the same regions (Barrera et al., 2011; Figueiredo, et al., 2009; Milks et al., 1997) or among cloned variants derived from a single wild-type virus (Cory et al., 2005; Ogembo et al., 2007). In addition, minor genetic differences can have significant consequences in the phenotypic characteristics (Maeda et al., 1993; Simón et al., 2012). Simón et al. (2012) found that minimal differences at nucleotide level detected in the sequence of the *sf58* genes of the defective SfMNPV-G and the complete SfMNPV-B genotypes produced changes in the aminoacid sequence and the predicted secondary structure, that have an adverse effect on the oral infectivity of SfMNPV-G genotype, being this genotype not orally infectious. In contrast, Maeda et al. (1993) expanded the host range of AcMNPV following recombination of a 0.6 kb DNA originating from *Bombyx mori* NPV (BmMNPV) genome. Later, Kamita and Maeda (1997) determined that two adjacent nucleotides (A and T) appeared to be the minimal essential sequence necessary to expand the host range of AcMNPV, which encoded a single amino acid different

between BmMNPV (Asp) and AcMNPV (Ser). So, determining minimal differences at genome level will be of special interest to clarify the reasons for the differences found in biological activity.

The Canary Islands variants although genetically very similar, displayed differences in pathogenicity. ChchSNPV-TF1 isolate was the most pathogenic variant tested and one of the most virulent strains, while homologous viruses from the Canary Islands and from the other geographical regions as ChchNPV-SP1 and ChchSNPV-NL were less pathogenic. Functional studies on the genes found within the variable genomic region will help to determine their association with the biological activity of ChchSNPV. *C. chalcites* larvae from the Canary Islands were markedly more susceptible to the indigenous isolate ChchSNPV-TF1 compared to isolates from Netherlands and Almería or heterologous viruses. Homologous viruses are normally more pathogenic than viruses from other host species (Kouassi et al., 2009) and native isolates tend to be more pathogenic to the local insect population than homologous isolates from other geographical regions (Erlandson et al., 2007). This fact made necessary to determine the natural diversity of ChchSNPV in the Canary Islands in order to select an isolate with higher insecticidal characteristics.

In conclusion, the highest pathogenicity, comparable to that of the most pathogenic baculoviruses currently commercialized as bioinsecticide products (Moscardi, 1999; Caballero et al., 2009), the fast speed of kill of ChchSNPV-TF1 and the fact that the OB productivity was similar among all the ChchNPV variants, suggest that the most prevalent and widespread strain in the Canary Islands prove useful as the active ingredient in the development of a biological insecticide for *C. chalcites* in banana crops in the Canary Islands.

5. ACKNOWLEDGEMENTS

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CHAPTER 3

A *Chrysodeixis chalcites* single nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival

ABSTRACT

A *Chrysodeixis chalcites* single nucleopolyhedrovirus wild-type isolate from the Canary Islands, Spain, named ChchSNPV-TF1 (ChchTF1-wt), appears to have great potential as the basis for a biological insecticide for control of this pest. An improved understanding of the genotypic structure of this wild-type strain population should facilitate the selection of genotypes for inclusion in a bioinsecticidal product. Eight genetically distinct genotypes were cloned *in vitro*: ChchTF1-A to ChchTF1-H. qPCR analysis confirmed that ChchTF1-A accounted for 36% of the genotypes in the wild-type population. In bioassays, ChchTF1-wt occlusion bodies (OBs) were significantly more pathogenic than any of the component single genotype OBs, indicating that genotype interactions were likely responsible for the pathogenicity phenotype of wild-type OBs. However, the wild-type population was slower-killing and produced higher OB yields than any of the single genotypes alone. These results strongly suggested that the ChchTF1-wt population is structured to maximize its transmission efficiency. Experimental OB mixtures and co-occluded genotype mixtures containing the most abundant and the rarest genotypes, in frequencies similar to those in which they were isolated, revealed a mutualistic interaction that restored the pathogenicity of OBs. In OB and co-occluded mixtures containing only the most abundant genotypes, ChchTF1-ABC, OB pathogenicity was even greater than that of wild-type OBs. ChchTF1-ABC co-occluded mixture killed larvae 33 h faster than the wild-type population and remained genotypically and biologically stable throughout five successive passages *in vivo*. In conclusion the ChchTF1-ABC mixture shows great potential as the active ingredient of a bioinsecticide to control *C. chalcites* in the Canary Islands.

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1. INTRODUCTION

Baculoviruses are highly effective control agents for a number of lepidopteran pests due to their host specificity and outstanding safety record (Szewczyk et al., 2006). Dozens of baculoviruses are currently produced on a commercial scale and applied to large areas of crops, such as the nucleopolyhedrovirus (NPV) of *Anticarsia gemmatalis* (AgMNPV) in Brazil (Moscardi, 1999), the NPV of *Helicoverpa armigera* in China (Zhang et al., 1994) or the granulovirus of *Cydia pomonella* in Europe (Lacey and Shapiro-Ilan, 2008). Considerable genetic heterogeneity is usually observed in natural baculovirus populations (Erlandson, 2009). However, wild-type baculoviruses that can be collected as natural isolates, are not patentable and genotypic characterization is required to select the genotype or mixture of genotypes with the insecticidal characteristics that favour their use as biological control agents.

This diversity has been demonstrated by the characterization of different geographical isolates of the same virus (Ogembo et al., 2007; Williams et al., 2011) and also within single isolates, which frequently comprise a mixture of genotypes (Cory et al., 2005; Redman et al., 2010; Rowley et al., 2011). In wild-type populations, genetic diversity seems to be an important aspect of virus survival under field conditions to allow adaptation to varying environmental conditions (Hitchman et al., 2007; Moscardi, 1999). Genotypic variability is commonly determined with the application of standard molecular tools such as restriction endonuclease analysis (REN), polymerase chain reaction (PCR) (Cory et al., 2005; Erlandson, 2009; Williams et al., 2011), or more recently, denaturing gradient gel electrophoresis (DGGE) (Baillie and Bouwer, 2012). Studies on genotypic heterogeneity within baculovirus populations using *in vitro* (Lynn et al., 1993; Simón et al., 2004) or *in vivo* techniques (Cory et al., 2005; Muñoz et al., 1998; Redman et al., 2010), or bacterial artificial chromosomes (BACs) (Wang et al., 2003), have revealed that intraspecific variability is due to genomic reorganizations, DNA deletions or insertions, recombination or mutations (Cory et al., 2005).

Infections with experimental mixtures of different genotypes of a given viral species or different viral species have reported antagonistic or synergistic effects in the phenotypic traits resulting from these interactions (Clavijo et al., 2010; Espinel-Correal et al., 2012; Read and Taylor, 2001). Such effects may include the

modification of biological parameters such as pathogenicity, speed of kill, or virus yield, among others. In some instances, the mixture of viral genotypes enhances the efficiency of the virus as a biological insecticide (López-Ferber et al., 2003; Shapiro and Shepard, 2006; Simón et al., 2005), but contrasting results have also been reported in other virus-host systems (Arends et al., 2005; Muñoz and Caballero, 2000). Interest in baculoviruses as biological insecticides has led to the search for effective isolates, comprising either single genotypes or genotype mixtures, making genotypic characterization of field isolates a key step in the process of active material selection during bioinsecticide development (Erlandson, 2009; Ogembo et al., 2007).

Chrysodeixis chalcites (Lepidoptera: Noctuidae) is a major polyphagous pest in many countries (Shepard et al., 2009; van Oers et al., 2005). In the Canary Islands, Spain, *C. chalcites* larvae can cause up to 30% production loss in banana crops (Del Pino et al., 2011). To date different geographical *Chrysodeixis chalcites* single nucleopolyhedrovirus (ChchSNPV) strains have been described: one from the Netherlands (ChchSNPV-NL) (van Oers et al., 2005; van Oers et al., 2004), another one from Almería, Spain (ChchNPV-SP1) (Murillo et al., 2000) and five from Tenerife, Canary Islands, Spain (ChchSNPV-TF1 to -TF5) (Bernal et al., 2013a). The ChchSNPV-TF1 isolate was recently selected for further development as a bioinsecticide for protection of banana crops due to its highest pathogenicity and rapid speed of kill against an insect population from the Canary Islands (Bernal et al., 2013a). The aim of the present study was, first, to evaluate the genetic structure of the ChchSNPV-TF1 wild-type isolate and, second, to determine the outcome of genotype interactions on the insecticidal phenotype of genotype mixtures by testing different OB and co-occluded mixtures. Finally, the genetic and biological stability of the most insecticidal genotypic mixture was evaluated through five serial passages *in vivo*.

2. MATERIAL AND METHODS

2.1. Insect, viruses and cells

C. chalcites larvae were obtained from pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007, and refreshed periodically with pupae from the Canary Islands. Previous qPCR studies indicated

that the insect colony did not harbor an inapparent nucleopolyhedrovirus infection (Bernal et al., 2013a). Larvae were reared at 25±1 ºC, 70±5% humidity and a photoperiod of 16:8 (light:dark) on a semisynthetic diet described by Greene et al. (Greene et al., 1976). Adults were fed 30% (w/v) diluted honey. The ChchSNPV-TF1 (abbreviated from here onwards as ChchTF1-wt) was originally isolated from a single larva collected during a natural epizootic in banana crops in Tenerife, Canary Islands, Spain. ChchTF1-wt occlusion bodies (OBs) were amplified once in fifth instar (L_5) *C. chalcites* larvae from the laboratory colony. The BTI-TN-5B1-4 *Trichoplusia ni* cell line, also known as High Five™ Cells (Invitrogen™, Life Technologies Ltd, Carlsbad, California, USA), was maintained in TNM-FH insect medium (Gibco® , Life Technologies Ltd) supplemented with 10% fetal bovine serum (FBS, Gibco®).

2.2. In vitro virus cloning and restriction endonuclease analysis

For the isolation of individual genotypes, L_5 larvae that had been inoculated with a 90% lethal concentration (LC_{90}, $5x10^7$ OBs/ml) of ChchTF1-wt OBs were surface decontaminated with 70% ethanol, and a hemolymph sample was obtained by bleeding at 48 h post infection. Each hemolymph sample was filtered throughout a 0.45 µm filter, serially diluted in TNM-FH medium and used to infect $2x10^6$ High Five cells as previously described (Simón et al 2004). After 10 days, clearly separated plaques were picked individually with a sterile Pasteur pipette and transferred to a vial containing 0.5 ml phosphate-buffered saline (PBS). A 100 µl volume of each plaque-PBS suspension was amplified in $5x10^5$ cells per well in six-well tissue culture plates. Ten days after infection, the medium and cells were collected and centrifuged (3,800 *g*, 5 min) to pellet the cells whereas the supernatant, containing the budded virions (BVs), was stored at 4 ºC. For clone amplification, 5 µl of these suspensions were injected into five L_5 *C. chalcites* larvae, which were then reared individually on diet. Mortality was recorded daily. Virus-killed larvae were individually transferred to a microcentrifuge tube and stored at -20 ºC until required.

OBs of the different clones were purified from dead larvae and DNA was purified as previously described (Bernal et al., 2013a). For restriction endonuclease

analysis, viral DNA was mixed with *Bgl*II as this enzyme allowed clear discrimination between the different ChchSNPV isolates (Bernal et al., 2013a).

2.3. Biological activity of single ChchTF1 genotypes

The insecticidal characteristics of the isolated genotypes were assessed in terms of mean lethal concentration (LC_{50}), mean time to death (MTD) and OB production (OBs/larva), as an expression of pathogenicity, virulence and OB yield, respectively, in *per os* insect bioassays (Hughes and Wood, 1981).

To determine OB pathogenicity, second instar *C. chalcites* (L_2) were starved for 8-12 h at 26 °C and then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs at one of the following five concentrations: $1x10^5$, $2x10^4$, $4x10^3$, $8x10^2$ and $1.6x10^2$ OBs/ml. This range of concentrations was previously determined to kill between 95 and 5% of the experimental insects (Bernal et al., 2013a; 2013b). Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed three times. Mortality results were subjected to logit analysis using the POLO-PC program (Le Ora Software, 1987).

To determine speed of kill, time-mortality analyses were performed in groups of 25 L_2 *C. chalcites* larvae inoculated orally with OB concentrations that resulted in ~90% larval mortality: $3.31x10^4$ OBs/ml for ChchTF1-wt, and $2.22x10^5$, $2.33x10^5$, $3.01x10^5$, $2.36x10^5$, $2.91x10^5$, $1.60x10^5$, $1.90x10^5$ and $1.87x10^5$ OBs/ml for genotypes ChchTF1-A, ChchTF1-B, ChchTF1-C, ChchTF1-D, ChchTF1-E, ChchTF1-F, ChchTF1-G and ChchTF1-H, respectively. Larval mortality was recorded every 8 h until the insects had either died or pupated. The whole bioassay was carried out three times. Time mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modelling (GLIM) program (Crawley, 1993).

Finally, infected larvae from the speed of kill bioassay were used to determined OB production from at least 20 larvae for each virus treatment in each replicate (total ~60 larvae per virus treatment). Each larva was thawed, individually homogenized in 1 ml distilled water and the total OB yield per larva was estimated by counting in triplicate in a Neubauer hemocytometer. OB yields were averaged for

each replicate and were subjected to analysis of variance using the SPSS v12 program (SPSS Inc., Chicago, IL).

2.4. Construction and biological activity of OB and co-occluded mixtures

Four OB mixtures were prepared using *in vitro* purified OBs of the different genotypes. These OBs were mixed in proportions similar to which they were isolated: 58% ChchTF1-A + 42% ChchTF1-B (ChchTF1-AB); 47% ChchTF1-A + 34% ChchTF1-B + 19% ChchTF1-C (ChchTF1-ABC); 47% ChchTF1-A + 34% ChchTF1-B + 18% ChchTF1-C + 1% ChchTF1-G (ChchTF1-ABCG); 46% ChchTF1-A + 33% ChchTF1-B + 19% ChchTF1-C + 1% ChchTF1-G + 1% ChchTF1-H (ChchTF1-ABCGH).

To produce co-occluded genotype mixtures, the OBs of each genotype were mixed in the desired proportions (identical to the OB mixtures), and fifth instars were orally inoculated with these mixtures ($5x10^7$ OBs/ml) by the droplet feeding method. OBs were purified from infected larvae and these OBs were considered co-occluded genotype mixtures. The co-occlusion of genotype mixtures was confirmed as described below.

To confirm the relative frequency of the different genotypes in the co-occluded mixtures and in the wild-type population, a quantitative real-time PCR (qPCR) was performed using genotype-specific primers. To design these primers five genomic regions of ChchTF1 were sequenced. These regions were highly variable according to the ChchSNPV-NL sequence information (GeneBank accession number: AY864330) (van Oers et al., 2005) and other reports (Bideshi et al., 2003; Le et al., 1997). These regions were outside the complete coding regions of *hoar* (*chch4* nucleotides 2,965 to 6,504 in ChchSNPV-NL genome), *bro-a* (*chch55* nt 48,823 to 50,376), *bro-b* (*chc69* nt 68,861 to 66954), *bro-c* (*chch70* nt 70,060 to 69,254) and *bro-d* (*chc114* nt 114,806 to 113,517) (Table 1). PCR products were amplified using a high fidelity Taq Polymerase (PrimeStar, Takara Bio Inc., Shiga, Japan), purified using the QIAquick PCR Purification Kit (Qiagen, Düsseldorf, Germany) and used for direct sequencing (Sistemas Genómicos S.L., Valencia, Spain). The recent availability of the complete sequences of the ChchTF1-A, -B, and –C confirmed that the most variable regions in these genomes are the *hoar* and *bro-d* genes. Multiple

Table 1. Specific PCR primers designed for the amplification of complete *hoar*, *bro-a*, *bro-b*, *bro-c* and *bro-d* genes and qPCR primers designed for the quantification of the relative proportions of ChchSNPV-TF1-A (ChchTF1-A), ChchSNPV-TF1-C (ChchTF1-C) and the rest of genotypes. Primer name, nucleotide sequence, position in the ChchSNPV-NL genome, PCR/qPCR fragment sizes (bp) and purpose are described.

Primer	Sequence (5'-….-3')	Position in ChchSNPV-NL genome	PCR/qPCR fragment (bp)						Amplification purpose
			NL*	A	B	C	G	H	
hoar.F	ttattctatttttttgaactg	2,965-2,984	3,540	3,636	3,804	3,900	3,540	3,396	Primers used to amplify the complete *hoar* gene
hoar.R	atgatacggaaatttaaatc	6,504-6,485							
bro-a.F	atgtctctctcaaaaataaatc	48,823-48,844	1,554	1,554	1,554	1,557	1,554	1,557	Primers used to amplify the complete *bro-a* gene
bro-a.R	ttattttctgcccgacgctttg	50,355-50,376							
bro-b.F	ttattcgttagaacttttattg	66,954-66,975	1,908	1,887	1,887	1,908	1,908	1,908	Primers used to amplify the complete *bro-b* gene
bro-b.R	atgactacatccaaaatcatg	68,861-68,841							
bro-c.F	ctacataatgttgcagttgag	69,254-69,274	807	807	807	807	807	807	Primers used to amplify the complete *bro-c* gene
bro-c.R	atgaattctaaagtcattacc	70,060-70,040							
bro-d.F	ttattctatagccaaggtatttg	113,517-113,539	1,290	1,611	828	420	828	420	Primers used to amplify the complete *bro-d* gene
bro-d.R	atggattatcgccccctgaaac	114,806-114,784							
A*bro-d*.F	cgagtatttaccagttttcc	114,462-114,481	-	217	-	-	-	-	Primers used to quantify the relative proportion of ChchTF1-A in the OB and co-occluded mixtures by qPCR.
A*bro-d*.R	gatggattatcgccccctg	114,678-114,659							
C*bro-d*.F	ggaatccagttttcctcaattc	114,881-114,902	-	-	-	212	-	-	Primers used to quantify the relative proportion of ChchTF1-C in the OB and co-occluded mixtures by qPCR.
C*bro-d*.R	cctaaatcaggacccccttg	115,092-115,073							
DNA-pol.F	ttaatgcgttcgctgctcac	54,174-54,193	91	91	91	91	91	91	Primers used to quantify the relative proportion of all genotypes in the OB and co-occluded mixtures by qPCR.
DNA-pol.R	atcacgccttttccaagtgc	54,264-54,245							

* ChchSNPV from Netherlands (van Oers et al., 2005)

sequence alignments were carried out with Clone Manager version 9 (Scientific & Educational Software, Cary, NC, USA).

Once sequences had been analyzed, different sets of primers were designed in the most variable region. Non-template controls (NTCs) were analyzed for each set of primers in order to verify the absence of non-specific background signal. Milli-Q water was used in all reactions as a negative control. Primers sets for each genotype (Table 1) were selected based on the presence of a single melting peak, a measure of specific amplification. A 1:100 dilution of DNA (1 µl) extracted from $1x10^8$ OBs of the different mixtures was used for qPCR. Wild-type genomic DNA was also included in the assay. All qPCR reactions were performed using SYBR Green Premix Ex Taq™ (Tli RNaseH Plus) (Takara Bio Inc., Shiga, Japan). A 1 µl

volume of CsCl-purified DNA of single genotypes was quantified using a UV-spectrophotometer (Eppendorf AG, Hamburg, Germany) and serial dilutions (10^{-1} to 10^{-8} ng/µl) were used as internal standards for each qPCR reaction. The identity of each genotype was previously confirmed by restriction endonuclease analysis using *Bgl*II. All reactions were performed under the following conditions: 2 min at 50 ºC; 30 s at 95 ºC; 45 cycles at 95 ºC for 5 s, 65 ºC for 30 s and 95 ºC for 15 s; 60 ºC for 15 s; 95 ºC for 15 s for the dissociation curve. qPCR results of each replicate were subjected to analysis of variance using the SPSS v12 program. All reactions were performed in duplicate and the experiment was repeated three times. To verify the co-occlusion of the different genotypes within a single OB, a qPCR analysis was performed with 100 L_2 larvae orally inoculated with $1.6x10^2$ OBs/ml of the ChchTF1-ABC co-occluded mixture. This dilution was expected to kill ~5% of the inoculated larvae. Previous studies indicated that L_2 larvae drank an average of 0.41 µl of suspension (A. Bernal, unpublished data), that is ~0.26 OBs/larva at the OB concentration used, which meant that almost all infections in virus-killed larvae occurred following the ingestion of a single OB. As such, qPCR detection of different genotypes from single larvae was considered to be a reliable indicator of their co-occlusion in mixtures within a single OB. The experiment was performed five times. A 1:100 dilution of DNA (1 µl) extracted from OBs purified from a single larva was subjected to qPCR analysis as described previously.Finally, the insecticidal activity of the OB mixtures and co-occluded genotype mixtures was compared with that of ChchTF1-wt OBs and the individual genotype OBs used to produce the mixtures. The pathogenicity, speed of kill and OB productivity were determined in L_2 as described previously. For wild-type and individual genotypes, the OB concentrations used for these assays were those used in single genotype assays described above. For OB mixtures and co-occluded mixtures, inocula concentrations to determine pathogenicity were the same as used above, whereas the following concentrations were used for speed of kill and OB productivity assays: $4.37x10^4$, $3.49x10^4$, $4.22x10^4$ and $4.37x10^4$ OBs/ml for the four OB mixtures (ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG and ChchTF1ABCGH, respectively), and $5.31x10^4$, $3.73x10^4$, $4.91x10^4$ and $5.19x10^4$ OBs/ml, for the co-occluded mixtures (ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG and ChchTF1-ABCGH, respectively).

2.5. Serial passage in vivo of a co-occluded genotype mixture

A serial passage experiment was performed three times with the selected mixture (ChchTF1-ABC) and ChchTF1-wt. A group of 50 *C. chalcites* L_5 larvae were inoculated per os with the corresponding LC_{90} of each inoculum and individually reared on diet until death or pupation. Virus killed cadavers were collected and pooled in 50 ml plastic centrifuge tubes, purified and used to infect the following batch of experimental larvae. The four subsequent passages were initiated with the same number of larvae and OB concentration to complete five passages. The relative frequency of the different genotypes from each passage was analyzed by qPCR as described above. Finally, the pathogenicity of ChchTF1-ABC and the wild-type was also determined for the OBs from the final passage and compared with that of OBs from the first passage, as previously described.

3. RESULTS

3.1. Genotypic structure of ChchTF1-wt

A total of 117 ChchTF1 clones were amplified in cell culture. The plaques they produced in *T. ni* cells, were small and all clones rendered similarly low replication titres compared with the plaques and replication titers produced by the type baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) in the same cells. Eight different genotypes were identified, named ChchTF1-A to ChchTF1-H, according to differences in their *Bgl*II restriction profiles (Fig. 1). The ChchTF1-A restriction profile was indistinguishable from that of ChchTF1-wt, and was the most frequently observed, representing 36% (N=42) of the plaque purified clones. ChchTF1-A together with ChchTF1-B (26%) and ChchTF1-C (14%). accounted for the majority (76%) of isolated clones. The other genotypes were present at lower frequencies: ChchTF1-F (11%), ChchTF1-E (8%) and ChchTF1-D (3%), whereas ChchTF1-G and ChchTF1-H each originated from a single clone (~1%). All genotypes showed restriction fragment length polymorphisms with respect to ChchTF1-wt or ChchTF1-A. ChchTF1-B, -C and -D genotypes each showed a unique profile, involving the marker fragments *Bgl*II-L, *Bgl*II-M and *Bgl*II- D, respectively. Genotypes ChchTF1-E and ChchTF1-F could be differentiated by two polymorphisms involving *Bgl*II-K and *Bgl*II-L, and *Bgl*II-D and *Bgl*II-L, respectively.

Finally, the restriction profiles of ChchTF1-G and ChchTF1-H showed three polymorphisms in *Bgl*II-A, *Bgl*II-L and *Bgl*II-P, and *Bgl*II-L, *Bgl*II-N, and *Bgl*II-P.

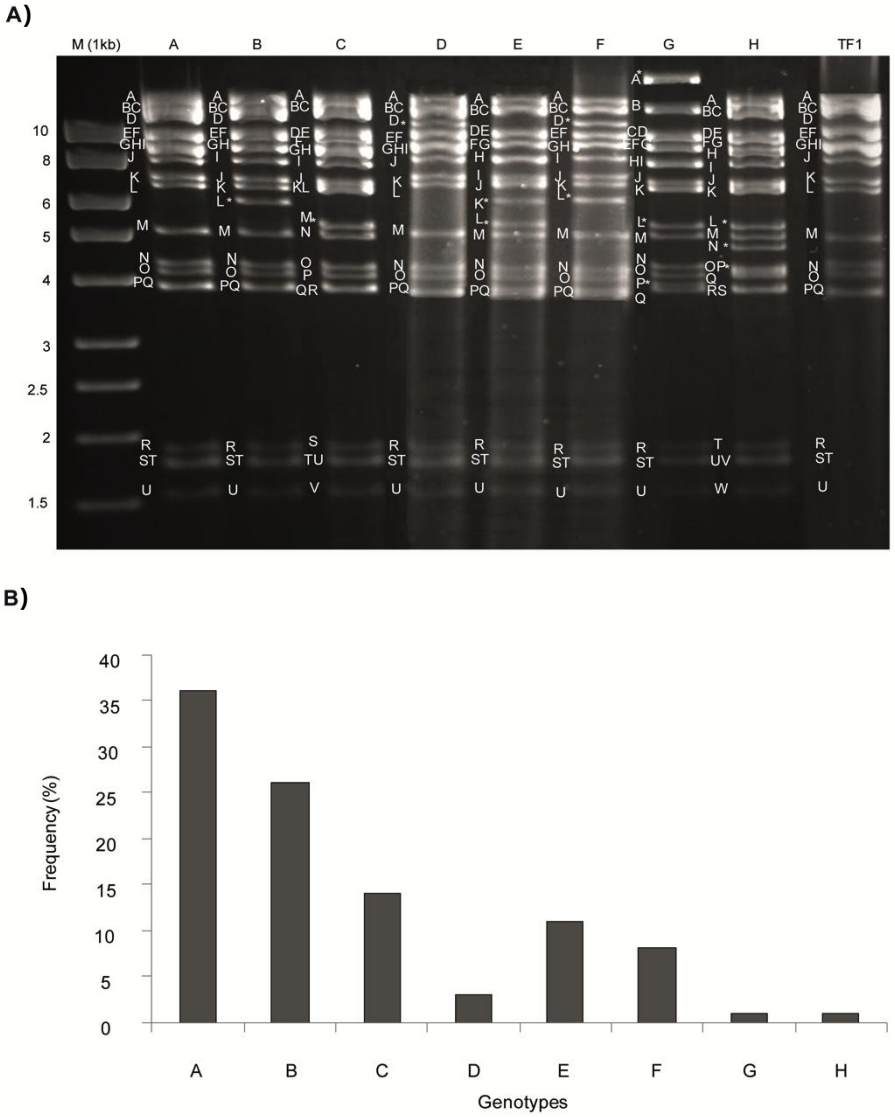

Figure 1. A) Restriction endonuclease profiles of the DNA of ChchTF1-wt (TF1) and its cloned genotypes ChchTF1-A to ChchTF1-H digested with *Bgl*II. Fragments were named alphabetically giving the letter A to the largest *Bgl*II fragment, respectively. No submolar bands were observed in these genotypes and the restriction profiles remained invariant for at least three passages in insects. The 1Kb DNA Marker Ladder (Stratagene) was used as a molecular size marker (kbp) (M). *Asterisks point out restriction fragment length polymorphisms (RFLPs). B) Frequencies at which the different genotypes were cloned.

3.2. Biological activity of individual ChchTF1 genotypes

The LC_{50} value of ChchTF1-wt was estimated at $1.61x10^3$ OBs/ml, and was 5.6 to 11.1-fold more pathogenic than any of its component genotypes in single infections (Table 2). The broadly overlapping 95% fiducial limits of the relative potency values of individual genotypes indicated that the OBs of these genotypes did not differ significantly in their insecticidal potency.

Table 2. Mean lethal concentration (LC_{50}), relative potency and mean time to death (MTD) values of wild-type ChchSNPV-TF1 (ChchTF1-wt) and its individual genotypes, ChchTF1-A to ChchTF1-H, in second instar *Chrysodeixis chalcites.*

Virus	LC_{50}	Relative	Fiducial limits (95%)		MTD	Fiducial limits (95%)	
inoculum	(OBs/ml)	Potency	Low	High	(h)	Low	High
ChchTF1-wt	$1.61x10^3$	1	-	-	131	126	136
ChchTF1-A	$1.27x10^4$	0.12	0.08	0.19	125	122	128
ChchTF1-B	$8.87x10^3$	0.18	0.11	0.28	117	115	119
ChchTF1-C	$8.92x10^3$	0.18	0.11	0.28	115	113	117
ChchTF1-D	$1.03x10^4$	0.15	0.10	0.24	120	118	122
ChchTF1-E	$1.69x10^4$	0.09	0.06	0.14	119	117	121
ChchTF1-F	$1.39x10^4$	0.11	0.07	0.17	126	121	129
ChchTF1-G	$1.69x10^4$	0.09	0.06	0.15	133	131	137
ChchTF1-H	$1.35x10^4$	0.11	0.07	0.18	129	127	131

Mean OB production values were normally distributed. OB yields per larva differed significantly between genotypes ($F_{(8,18)}$=9.730; P<0.001). ChchTF1-wt was the most productive ($7.80x10^6$ OBs/larva), whereas single genotypes yielded an average of between $7.22x10^6$ and $5.23x10^6$ OBs/larva (Fig. 2). Only the ChchTF1-A genotype was as productive as the wild-type strain (Tukey P=0.952), while the other single genotypes were ~1.5-fold less productive than ChchTF1-wt. Interestingly, ChchTF1-B, ChchTF1-C, ChchTF1-D and ChchTF1-E were as productive as the other single genotypes, despite their faster speed of kill (Fig. 2). In contrast, ChchTF1-F, ChchTF1-G and ChchTF1-H, which were as fast killing as the wild-type population, were among the least productive genotypes.

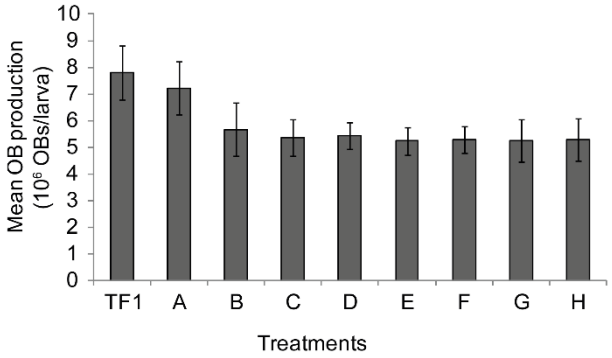

Figure 2. Mean OB yields obtained from infection of *Chrysodeixis chalcites* second instars with the LC_{90} of: ChchTF1-wt (TF1), ChchTF1-A (A), ChchTF1-B (B), ChchTF1-C (C), ChchTF1-D (D), ChchTF1-E (E), ChchTF1-F (F), ChchTF1-G (G) and ChchTF1-H (H).

3.3. Genomic variability between ChchTF1 genotypes

Sequencing of the variable genomic regions of the ChchTF1 genotypes revealed a greater variability in the *hoar* and the *bro-d* genes, whereas *bro-a*, *bro-b* and *bro-c* were less variable and had 99-100% homology to those of ChchSNPV-NL. Recently, the complete genome sequences of ChchTF1-A (accession number JX535500), ChchTF1-B (JX560540) and ChchTF1-C (JX560539) genotypes was achieved and confirmed that the most variable regions within these viruses were the *hoar* and *bro-d genes*. In the *hoar* gene, all genotypes showed deletions or insertions, so that *hoar* gene lengths differed in all genotypes compared to that of ChchSNPV-NL (Table 1). ChchTF1-A, ChchTF1-B and ChchTF1-C had insertions of 96, 264 and 360 nt, respectively. ChchTF1-G had no deletions or insertions, whereas ChchTF1-H had a 144 nt deletion compared to ChchSNPV-NL (Table 1). As a result, ChchTF1-A to ChchTF1-G were 96, 92, 87, 99 and 94% homologous to the ChchSNPV-NL *hoar* gene, respectively. The *bro-d* genes of ChchTF1 single genotypes also presented variations with respect to that of ChchSNPV-NL: a 321 nt insertion in ChchTF1-A, a 462 nt deletion in ChchTF1-B and ChchTF1-G, and 870 bp deletions in ChchTF1-C and ChchTF1-H. Sequence homology to ChchSNPV-NL varied from 80% for ChchTF1-A, to 38% for ChchTF1-B and ChchTF1-G and to just 21% for ChchTF1-C and ChchTF1-H (Table 1). This gene was selected for primer design as it allowed differentiation of genotypes.

3.4. Genotype abundance in OB mixtures and co-occluded mixtures

For the OB mixtures, genotypes were mixed in frequencies similar to those in which they were isolated *in vitro*: ChchTF1-AB (58%:42%), ChchTF1-ABC (47%:34%:19%), ChchTF1-ABCG (47%:34%:18%:1%) and finally ChchTF1-ABCGH (46%:33%:19%:1%:1%). OB progeny obtained following after oral inoculation of larvae with these OB mixtures were confirmed to be co-occluded mixtures.

Of the different sets of primers designed in *bro-d* gene, only those that differentiated ChchTF1-A and ChchTF1-C could be used in the qPCR. This was mostly due to the fact that all primers designed in the *bro-d* variable region of the other genotypes produced more than one melting peak in the qPCR reaction or dimers, and lacked specificity. Due to the unavailability of specific primers for the ChchTF1-B and other genotypes, a set of primers common to all genotypes were designed in the *DNA-polymerase* gene (Table 1). Therefore, the relative proportion of ChchTF1-B was estimated by the difference between the amplification obtained for the *DNA-polymerase* common to all genotypes (being 100%) and those obtained specifically for ChchTF1-A and -C.

Analysis by qPCR confirmed that the genotype ratio in the OB mixtures reflected the frequency of isolation in tissue culture from the original inocula. In the ChchTF1-AB mixture, the two genotypes were present at a prevalence of 58% and 42%, respectively, closely reflecting their abundance in the plaque-purified clones (36%A:26%B, for a total of 62%), i.e., 58% of 62 is 36, and 42% of 62 is 26. In the ChchTF1-ABC mixture, the frequencies of the three genotypes were estimated at 47%, 38% and 14%, respectively, which also resembled their cloning frequency *in vitro* (36%A:26%B:14%C, for a total of 76%), i.e. 47% of 76 is 36, 38% of 76 is 29, and 14% of 76 is 11. In the ChchTF1-ABCG mixture, the frequency of ChchTF1-A was estimated at 47%, the frequency of ChchTF1-B+G was estimated at 40%, and the frequency of ChchTF1-C was estimated at 13%. The 2% increase in the *DNA-polymerase* amplification with respect to that in mixture ChchTF1-ABC, was attributed to the addition of ChchTF1-G to the initial inoculum at a frequency of 1%, although in this case, no direct estimate could be performed due to lack of suitable specific primers. As such, these frequencies closely matched the genotype prevalence in the plaque-purified clones (36%A:26%B:14%C:1%G, for a total of

77%). Similarly in the ChchTF1-ABCGH mixture, the frequency of ChchTF1-A was estimated at 46%, ChchTF1-B+G+H at 41% and ChchTF1-C at 13%. A 1% increase in the *DNA-polymerase* amplification signal with respect to that in the mixture ChchTF1-ABCG, was attributed to the presence of ChchTF1-H at a frequency of 1%. As a result, the genotype frequencies in this mixture closely mimicked the frequencies estimated from *in vitro* cloning (36%A:28%B:14%C:1%G:1%H, for a total of 78%). Similarly, several ChchTF1-wt samples were analyzed and the prevalence of ChchTF1-A and ChchTF1-C was estimated at 34%A:12%C, respectively, which was also similar to estimates generated from *in vitro* cloning, whereas 54% of amplifications reflected the presence of the remaining genotypes.

Co-occlusion of different genotypes in a single OB was also confirmed by qPCR. In total 23 larvae died after having consumed a single OB; both ChchTF1-A and ChchTF1-C were successfully amplified from all OB samples taken from each cadaver, indicating that both genotypes had replicated in these insects and were present in OBs. The frequencies of the three genotypes after infection (49%A:34%B:17%C) were similar to that in which they were inoculated (47%:34%:19%).

3.5. Biological activity of different OB and co-occluded genotypic mixtures

Dose-response bioassays revealed that the pathogenicity values of ChchTF1-AB, ChchTF1-ABCG, and ChchTF1-ABCGH OBs, in both OB mixtures and co-occluded mixtures, were restored to that of the wild-type population, or were ~2-fold lower (in ChchTF1-ABC as both OB and co-occluded mixtures) than the wild-type OBs (Table 3).

Table 3. Mean lethal concentration (LC_{50}), relative potency and mean time to death (MTD) values of the wild-type ChchSNPV-TF1 (ChchTF1-wt); its individual genotypes: ChchTF1-A, ChchTF1-B, ChchTF1-C, ChchTF1-G, and ChchTF1-H; and OB and co-occluded mixtures of cloned genotypes, ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH in second instar *Chrysodeixis chalcites.*

Virus inoculum		LC_{50} (OBs/ml)	Relative Potency	Fiducial limits (95%)		MTD (h)	Fiducial limits (95%)	
				Low	High		Low	High
Wild-type	ChchTF1-wt	$1.34x10^3$	1	-	-	126	121	131
Individual genotype	ChchTF1-A	$9.14x10^3$	0.19	0.08	0.26	123	120	126
	ChchTF1-B	$5.24x10^3$	0.26	0.14	0.46	116	114	118
	ChchTF1-C	$1.12x10^4$	0.12	0.07	0.21	113	109	117
	ChchTF1-G	$1.73x10^4$	0.08	0.04	0.15	127	121	133
	ChchTF1-H	$9.42x10^3$	0.21	0.12	0.37	125	120	130
OB mixtures	ChchTF1-AB	$1.34x10^3$	1.01	0.56	1.80	115	110	120
	ChchTF1-ABC	$5.75x10^2$	2.34	1.17	4.70	112	105	119
	ChchTF1-ABCG	$9.09x10^2$	1.48	0.83	2.66	113	106	120
	ChchTF1-ABCGH	$1.96x10^3$	0.69	0.40	1.19	111	104	120
Co-occluded mixtures	ChchTF1-AB	$2.13x10^3$	0.63	0.36	1.10	105	97	113
	ChchTF1-ABC	$6.44x10^2$	2.09	1.06	4.13	93	90	96
	ChchTF1-ABCG	$1.93x10^3$	0.70	0.40	1.22	99	96	102
	ChchTF1-ABCGH	$1.77x10^3$	0.76	0.42	1.35	98	95	101

Speed of kill analysis revealed three distinct groups: the fastest killing group comprised the co-occluded mixtures ChchTF1-ABC, ChchTF1-ABCG and ChchTF1-ABCGH, with MTD values of 93, 99 and 98 hpi, respectively. The second group comprised ChchTF1-B, ChchTF1-C, ChchTF1-AB OBs and co-occluded mixtures and ChchTF1-ABC, ChchTF1-ABCG and ChchTF1-ABCGH OB mixtures, which had intermediate MTD values ranging from 105 to 116 h. Finally, ChchTF1-wt, ChchTF1-A, ChchTF1-G and ChchTF1-H constituted the slowest killing inocula (Table 3). Considering both parameters (OB pathogenicity and speed of kill), the ChchTF1-ABC co-occluded mixture was selected as the active material as the basis for a biological insecticide based on OB potency and virulence properties.

Significant differences were observed in OB yields in insects infected by the different inocula ($F_{(13,28)}$=20.755; P<0.001). ChchTF1-wt and ChchTF1-A were the most productive treatments with $8.26x10^6$ and $7.67x10^6$ OBs/larva, respectively (Tukey P=0.236), followed by the ChchTF1-AB OB mixture, which was as

productive as ChchTF1-A (Tukey *P*=0.060) but significantly less productive than ChchTF1-wt (Tukey *P*<0.05). The other single genotypes and mixtures were significantly less productive, with means ranging from $5.62x10^6$ to $4.32x10^6$ OBs/larva (Fig. 3).

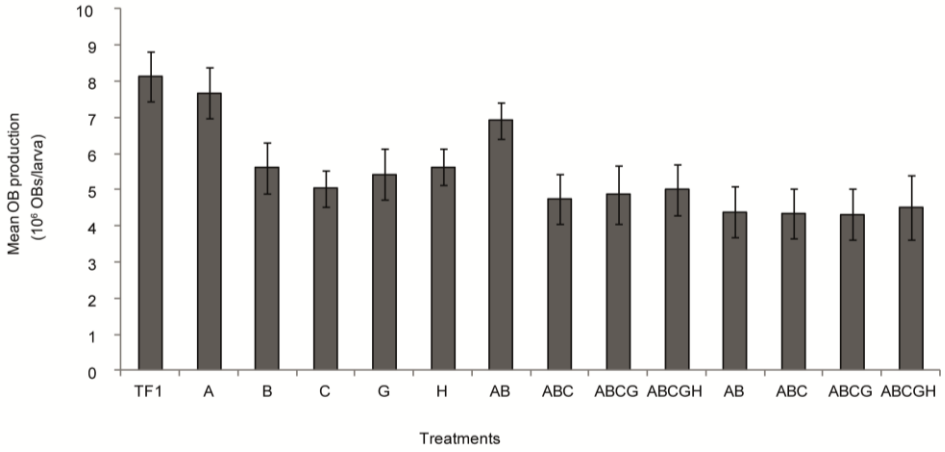

Figure 3. Mean OB yield values obtained from infection of *Chrysodeixis chalcites* second instars with the LC_{90} of ChchTF1-wt (TF1), its individual genotypes ChchTF1-A, ChchTF1-B, ChchTF1-C, ChchTF1-G and ChchTF1-H, the OB mixtures ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG and ChchTF1-ABCGH and the co-occluded genotype mixtures ChchTF1-AB, ChchTF1-ABC, ChchTF1-ABCG, and ChchTF1-ABCGH.

3.6. Stability of the ChchTF1-ABC co-occluded mixture throughout serial passage

The relative frequency of ChchTF1-A ($F_{(5,12)}$=2.453; *P*=0.094), ChchTF1-B ($F_{(5,12)}$=1.965; *P*=0.157) and ChchTF1-C ($F_{(5,12)}$=0.366; *P*=0.862) in the ChchTF1-ABC co-occluded mixture remained did not differ significantly over five successive passages. The average frequencies of these genotypes in the mixture were 48, 34 and 18%, respectively, compared with the starting frequencies 36%A:26%B:14%C (Fig. 4A). The frequencies of ChchTF1-A ($F_{(5,12)}$=2.640; *P*=0.078) and ChchTF1-C ($F_{(5,12)}$=1.450; *P*=0.276) in the wild-type population also did not differ significantly over five successive passages, at 34% and 12%, respectively (Fig. 4B). This genetic stability was also reflected in the pathogenicity values of the corresponding mixed genotype OBs. The LC_{50} values of ChchTF1-wt OBs and the ChchTF1-ABC co-occluded mixture OBs at the fifth passage were $1.26x10^3$ and $7.59x10^2$ OBs/ml,

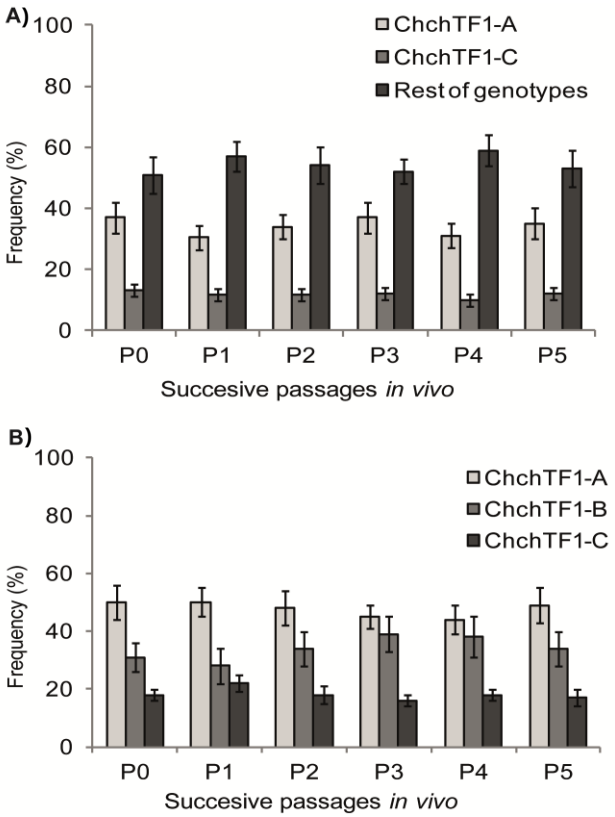

Figure 4. Percentage of relative frequencies of individual genotypes in: the ChchTF1-ABC co-occluded mixture (A) and the wild-type ChchTF1 population (B), across five serial passages *in vivo* (P_0-P_5).

respectively, that did not differ significantly from values estimated at the first passage (Table 3). Similarly, ChchTF1-ABC OBs from the fifth passage were 1.67-fold more potent than wild-type OBs from the same passage, as observed with the initial inocula (Table 2).

4. DISCUSSION

The genetic structure of the most prevalent and pathogenic isolate of ChchSNPV from the Canary Islands, ChchTF1-wt, and the interactions among its genotypes were examined with the aim of selecting a genotype or mixture of genotypes with valuable insecticidal traits. Eight different genotypes were identified in the ChchTF1-wt population. The *Bgl*II profile of the single genotype ChchTF1-A was the same to that of ChchTF1-wt, suggesting it is likely to be an abundant

genotype in the natural population. The abundance of this genotype may have masked submolar bands from other less frequent genotypes (Bernal et al., 2013a). The dominance of ChchTF1-A was confirmed by qPCR analysis, and was present in ~36% of the plaque isolates from the wild-type population. The presence of particular dominant genotype in nucleopolyhedrovirus populations has also been reported in *S. exigua* MNPV (Muñoz et al., 1998; Gelenter and Federici, 1990) and *S. frugiperda* MNPV (Simón et al., 2004). Pure genotypes ChchTF1-C, ChchTF1-G and ChchTF1-H presented *Bgl*II profiles similar to those of previously described isolates: ChchSNPV-TF3, ChchSNPV-NL and ChchSNPV-TF2, respectively (Bernal et al., 2013a). The fact that the ChchTF1-G genotype presented the same profile as the Dutch strain suggests both a phylogenetic relationship between the Dutch and Canary Island populations, and also underlines the importance of differences in the contribution of each genotype to the survival of nucleopolyhedroviruses in genetically distinct host populations and differing environmental conditions. Genome sequencing and phylogenetic, evolutionary and selection analyses would be necessary to support these hypothesis.

Phenotypic differences are derived from changes at the genome level (Kamiya et al., 2004; Wu et al., 2000) and are important for virus adaptation and survival (Cory et al., 2005; Hodgson et al., 2001). The major source of intraspecific variability among genotypes can be found in homologous repeat regions (*hrs*) Erlandson. 2009, the *bro* gene family (Erlandson, 2009; Hitchman et al., 2007; Bideshi et al., 2003) and the *hoar* gene (Le et al., 1997). For ChchTF1 genotypes, the greatest variability was observed within the *hoar* and *bro-d* genes. Recently, the complete genome sequences of these genotypes was determined and confirmed that the region of highest variability is located in these two genes. The fastest-killing genotypes, ChchTF1-B and ChchTF1-C, had an insertion in the *hoar* gene, whereas the most productive genotype, ChchTF1-A, had an insertion in the *bro-d* gene. The *bro* genes, or baculovirus repeated ORFs, play an important role in viral replication due to their nucleic acid binding activity and association with nucleosomes (Zemskov et al., 2000). Recombinant genotypes with these genes deleted or interchanged are currently being constructed with the aim of determining their role in the observed phenotype of ChchSNPV genotypes.

Phenotypic characterization revealed that the genotypic structure of ChchTF1-wt maximized viral pathogenicity and transmissibility as none of the pure genotypes was more pathogenic or produced more OBs per insect than the wild-type population. The ChchTF1-wt was 8.3-fold more pathogenic than the most prevalent ChchTF1-A genotype. Single genotypes had similar levels of OB pathogenicity, but differed markedly in speed of kill, as observed in other nucleopolyhedrovirus populations (Ogembo et al., 2007). Fast-killing genotypes tend to produce fewer OBs, probably because insects die soon after infection and have insufficient time to grow as large as larvae infected by slower killing genotypes. However, this trade-off is not always observed in single genotypes (Hodgson et al., 2001). In the case of ChchTF1-F, ChchTF1-G and ChchTF1-H, these genotypes were as fast-killing as the wild-type but yielded significantly fewer OBs from each infected larva, suggesting the existence of a genetic basis for this important trait.

The fact that ChchTF1-wt OBs were more pathogenic than any of the single genotypes strongly suggested that interactions between the individual genotypes increased the pathogenicity of the wild-type population. Indeed, in OB and co-occluded mixtures containing the most abundant genotypes, together with the least common ones, ChchTF1-ABCHG and ChchTF1-ABCGH, in frequencies similar to those in which they were isolated, the pathogenicity of the viral population was restored. Although constraints on the design of specific primers for rare genotypes limited our ability to generate direct evidence of the presence of rare genotypes in experimental mixtures, the qPCR reactions based on the viral *DNA-polymerase* gene provided indirect but consistent evidence of the presence of the rare genotypes in experimental genotype mixtures at approximately the proportions in which they were present in inocula used to initiate infections.

Similarly, studies with complete and defective genotypes of *Spodoptera frugiperda* MNPV revealed that, when they were mixed in near natural frequencies, and co-occluded into OBs, the pathogenicity of the wild-type population was restored, demonstrating cooperation between genotypes (López-Ferber et al., 2003). Interestingly, in the present study, OB mixtures and co-occluded mixtures containing only the most abundant genotypes (ChchTF1-ABC) resulted in an increase in the pathogenicity of OBs with respect to ChchTF1-wt by two-fold.

Synergistic effects between genotypes have also been observed in other wild-type nucleopolyhedrovirus populations (Hodgson et al., 2004; Simón et al., 2004).

In terms of virulence, determined as speed of kill, mixtures that included two of the fastest killing genotypes (ChchTF1-B and ChchTF1-C) were significantly more virulent than the wild-type population, which was associated with decreased OB production. However, genotypes ChchTF1-A, ChchTF1-G and ChchTF1-H, despite being the least virulent genotypes, had no significant influence on the speed of kill of mixtures including those that included ChchTF1-B and ChchTF1-C. When more genotypes were added to the ChchTF1-ABC mixture, OB pathogenicity was restored to that of the wild-type population, however virulence and OB productivity were not affected, suggesting that interactions involving other minor genotypes not included in the experimental mixtures, such as ChchTF1-D or ChchTF1-E, or other genotypes that we failed to isolate, could attenuate the speed of kill of ChchTF1-wt and increase OB productivity. The capacity to infect a host (OB pathogenicity) is crucial for virus survival, as well as the production of progeny OBs for efficient transmission (Cory and Myers et al., 2003). In this kind of obligate-killing parasites, there is often a trade-off between virulence and progeny production (Hodgson et al., 2004; Cory and Myers et al., 2003: Buckling and Brockhurst, 2008) and ChchTF1-wt provides a further example of this balance. Even though single genotypes were more virulent, the ChchTF1-wt population seems to be genotypically structured to exploit maximally host resources, reflected in investment in OB production, thus increasing the likelihood of transmission under natural conditions.

One drawback in the commercialization of these viruses as biological insecticides is their slow speed of kill compared to most synthetic insecticides. However this trait can be significantly improved by manipulation of genotype composition (Simón et al., 2008). Improvement of the speed of kill has been one of the major research objectives in the development of recombinant baculoviruses as the basis for bioinsecticidal products. Two main approaches have been employed; the expression of insecticidal toxins, enzymes or hormones (Gramkow et al., 2010; Kamita et al., 2010; Shim et al., 2013), the deletion of genes affecting life-stage (O'Reilly and Miller, 1991), or a combination of both (Bonning et al., 1995). In the present study, we demonstrated that co-occlusion of certain genotypes within the same OB resulted in a significant improvement in speed of kill. The reasons for the

higher speed of kill of co-occluded mixtures compared to OB mixtures have not been investigated but we hypothesize that because co-occlusion results in an intimate physical proximity of occlusion derived virions, even in single NPVs, this may favor the entry of different genotypes in midgut cells in similar frequencies as they were present in the inoculum, which is probably less likely to occur when genotypes that are segregated among different OBs.

Notably, the co-occluded genotype mixtures of ChchSNPV maintained their genotype frequencies and insecticidal characteristics over five successive passages. In contrast, in studies with genotype populations of multicapsid NPVs that had been mixed in non-natural frequencies, genotypic mixtures rapidly converged to natural frequencies during serial passage and only maintained stable genotype frequencies once the proportions of genotypes in the natural population had been reached (Clavijo et al., 2009; Simón et al., 2006).

Baculovirus based bioinsecticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest, without the explicit requirement for virus transmission from progeny OBs produced by the first wave of infected larvae. Therefore, the selection of active material in insecticide development is usually focused on the isolate(s) with the most favorable pathogenicity and virulence characteristics with the aim of rapidly achieving high levels of lethal infection of the insect pest following the application of the lowest effective concentration of OBs, with the aim of reducing the quantity of OBs, and therefore the costs associated with each virus application. In this respect, the ChchTF1-ABC co-occluded mixture of genotypes presented the most suitable combination of characteristics for use as a biological insecticide that were better than those of the wild-type isolate. For the first time we demonstrated that just co-occluding three genotypes the insecticidal properties were improved compared to the wild-type population. In conclusion, the co-occluded mixture ChchTF1-ABC was found to have insecticidal characteristics, and genetic and biological stability that favor its use as the basis for a biological insecticide for control of *C. chalcites* in the Canary Islands. This genotypic mixture is now undergoing field trials and virus production studies with the aim of producing a commercially viable product in the near future.

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CHAPTER 4

Complete genome sequence of five *Chrysodeixis chalcites* nucleopolyhedrovirus genotypes from a Canary Islands isolate

ABSTRACT

The *Chrysodeixis chalcites* single nucleopolyhedrovirus (ChchSNPV) infects and kills *C. chalcites* larvae, an important pest of banana crops in the Canary Islands. Five genotypes present in the most prevalent and widespread isolate in the Canary Islands were sequenced, providing genetic data relevant to the genotypic and phenotypic diversity of this virus.

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The *Chrysodeixis chalcites* single nucleopolyhedrovirus, ChchSNPV (*Baculoviridae*: *Alphabaculovirus*) has great potential to be a complement or alternative to chemical control of its natural host, *C. chalcites* (Lepidoptera: Noctuidae), as occurs with many other baculovirus-host systems (Moscardi, 1999), particularly for banana protection in the Canary Islands (Spain). A Canarian isolate, ChchSNPV-TF1wt (ChchTF1), obtained from a *C. chalcites* infected larva collected during a natural epizootic in banana crops, is the most prevalent and widespread isolate in these Islands, and displays the highest pathogenicity and virulence values compared to previously described strains from Netherlands (van Oers, et al., 2005) or Spain (Bernal et al., 2013a; Murillo et al., 2000). This strain is composed of multiple genotypes, which have been cloned *in vitro* (Bernal et al., 2013b). Complete genome sequencing of the three most abundant genotypes, namely ChchTF1-A, -B, and -C, and the two scarcest ones, ChchTF1-G and -H, was determined by 454 sequencing, assembled with Newbler v2.3 software and then checked in detail manually.

The genomes of ChchTF1-A, -B, -C, -G, and -H were 149,684, 149,080, 150,079, 149,039, 149,624 bp long, respectively, very similar to the ChchSNPV-type isolate, ChchSNPV-NL (149,622 bp) (Acc. no. AY864330), from The Netherlands. All five of them had a 39% GC content, also similar to that of ChchSNPV-NL (van Oers et al., 2005). The unique ChchSNPV gene, ORF 53, was not identified in the ChchTF1-A, -B, -C, -H genomes due to a single nucleotide mutation in the start codon (TGC). Hence, a total of 150 ORFs were predicted in ChchTF1-A, -B, -C, -H, and 151 in the ChchTF1-G genome. Fifty-eight ORFs were 100% homologous in the six ChchSNPV genomes sequenced to date. The 62 genes conserved in other lepidopteran baculoviruses were all present (Herniou et al., 2003). As previously described for ChchSNPV-NL (van Oers et al., 2005), no typical homologous regions (*hrs*) were identified in the ChchTF1 genotypes. A whole genome sequence alignment between ChchTF1A, -B, -C, -G and -H and ChchSNPV-NL showed 98-99% homology at the nucleotide level. This analysis also demonstrated that variable genomic regions were located principally in the *hoar* and *bro-d* genes, which represent a major source of intra-specific variability among genotypes in many baculoviruses (Bideshi et al., 2003; Erlandson, 2009; Le et al., 1997). Finally, phylogenetic analysis grouped the five Spanish and the Dutch

genotype in three pairs of clusters: ChchSNPV-NL with ChchTF1-G; ChchTF1-A with ChchTF1-B; and ChchTF1-C with ChchTF1-H.

In all, our work will be helpful for further exploring the genetic diversity of this virus and the genes involved in insecticidal traits.

NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The complete genome sequences of ChchSNPV-TF1-A, -B, -C, -G and -H were submitted to GenBank under the accession numbers JX535500, JX560540, JX560539, JX560541 and JX560542, respectively.

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CHAPTER 5

Boosting *in vivo* mass-production of *Chrysodeixis chalcites* nucleopolyhedrovirus in its homologous host

ABSTRACT

Commercialization of a Canarian *Chrysodeixis chalcites* nucleopolyhedrovirus isolate, ChchSNPV-TF1, with outstanding performance as a bioinsecticide in banana field trials against its natural host pest populations, requires an efficient large-scale production system. This study attempted to maximize ChchSNPV-TF1 occlusion body (OB) yields using its homologous host as a biofactory. The most suitable larval instar for virus inoculation was the sixth instar (L_6), as it recorded higher virus productivity ($1.80x10^{11}$ OBs/larva) and showed lower cannibalism rates (5.3%) than L_5 and L_4 instars. The best inoculation time was 24 h post L_6 molting, which produced about six times more OBs ($5.72x10^{11}$ OBs/larva) than newly molted L_6 larvae. Five different viral concentrations, ranging from lethal concentration causing 90% mortality (LC_{90}) to LC_{50} were evaluated, but no differences on OB production *per* larva or mg of larva or mean time to death (MTD) were observed. Hence, the LC_{90}, which killed the highest number of larvae, was selected, as it allowed the greatest OB yields *per* number of inoculated larvae. In larval density assays, yielded greater OB productions (average $8.07x10^{13}$ OBs/container), than lower (1, 25, 50 and 100) densities and similar to those yielded in the highest (200) larval density. Given the low virus concentrations predicted for efficient banana protection from *C. chalcites*, we hypothesize a profitable commercialization of ChchSNPV-TF1 using the production method optimized here.

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1. INTRODUCTION

Chrysodeixis chalcites (Esper, 1789) (Lepidoptera: Noctuidae) is considered as one of the most serious lepidopteran pests in many countries (Del Pino et al., 2011; Shepard et al., 2009; van Oers et al., 2004). In Spain, major damages have been reported in banana crops in the Canary Islands (Del Pino et al., 2011) and in horticultural greenhouse crops in Almería (Cabello and Belda, 1994). A previous research showed that an indigenous *C. chalcites* simple nucleopolyhedrovirus isolate from Southern Tenerife (Canary Islands), ChchSNPV-TF1, had a great potential as a bioinsecticide for the control of *C. chalcites* pest populations (Bernal et al., 2012a, 2012b). The pathogenicity and virulence of this strain is comparable to that of the most pathogenic and virulent baculoviruses currently commercialized as bioinsecticide products (Caballero et al., 2009; Moscardi, 1999).

One of the greatest limitations to the use of baculoviruses as biocontrol agents is an efficient and economically viable mass production system. As obligate pathogens, viruses require living hosts for replication, and this can be achieved only in host larvae (*in vivo*) or in cell culture (*in vitro*). Costs associated with both these systems make it difficult to offer viral products with marketable prices (Grzywacz et al., 1998). In addition, *in vitro* system techniques, firstly developed for protein production using recombinant baculoviruses and currently used for vaccine development, drug screening, and gene therapy (van Oers, 2011), have not been sufficiently developed for large-scale viral production (Claus and Sciocco de Cap, 2001) and face important technical constraints. For instance, cell culture production can lead to a variety of mutations or yield defective viral populations due to the passage effect that can reduce the biological activity of insect viruses (Dai et al., 2000; Pijlman, et al., 2001, 2004; Sun et al., 2005). Also, Bonning et al. (1995) found that AcMNPV OBs produced in *Trichoplusia ni* (Lepidoptera: Noctuidae) and *Heliothis virescens* (Lepidoptera: Noctuidae) larvae caused mortality significantly faster than OBs derived from Sf21 or TniSB-1 insect cell lines, due to a host protease that is incorporated in the larval-produced OBs. Moreover, *in vitro* production costs are higher than *in vivo* (Gupta et al., 2007).

At present the only viable option for large-scale production of baculoviruses is *in vivo*, most often in the homologous permissive host reared on artificial diet (Kumar et al., 2005; Monobrullah and Nagata, 2000; Rabindra et al., 2003). The

cost of *in vivo* production systems can be reduced if aspects like the larval stage and viral inoculum concentration are optimized. These two parameters are precisely adjusted for each virus-host system to achieve full larval growth, which, in turn, maximizes OB production (Gupta et al., 2007; Ignoffo and Couch, 1981). Another key factor in optimizing mass-production is determining larval density per rearing container. In general, when cannibalism is not a problem, larvae are rather reared at the highest densities possible to reduce handling times, while keeping larval stress to a minimum to avoid reduced host weights. In the present study our aim was to evaluate all of these parameters to optimize ChchSNPV-TF1 OB production in *C. chalcites* larvae.

2. MATERIAL AND METHODS

2.1. Insect source, rearing and virus strain

C. chalcites larvae were obtained from pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007, and refreshed periodically with pupae from the Canary Islands. Larvae were reared at 25±1ºC, 70±5% humidity, and a photoperiod of 16:8 (light:dark), on a semi-synthetic diet described by Greene et al. (1976). Adults were fed 30% w/v diluted honey. ChchSNPV-TF1 strain was originally isolated from a single larva collected during a natural epizootic in banana crops in the Southern Tenerife, Canary Islands (Spain) (Bernal et al., 2012a). ChchSNPV-TF1 was selected among other strains due to its highest pathogenicity and virulence. ChchSNPV-TF1 OBs were amplified in a single passage through fifth instar (L_5) *C. chalcites* larvae. OBs from virus-died larvae were extracted and filtered through cheesecloth. These were washed twice with 0.1% SDS and once with 0.1 M NaCl and finally resuspended in double-distilled-water. Purified OBs were stored at 4ºC.

2.2. Bioassays to test the effect of larval instar on virus production

Fifty *C. chalcites* larvae of each of the three latter instars, L_4, L_5 and L_6, were inoculated with ChchSNPV-TF1 using the droplet feeding method described by Hughes and Wood (1981) and were placed individually in 25 ml plastic cups with artificial diet until larvae died or reached the pupal stage. A single concentration producing ~90% larval mortality (LC_{90}) was used to inoculate each instar: $5.56x10^6$, $5.00x10^7$ and $9.02x10^8$ OBs/ml for L_4, L_5 and L_6, respectively (Bernal et al., 2012b).

Larval mortality was recorded every 24 h. Bioassays were carried out three times. All NPV-killed larvae (at least 40 for each larval stage and replication) were frozen in their individual cup and once frozen transferred individually to 1.5 ml tubes, which were stored at -20ºC until titration was accomplished. The average OB production from each replication was compared by ANOVA using the SPSS v12.0 program.

2.3. Bioassays to test the effect of larval instar on cannibalism

Fifty L_4, L_5 and L_6 *C. chalcites* larvae were inoculated with their corresponding ChchSNPV-TF1 LC_{90}, as described previously, and placed in 1.5 l plastic containers. Larvae reaching the pupal stage, cannibalized larvae (those partially or totally consumed), and virus-killed larvae were recorded daily. The bioassay was carried out three times. The average number of pupae, cannibalized larvae and NPV-killed larvae were analyzed by contingency table analysis using the SPSS v12.0 program.

2.4. Bioassays to test the effect of inoculation time on virus production

Twenty-five *C. chalcites* larvae were inoculated with the ChchSNPV-TF1 LC_{90} ($9.02x10^8$ OBs/ml) (Bernal et al., 2012b) at two different physiological times during the L_6 instar: i) newly molted and ii) 24 h after molting had occurred (24 h old L_6). The bioassay was carried out three times and larval mortality was recorded every 24 h. Larvae were weighed when they were moribund (one day before dying), at which time they were collected in two different 50 ml falcon tubes for each physiological time and titrated. The mean final larval weights and mean OB production values of each replica were normally distributed and were analyzed by Student t-test using the SPSS v12.0 program. Values of OBs *per* mg of larvae were also subjected to Student t-test in SPSS v12.0 program.

2.5. Bioassays to test the effect of viral concentration on virus production

Five groups of 25 larvae (24 h old L_6), were inoculated with one the following ChchSNPV-TF1 concentrations: $9.02x10^8$, $6.82x10^8$, $4.62x10^8$, $2.42x10^8$ and $2.20x10^7$ OBs/ml. These concentrations had previously been calculated to kill 90, 80, 70, 60 and 50% larvae, respectively (Bernal et al., 2012b). Larvae were placed in 1.5 l plastic containers. To determine the influence of viral concentration in the mean time to death, larval mortality was recorded every 8 hours. Larvae were

weighed when they were moribund (one day before dying), at which time they were collected in five different 50 ml falcon tubes for each treatment and titrated. The bioassay was carried out six times. The mean final larval weights and mean OB production values (in OBs *per* mg of larvae), of each replica were normally distributed and were analyzed by ANOVA using the SPSS v12.0 program. Mean time to death values were subjected to Weibull survival analysis using the Generalizer Linear Interactive Modeling (GLIM) program (Crawley, 1993).

2.6. Bioassays to test the effect of larval density on virus production

C. chalcites larvae (24 h old L_6) were orally inoculated with the ChchSNPV-TF1 LC_{90} ($9.02x10^8$ OBs/ml) using the droplet-feeding method and distributed in groups of 1, 25, 50, 100, 150 and 200 in 1.5 l plastic containers. Larvae were weighed one day prior to death, at which time they were transferred into 50 ml falcon tubes. Each container was checked daily for 8 days post-inoculation to collect larvae before dying, in order to avoid OB production losses due to larval cannibalism. Virus-killed larvae were carefully collected daily and stored at -20ºC. On the 8th day, when virtually all larvae had died and to avoid losses of OBs due to insect integument lysis, each container was placed at -20ºC and frozen larvae were collected and placed in the falcon tubes. The bioassay was carried out ten times. Results on larval weight were analyzed by ANOVA, those on the percentage of larvae reaching the pupal stage, larval cannibalism and virus-killed larvae were subjected to contingency table analysis, and finally, productivity in terms of OBs *per* mg of larval weight and total OB production per container were analysed by ANOVA after a square root transformation, all of them using the SPSS v12.0 program. Transformed data satisfied the assumption of normality required.

2.7. Titration of OBs

OB production was determined from single or from groups of infected larvae, which were collected when they were moribund or dead and kept at -20ºC. Before titration, single larvae were homogenized in 1 ml distilled water, while groups of larvae were homogenized in variable volumes (1 ml per larvae) according to the number of larvae collected. OBs were extracted from dead larvae by filtration and differential centrifugation. Appropriate dilutions were prepared for OB counting in an improved Neubauer hemocytometer (Hawksley, Laucing, UK) under phase contrast

microscopy at x400. Titration of OBs was performed twice from each larva or groups of larvae.

3. RESULTS

3.1. Effect of larval instar on OB production and cannibalism rates

A significant influence of larval stage in OB yield was observed ($F_{(2,6)}$=33.637; *P*<0.001). L_6 instars significantly recorded the highest OB production, with 1.80x10^{11} OBs/larva (Fig. 1 A). As for cannibalism rates, these were lowest in L_6 larvae (5.3%) and increased with decreasing larval development (χ^2=98.266; d.f.=4; *P*<0.001) (Fig. 1 B). According to these results, L_6 larvae were selected as the most appropriate stage for ChchSNPV-TF1 mass production.

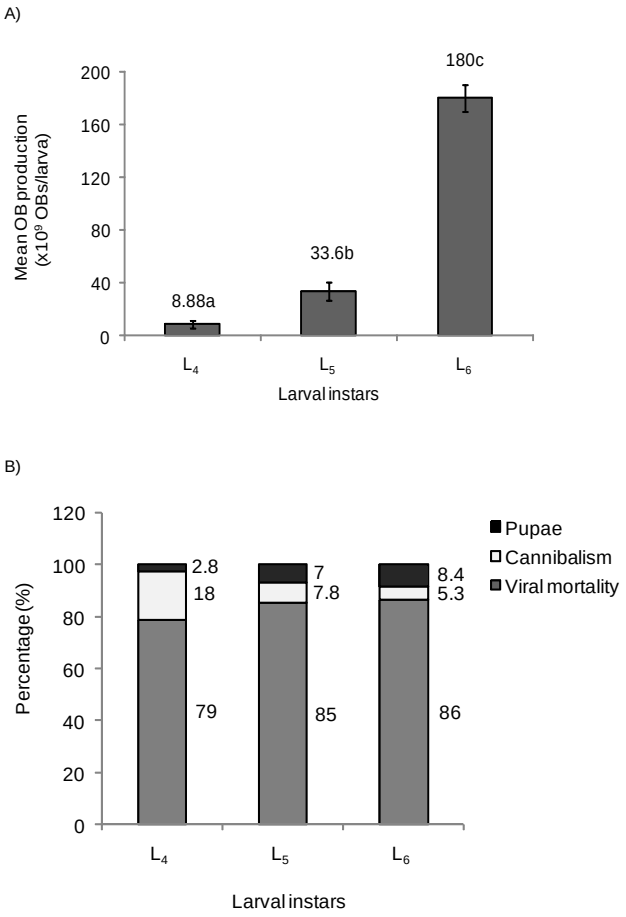

Figure 1. A) Mean OB production of ChchSNPV-TF1 in L_4, L_5, and L_6 *Chrysodeixis chalcites* larvae. Values above bars represent average OB production. Different letters accompanying values indicate significant differences between them (*P*<0.05). B). Larval percentage reaching the pupal stage, larval cannibalism and virus-killed larvae in L_4, L_5 and L_6 *C. chalcites* instars inoculated with their corresponding ChchSNPV-TF1 LC_{90} values.

3.2. Effect of inoculation time on OB production

Twenty-four h old L_6 larvae weighed 1.4 times more than newly molted L_6 (t=13.643; d.f.=5; *P*<0.001), being their average weights 315 and 230 mg, respectively. However this increase in larval weight was lower than that observed in OB production, as 24 h old L_6 larvae produced almost six times more ChchSNPV-TF1 OBs ($5.72x10^{11}$ OBs/larva) than newly molted L_6 ($1.00x10^{11}$ OBs/larva) (t=14.008; d.f.=5; *P*<0.001) (Fig. 2 A). When relating OB production with larval weight, a 4.2-fold increased was still observed (t=6,708; d.f.=5; *P*=0.001) (Fig. 2 B).

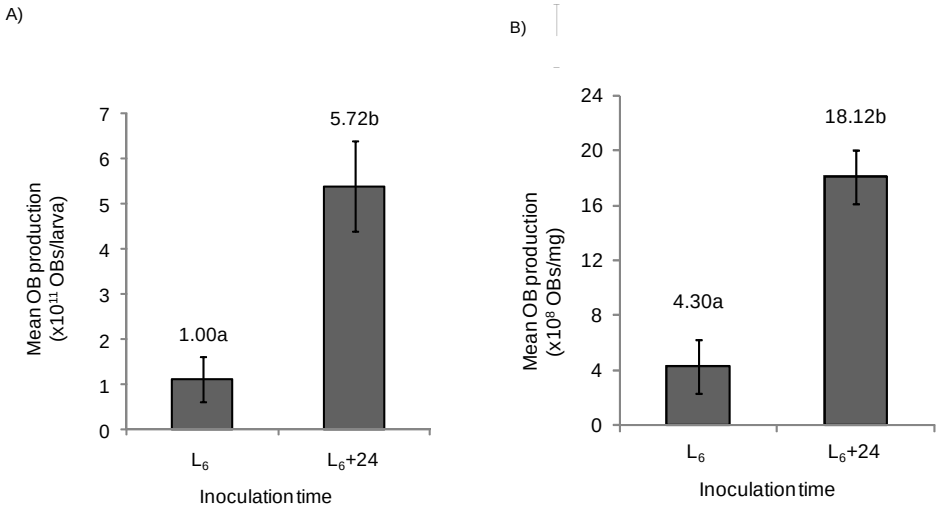

Figure 2. A) Mean OB production *per* larva of ChchSNPV-TF1 (OBs/larva) and B) mean OB production *per* mg of larva of ChchSNPV-TF1 (OBs/mg) in *Chrysodeixis chalcites* L_6, newly molted (L_6) or 24 h after molt to L_6 had occurred (L_6+24). Values above bars represent OB production average. Different letters accompanying values indicate significant differences between these values (*P*<0.05).

3.3. Effect of virus concentration on MTD and OB production

No differences were observed in final larval weights among the different concentration tested ($F_{(4,\ 25)}$=0.905; *P*=0.476). In addition, analysis of MTD values revealed no significant differences between larvae infected with the different ChchSNPV-TF1 concentrations. All inoculated larvae died between 165-175 h post-infection (Fig. 3 A). Similarly, no significant differences were observed in OB production per larva ($F_{(4,25)}$= 2.932; *P*= 0.041) (Fig. 3 B), and so, in OB production *per* mg of larva among the five concentrations tested ($F_{(4,25)}$= 2.932; *P*= 0.041) (Fig.

3 C). However, the highest concentration ($9.08x10^8$ OBs/ml) allowed recovery of a greater number of virus-killed larvae and therefore produced the greatest total OB yields, $7.17x10^{12}$ OBs obtained from 15 virus-killed larvae.

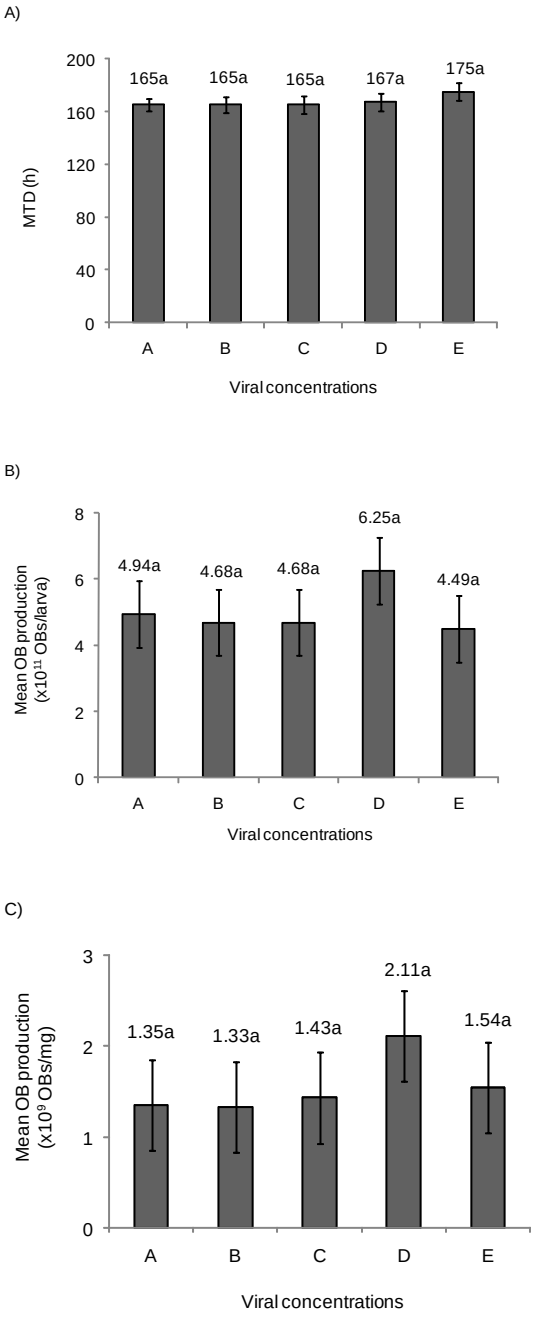

Figure 3. A) Mean time to death (MTD) values of 24 h old L_6 *Chrysodeixis chalcites* inoculated with one of the following ChchSNPV-TF1 concentrations: $9.02x10^8$ OBs/ml (A), $6.82x10^8$ OBs/ml (B), $4.62x10^8$ OBs/ml (C), $2.42x10^8$ OBs/ml (D) and $2.20x10^7$ OBs/ml (E). Values above bars indicate means. Values followed by identical letters did not differ significantly. B) Mean OB production *per* larva of ChchSNPV-TF1 (OBs/larva) and C) mean OB production *per* mg of larva of ChchSNPV-TF1 (OBs/mg) in 24 h old L_6 *Chrysodeixis chalcites* inoculated with the five different ChchSNPV-TF1 concentrations described above. Values over the bars represent OB production averages and those labelled with the same letters indicate no significant differences ($P>0.05$).

The 100-larva container was significantly less productive with $3.64x10^{13}$ OBs/container obtained from 83 virus-killed larvae (Tukey $P<0.001$). The 50 and 25-larva containers were equally productive, with $1.41x10^{13}$ OBs from 41 larvae and $6.70x10^{12}$ OBs from 21 larvae, respectively (Tukey $P=0.931$), but significantly less productive than the 150 or 200-larva containers (Tukey $P<0.001$).

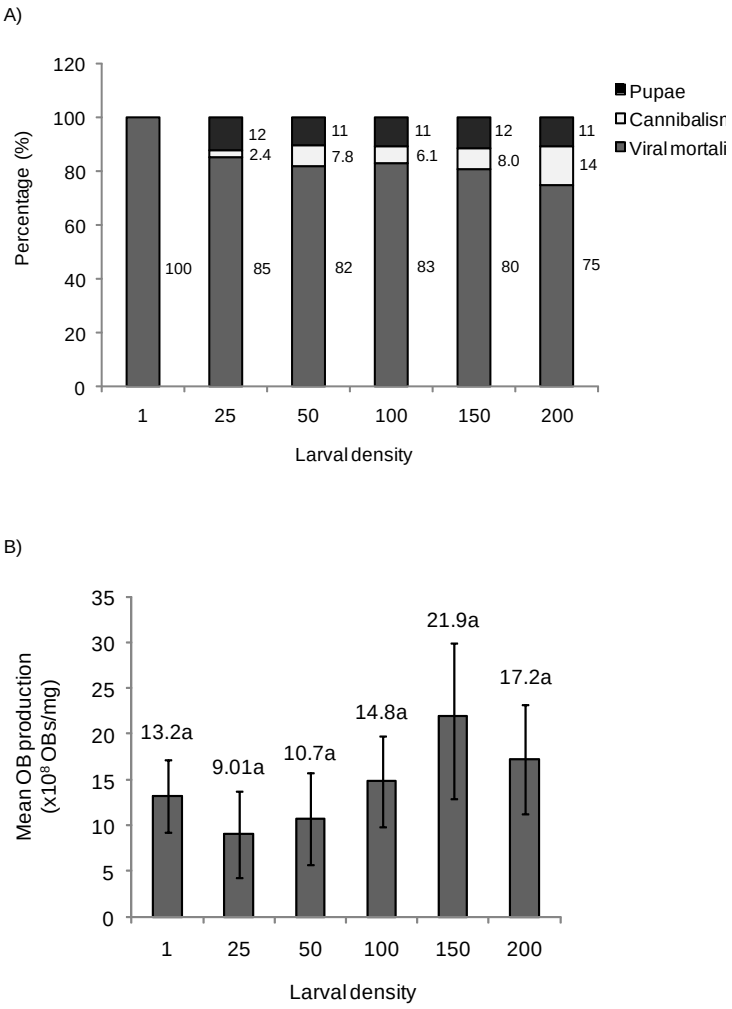

Figure 4. A) Larval percentage reaching the pupal stage, larval cannibalism and virus-killed larvae reared at larval densities: 1, 25, 50, 100, 150 and 200 in 1.5 l plastic containers. Twenty-four h old L_6 *C. chalcites* larvae were inoculated with the ChchSNPV-TF1 LC_{90} ($9.02x10^8$ OBs/ml). B) Mean OB production *per* mg of larva (OBs/mg) and C) total OB production per container in 24 h old L_6 *C. chalcites* larvae in 1.5 l plastic containers at larval densities: 1, 25, 50, 100, 150 and 200. Values above bars represent average OB production. Same letters accompanying values indicate no significant differences between them ($P>0.05$).

4. DISCUSSION

Feasibility of *in vivo* NPV large-scale production systems has been demonstrated throughout a wide variety of host-pathogen systems. In the present study we describe an effective ChchSNPV-TF1 mass production method in its homologous host by optimizing parameters such as: larval instar for infection, timing of inoculation, virus inoculum concentration, and larval density per container.

Undoubtedly, L_6 instar *C. chalcites* larvae constituted the most suitable instar for ChchSNPV-TF1 production since OB yields 20 and five-fold higher than in L_4 and L_5 instars were obtained, respectively. Exponential increase in OB yields as larvae grow has been found in many other host-virus systems (Kalia et al., 2001; Gupta et al., 2007; Monobrullah and Nagata, 2000; Shieh, 1989). This is due to the direct correlation existing between the insect host weight at death and OB production, which is well dependent on the initial weight of the larvae and on the increase in larval weight during infection (Shapiro, 1986). Thus, the latest larval instar, and in some instances also supernumerary instars induced with juvenile hormones analogs (JHA) (Glen and Payne, 1984; Lasa et al., 2007a; Shieh, 1989), are usually employed to maximize yields.

Another important consideration for the choice of larval instar in OB production is cannibalism. This is a frequent behavior in many species of Lepidoptera during the larval stage and is stage-dependent in some species, with later instars often showing a greater tendency for intraspecific predation compared to their younger conspecifics (Chapman et al., 1999; Polis, 1981). In *C. chalcites* however, the opposite occurred, L_6 larvae were less prone to aggressiveness than L_5, and these less than L_4. A similar tendency was observed in *S. exigua* when L_5 and JHA-induced supernumerary L_6 instars were compared (Elvira et al., 2010). Reportedly, L_6 instars were less agile and less willing to engage in aggressive interactions that L_5s, as occurred with *C. chalcites* L_6 compared to L_5 larvae.

The inoculation time was also adjusted for maximum OB production. Our results showed that 24 h old L_6 larvae produced almost six times more OBs than newly molted L_6 larvae. This could be related to the increased larval weight, as previous studies demonstrated that higher larval weights resulted in higher OB productions (Kalia et al., 2001; Lasa et al., 2007b; Monobrullah and Nagata, 2000; Takatsuka et al., 2007). However this 1.4-fold increase in larval weight was lower

than the 4-fold increase observed in OB production *per* mg of larva. In the mass production system used here, $1.81x10^9$ OBs/mg were obtained, greater by 138-fold than in the SeMNPV-*S. exigua* system ($1.31x10^7$ OBs/mg) (Lasa et al., 2007b) or by 52.9-fold than in the *Helicoverpa armigera* NPV-*H. armigera* system ($3.42x10^7$ OBs/mg) (Kalia et al., 2001). In the SeMNPV-*S. exigua* system JHA were used to achieve such productions rates (Lasa et al., 2007b). It seems that in the ChchSNPV-*C. chalcites* host-pathogen system, other natural factors might influence the highest OB production values obtained. A more efficient use of host tissues by the virus may be one of them.

The concentration of the viral inoculum is another crucial aspect in optimizing large-scale production and has to be determined for each production system. Usually, when the concentration of the viral inoculum is too high, the infectious time process is reduced and larvae die in earlier developmental stages, producing less OBs (Kumar et al., 2005). On the other hand, if the inoculum concentration is too low, a relatively large proportion of insects do not acquire a lethal dose, resulting in suboptimal production. In this study, the five different concentrations of ChchSNPV-TF1 used as inocula, ranging from LC_{90} to LC_{50}, had no effect in the infection rate, as observed by statistically similar MTD values, hence allowing the hosts to reach a similar developmental stage and similar OB/larva yields. Still, the highest concentration resulted in a significant increase in the final OB production as a whole (OB yield from all inoculated larvae) due to the larger number of larvae that became successfully infected. In most baculovirus mass-producing systems, such as SeMNPV-*S. exigua* and SpexMNPV-*S. exempta* (Cherry et al., 1997), AgMNPV-*Anticarsia gemmatalis* (Moscardi et al., 1997), SpliNPV-*S. littoralis* (Grzywacz et al., 1998) and HaSNPV-*H. armigera* (Sun et al., 2005), the highest OB yields were also obtained with inocula doses close to LC_{95}.

Density effects on larval development and weight are well documented. Goulson and Cory (1995) observed that larvae reared at high densities had a quicker larval development, a smaller size and were more susceptible to disease. The six larval densities tested here had no significant influence on larval growth, since larvae reached similar weights, or on their physiological development, as observed by the number of larvae reaching the pupal stage, which did not vary. However, they did have an effect on cannibalistic behavior. Although cannibalism

rates were not as high in *C. chalcites* larvae as those recorded for other lepidopteran species, like *H. armigera* (Kakimoto et al., 2003), *S. exigua* (Lasa et al., 2007a) and *S. frugiperda* (Chapman et al., 1999), where they were increased with increasing larval densities. This has previously been observed in other systems (Chapman et al., 1999) and explains why the 200-larva container yielded a similar OB production than that of the 150-larva container. Densities lower than 150 larvae yielded significantly lower OBs, making the 150 larval density preferred over the 200 larval density, as it represented a lower initial cost (less larvae and diet, and easier handling). Therefore, the 150-larva container was selected as the most appropriate density.

To sum up, yields as high as $8.07x10^{13}$ OBs can be obtained in 150-larva containers by inoculating 24 h L_6 *C. chalcites* larvae with the ChchSNPV-TF1 LC_{90}. This represents a 59,645-fold increase over the inoculated concentration and beats by far the OB productions of other host-baculovirus systems. For example, Gupta et al. (2007) recorded a production of $7.1x10^{8}$ OBs/larva in fifth instar *H. armigera* larvae (which have been reared individually). The *S. exigua*-SeMNPV system (Lasa et al. 2007a) reached $1.1\text{-}1.4x10^{11}$ OBs in 100 *S. exigua* larvae containers. Sun et al. (2005) obtained an OB production of $1.04x10^{9}$ OBs/larva inoculating fifth instar *H. armigera* larvae with a wild type HaSNPV. In our view, the key to achieve such a greatly increased production in *C. chalcites* was the inoculation of the host insect in the later sixth instar added to the efficiency showed by this pathogen-host system. At that stage, larvae have already reached a much higher weight and are thus able to produce significantly greater quantities of OBs compared with their fifth instar conspecifics or other lepidopteran species that only reach the fifth instar in laboratory rearing or sixth instar using JHAs. However not only the inoculation time but also other factors might influence the great efficacy of ChchSNPV-*C. chalcites* mass production system, as OB production increase was higher than the increase in weight. This increased efficacy might be due to a more efficient use of host resources by the virus, which in turn, produced many more OBs/mg larva compared to other systems.

In field applications, between $2x10^{11}\text{-}1x10^{12}$ OBs/ha are generally required for effective insect mortality (depending on caterpillar species, virus strain and formulation) (Moscardi, 1999). However, we have taken the *S. exigua*-SeMNPV

system as a reference since SeMPNV and ChchSNPV-TF1 presented similar pathogenicity and virulence against their homologous pests (Caballero et al., 2009). Lasa et al. (2007a) achieved 80-90% *S. exigua* larval mortality with doses of $5x10^{11}$ OBs/ha in greenhouse sweet pepper crops. If we could translate this into banana crop protection from *C. chalcites*, in which 1600-2000 l/ha are used in phytosanitary applications (Domínguez et al., 2012), the ChchSNPV-TF1 application rates would vary between $8x10^{11}$-$1x10^{12}$ OBs/ha. The practical NPV field application rate would thus equal 1-2 virus infected larvae/ha, and a single 150-larva container, would allow treatment of as many as 80-100 ha. This is merely a prediction, which needs to be confirmed with field trials, but even though application rates were raised by 10 or even 100 fold, the high production yields that can be obtained in *C. chalcites* would still make ChchSNPV-TF1 profitable. At the same level as other NPVs, which require e.g. four 100 *S. exigua* larvae containers/ha in sweet peeper greenhouse crops (Caballero et al., 2009) or 625-1250 SfMNPV-infected larvae/ha ($2.5x10^{12}$ OBs/ha) for cabbage protection against *S. frugip*erda (Behle and Popham, 2012).

Optimizing inocula doses, the instar and physiological stage of larvae at inoculation and the density of rearing had a significant impact on NPV productivity and represent key factors in making the commercial production of this virus economically feasible.

5. ACKNOWLEDGMENTS

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CHAPTER 6

Stage-specific insecticidal characteristics of a nucleopolyhedrovirus isolate from *Chrysodeixis chalcites* enhanced by optical brighteners

ABSTRACT

Chrysodeixis chalcites is a major noctuid pest of banana crops in the Canary Islands. The stage-specific susceptibility of this pest to *C. chalcites* single nucleopolyhedrovirus (ChchSNPV-TF1) was determined, as well as the effect of selected optical brighteners as enhancers of primary infection. Susceptibility to ChchSNPV-TF1 occlusion bodies (OBs) decreased as larval stage increased; second instars (L_2) were 10,000-fold more susceptible than sixth instars (L_6). Virus speed of kill was 42 h faster in L_2 than in L_6. OB production increased in late instars; L_6 larvae produced 23-fold more OBs than L_4. Addition of 10 mg/ml Tinopal enhanced OB pathogenicity by 4.43 to 397-fold depending on instar, whereas 10 µl/ml Leucophor resulted in potentiation of OB pathogenicity from 1.46 to 143-fold. Mean time to death decreased by 14 to 26 h when larvae consumed OBs in mixtures with 10 mg/ml Tinopal, or 10 µl/ml Leucophor, although in these treatments OB yields were reduced by up to 8.5 fold (Tinopal) or up to 3.8 fold (Leucophor). These results have clear applications for the use of ChchSNPV-TF1 as a biological insecticide in control programs against *C. chalcites* in the Canary Islands.

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1. INTRODUCTION

The golden twin spot tomato looper, *Chrysodeixis chalcites* (Esper) (Lepidoptera: Noctuidae), is an important polyphagous pest of crops including tobacco, tomato, cotton, crucifers, legumes, maize, soybean, potato, artichoke, cauliflower and ornamental crops (van de Veire and Degheele, 1994; van Oers et al., 2004). In Spain this pest has been responsible for major losses (>30% of total production) in banana crops in the Canary Islands (Del Pino et al., 2011), and in vegetable crops over a large area of greenhouses in Almeria, southern Spain (Cabello and Belda, 1994). Chemical-based control measures against this pest currently require multiple applications of insecticides that tend to increase production costs and can hamper the commercialization of products that may contain pesticide residues (Perera and Molina, 2007), hence the need to assess alternative methods to control this pest.

Baculoviruses are promising control agents for a number of lepidopteran pests due to their favorable insecticidal properties, host specificity and outstanding safety record (Szewczyk et al., 2006). A number of baculoviruses are currently produced on a commercial scale and applied to large areas of crops, such as the nucleopolyhedrovirus of *Anticarsia gemmatalis* (AgMNPV) in Brazil (Moscardi, 1999). Due to its high pathogenicity and virulence, a singly-encapsidated strain of *C. chalcites* nucleopolyhedrovirus (ChchSNPV, family Baculoviridae, genus *Alphabaculovirus*) isolated from a single larva collected from banana crops in southern Tenerife (Canary Islands, Spain), called ChchSNPV-TF1, was selected from among other ChchSNPV strains from the Canary Islands and other regions including Almería or The Netherlands (Bernal et al., 2013). The pathogenicity and virulence of this strain is comparable to that of the most pathogenic and virulent baculoviruses currently commercialized as bioinsecticide products (Moscardi, 1999; Caballero et al., 2009).

To determine the likely efficacy of a virus pathogen as the basis for a bioinsecticide product, the susceptibility of the different pest instars to the virus strain should be determined. Host stage can affect the characteristics of the concentration-mortality response and survival time of virus infected hosts (Erlandson, 2009; Milks, 1997). A mixture of larval stages of *C. chalcites* is likely to be present at any one time in the field due to overlapping pest generations, making

the determination of instar-related pathogenicity and virulence of practical importance for optimal timing of virus insecticide applications.

Certain components of the formulation may increase the insecticidal activity of the pathogen. Optical brighteners can enhance insect susceptibility to virus infection by disrupting the peritrophic membrane (Okuno et al., 2003; Wang and Granados, 2000) or by inhibiting the sloughing of infected midgut cells (Washburn et al., 1998). Previous studies have demonstrated that these compounds increase OB pathogenicity in laboratory bioassays (Shapiro, 2000; Shapiro and Shepard, 2008) or when applied to crops (Lasa et al., 2007).

In the present study, the susceptibility of different *C. chalcites* instars to an NPV isolated from *C. chalcites* larvae, ChchSNPV-TF1, alone and in mixtures with selected optical brighteners, and the influence of these compounds on speed of kill and OB production characteristics, were determined. The results of these studies provide valuable information for the development of this virus as a biological control agent against *C. chalcites* in the Canary Islands.

2. MATERIAL AND METHODS

2.1. Insect source and viruses

C. chalcites larvae were obtained from a laboratory colony at the Universidad Pública de Navarra, Spain that was established with pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007, and refreshed periodically with pupae from the Canary Islands. Larvae were reared at 25±1°C, 70±5% humidity, with a photoperiod light:dark (16:8), on a semi-synthetic diet described by Greene et al., 1976. Adults were fed with 300 mg/ml honey solution.

The ChchSNPV-TF1 strain used in this study was isolated from a single infected *C. chalcites* larva during a viral epizootic in banana crops in the Canary Islands (Bernal et al., 2013). Occlusion bodies (OBs) used in bioassays, were amplified in a single passage through fourth instars (L_4) of *C. chalcites*. For this, over-night starved larvae that had molted in the previous 12 h, were inoculated orally with an OB suspension (10^6 OBs/ml) and reared until death. OBs from virus-killed larvae were extracted and filtered through cheesecloth. OBs were washed twice with 1 mg/ml sodium dodecyl sulfate (SDS) and once with 0.1 M NaCl and

finally resuspended in double-distilled water. OB suspensions were quantified using an improved Neubauer hemocytometer (Hawksley, Laucing, UK) under phase contrast microscopy at x400, and stored at 4°C.

2.2. Virus identification

The identity of the virus was determined by sequencing amplicons generated using degenerate oligonucleotide primers (Jehle et al., 2006). DNA was extracted from purified OBs by releasing virions from 100 µl OB suspension (10^9 OBs/ml) by treatment with 100 µl 0.5 M sodium carbonate, 50 µl 10% (w/v) sodium dodecyl sulfate in a final volume of 500 µl and incubating for 10 min at 60° C. Undissolved OBs and other debris were removed by low-speed centrifugation (3,800 x *g*, 5 min). The supernatant containing the virions was treated with 25 µl proteinase K (20 mg/ml) for 1 hour at 50°C. Viral DNA was extracted with saturated phenol-chloroform, subjected to alcohol precipitation and resuspended in 0.1xTE buffer (Tris-EDTA, pH 8). DNA concentration was estimated at A_{260}. PCR reactions were performed followed standard procedures using the degenerate oligonucleotides for *polyhedrin*, *lef-8* and *lef-9* genes (Jehle et al., 2006) and a High Fidelity Taq Polymerase (Prime Star HS DNA polymerase, Takara, Japan). PCR amplifications were purified using a QIAquick gel extraction kit (QIAGEN, JHilden, Germany) and purified products were cloned into pGEM-T Easy vector (Promega, Fitchburg, Wisconsin). Nucleotide sequences were determined in an ABI PRISM 377 automated DNA sequencer (Sistemas Genómicos S.A., Valencia, Spain), employing standard M13 and M13 reverse primers. Finally, a blast search was performed using the NBCI database.

2.3. Susceptibility of C. chalcites instars to ChchSNPV-TF1 OBs

Bioassays were carried out on L_2, L_3, L_4, L_5 and L_6 instars of *C. chalcites* to determine instar-specific responses to ChchSNPV-TF1 OBs. The mean lethal concentration (LC_{50}), mean time to death (MTD) and OB production (OBs/larva) were determined following *per os* inoculation, carried out using the droplet-feeding method (Hughes and Wood, 1981).

Pre-molt *C. chalcites* larvae were starved for 8 to 12 h at 25±1°C, visually checked to have molted to the correct instars, and then allowed to drink from an aqueous suspension containing 100 mg/ml sucrose, 0.01 mg/ml Fluorella blue and

OBs at one of five different concentrations. These were 160, 800, $4x10^3$, $2x10^4$, and $1x10^5$ OBs/ml for L_2; 320, $1.6x10^3$, $8x10^3$, $4x10^4$ and $2x10^5$ OBs/ml for L_3; $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$ and $2x10^6$ OBs/ml for L_4, $3.2x10^4$, $1.6x10^5$, $8x10^5$, $4x10^6$ and $2x10^7$ OBs/ml for L_5; $3.2x10^5$, $1.6x10^6$, $8x10^6$, $4x10^7$ and $2x10^8$ OBs/ml for L_6. For all instars OB concentrations were obtained using 5-fold dilution series from the highest to the lowest concentrations. These concentration ranges were previously determined to kill between 95 and 5% of the experimental insects in each instar. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 25-well tissue culture plate with a semisynthetic diet plug. Bioassays with 25 larvae per virus concentration and 25 larvae as negative controls were performed three times. Larvae were reared at 25±1°C, and larval mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality was subjected to logit analysis using the POLO-PC program (LeOra Software, 1987). Relative potencies were calculated as the ratio of effective concentrations relative to L_2 instars (Robertson et al., 2007).

Time mortality results were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling program GLIM 4 (Crawley, 1993). OB concentrations used for the time mortality analysis were those that resulted in ~90% larval mortality namely: $5.13x10^4$ OBs/ml, $2.36x10^5$ OBs/ml, $5.56x10^6$ OBs/ml, $5.00x10^7$ OBs/ml and $9.02x10^8$ OBs/ml for L_2, L_3, L_4, L_5 and L_6, respectively. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times. The time mortality distribution among the different instars was analyzed graphically. Larval mortality was recorded at 8 h intervals until the insects had either died or pupated. Only individuals that died from polyhedrosis disease, confirmed by the microscopic observation of OBs, were included in the analyses.

OB production was determined in L_4, L_5 and L_6. Larvae were inoculated with the OB concentration that resulted in ~90% larval mortality, as described in the time mortality study. Groups of 25 larvae were inoculated for each treatment and the whole study was performed three times. All the larvae that died of virus disease (minimum 50 larvae per virus treatment) were collected and stored at -20°C until used for OB counting. For this, each larva was thawed, homogenized in 1 mL distilled water, and the number of OBs per larva was determined by counting in

triplicate. The average values of the OB counts from each replicate were analyzed by ANOVA using the SPSS v12 program.

2.4. Selection of optical brighteners

The degree of enhancement of OB activity by optical brighteners depends on the host-pathogen system, the chemical composition of the optical brightener and the instar and concentration used (Martínez et al., 2003; Shapiro and Shepard, 2008). To select the most effective optical brightener, preliminary tests were performed on L_2 and L_4 *C. chalcites* using a total of eight optical brighteners from three different chemical groups (Table 1). All compounds were dissolved in double-distilled water at a concentration of 10 mg/ml for powder brighteners (Tinopal UNPA-GX and Tinopal UNPA-GX free acid), or 10 µl/ml for liquid brighteners (Blankophor CLE, Leucophor AP, Leucophor SAC, Leucophor UO, Blankophor ER, Hostalux SN).

Table1. Chemical composition of eight optical brightener from three different chemical groups.

Chemical group Product	Chemical composition (% active component)	Supplier
Stilbene acid derivatives:		
Blankophor CLE	$C_{30}H_{20}N_6Na_2O_6S_2$ (91%)	Clariant, Barcelona, Spain
Leucophor AP	$C_{40}H_{42}N_{12}O_{10}S_{2.2}Na$ (90%)	Clariant, Barcelona, Spain
Leucophor SAC	$C_{48}H_{42}O_{24}S_6$ (90%)	Clariant, Barcelona, Spain
Leucophor UO	$C_{48}H_{42}O_{24}S_4$ (90%)	Clariant, Barcelona, Spain
Tinopal UNPA-GX	$C_{40}H_{44}N_{12}O_{10}S_2$ (90%)	Sigma Chemical CO, St. Louis, MO, USA
Tinopal UNPA-GX free acid	$C_{40}H_{44}N_{12}O_{10}S_2$ (90%)	Sigma Chemical CO, St. Louis, MO, USA
Styryl-benzenic derivative:		
Blankophor ER	$C_{24}H_{16}N_2$ (91%)	Clariant, Barcelona, Spain
Pyrazoline derivative:		
Hostalux SN	$C_{21}H_{26}ClN_3O_3S$ (90%)	Clariant, Barcelona, Spain

A single OB concentration that corresponded to the LC_{50} for each instar: $1.45x10^3$ and $1.95x10^5$ OBs/ml for L_2 and L_4, respectively, was used to inoculate larvae by the droplet feeding method. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times. Larvae were .reared .at 25±1°C, .and .larval .mortality .was .recorded

every .12 .h .until .theinsects had either died or pupated. The results were analyzed by fitting generalized linear models with a binomial error structure specified in GLIM 4 (Crawley, 1993).

2.5. Effects of Tinopal UNPA-GX and Leucophor UO on the insecticidal properties of ChchSNPV-TF1 OBs

Based on the results of preliminary tests, Leucophor UO and Tinopal UNPA-GX were selected to determine their effects on the insecticidal activity of ChchSNPV-TF1 OBs against the different instars of *C. chalcites*. Two different concentrations were tested; 1 and 10 mg/ml for Tinopal UNPA-GX or 1 and 10 µl/ml for Leucophor UO. Bioassays were performed on L_2, L_3, L_4, L_5 and L_6 using the droplet-feeding method. Batches of 25 larvae of each instar were starved for 8 to 12 h at 25±1°C and then allowed to drink from an aqueous suspension containing 100 mg/ml sucrose, 0.01 mg/ml Fluorella blue, optical brighteners at two different concentrations and OBs. An identical number of larvae were fed with identical solutions without OBs, as controls. For viral treatments alone, the concentrations for each instar were those used in susceptibility assays, whereas for the OB suspensions that included optical brighteners, the concentration ranges used were based on the following 5-fold dilution series: 16, 80, 400, $2x10^3$, and $1x10^4$ OBs/ml for L_2; 32, 160, 800, $4x10^3$, and $2x10^4$ OBs/ml for L_3; 320, $1.6x10^3$, $8x10^3$, $4x10^4$, and $2x10^5$ OBs/ml for L_4; $3.2x10^3$, $1.6x10^4$, $8x10^4$, $4x10^5$, and $2x10^6$ OBs/ml for both L_5 and L_6. Each bioassay was performed three times. Larvae were reared at 25±1°C and larval mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality was subjected to logit analysis using the POLO-PC program (LeOra Software, 1987).

Time mortality, data subjected to Weibull survival analysis, was only performed for viral treatments including OBs and 10 mg/ml Tinopal UNPA-GX or 10 µl/ml Leucophor UO. The OB concentrations used for the time mortality analysis were those that resulted in ~90% larval mortality, that were the same concentrations described in the susceptibility assay for OB alone treatments. For OB suspensions containing 10 mg/ml Tinopal UNPA-GX the OB concentrations used were $7.50x10^3$, $1.24x10^4$, $9.58x10^4$, $5.11x10^5$ and $1.54x10^6$ OBs/ml, for L_2, L_3, L_4, L_5 and L_6, respectively. In mixtures with 10 µl/ml Leucophor UO the concentrations that

produced ~90% mortality were $3.32x10^4$, $1.07x10^5$, $5.67x10^5$, $9.61x10^5$ and $7.66x10^6$ OBs/ml, for L_2, L_3, L_4, L_5 and L_6, respectively. Larval mortality was recorded every 8 h and only individuals that died from polyhedrosis disease, confirmed by the microscopic observation of OBs, were included in the analyses. Bioassays with 25 larvae per treatment and 25 larvae as negative controls were performed three times.

Finally, OB production in the three treatments; OBs alone, OBs in mixtures with 10 mg/ml Tinopal UNPA-GX and OBs in mixtures with 10 µl/ml Leucophor UO, was determined in L_4, L_5 and L_6 that died in the previous speed of kill assay. All the larvae that died of virus disease (minimum 20 larvae per virus treatment) were collected and stored at -20ºC until used for OB counting. Each larva was thawed homogenized in 1mL distilled water and the number of OBs $larvae^{-1}$ was determined by counting in triplicate. The experiment was performed three times. The average OB counts from each replicate were normalized by logarithmic transformation prior to ANOVA using the SPSS v12 program.

3. RESULTS

3.1. The virus used in this study belongs to ChchNPV species

Sequence analysis revealed that the ChchSNPV-TF1 isolate from a *C. chalcites* larva in Tenerife is a variant of *Chrysodeixis chalcites single nucleopolyhedrovirus* (ChchSNPV). The amplified sequences had the highest identity with the ChchSNPV isolate from The Netherlands (Genbank accession number AY864330.1) (Fig. 1A). The amplified *polyhedrin* gene sequence was 541 bp long with 99% identity (533/541 nt identical) to the Dutch ChchNPV isolate (Fig. 1S A) and 98% identity with *Trichoplusia ni* NPV (range 532/541). Amplification of the *lef-8* gene resulted in a product of 714 bp, with >99% sequence identity with ChchNPV (722/725 nt identical) (Fig. 1B) and 87% with TnSNPV (628/725 nt identical). Finally, the PCR fragment for *lef-9* was 293 bp long with >99% sequence identity with ChchSNPV (295/296 nt identical) (Fig. 1C) and 92% with TnSNPV (272/296 nt identical). These results confirmed that the TF1 isolate from the Canary Islands was indeed a variant of ChchSNPV.

A)

Chrysodeixis chalcites nucleopolyhedrovirus, complete genome

Sequence ID: gb|AY864330.1| **Length:** 149622 **Number of Matches:** 1

Range 1: 168 to 708 GenBank Graphics Next Match Previou

Score	Expect	Identities	Gaps	Strand
955 bits(517)	0.0	533/541(99%)	0/541(0%)	Plus/Plus

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Query  1    AGCTGAAGATCCTTTCCTGGGACCCGGTAAGAACCAAAAACTCACTTTGTTTAAAGAGAT  60
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Sbjct  168  AGCTGAAGATCCTTTCCTGGGACCCGGTAAGAACCAAAAACTCACTTTGTTTAAAGAGAT  227

Query  61   CCGTAATGTAAAGCCCGATACCATGAAGCTTGTCGTTAACTGGAGCGGCAAAGAGTTTCT  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  228  CCGTAATGTAAAGCCCGATACCATGAAGCTTGTCGTTAACTGGAGCGGCAAAGAGTTTCT  287

Query  121  CAGGGAAACTTGGACCCGCTTCATGGAGGACAGCTTCCCCATCGTTAACGACCAAGAAAT  180
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Sbjct  288  CAGGGAAACTTGGACCCGCTTCATGGAGGACAGCTTCCCCATCGTTAACGACCAAGAAAT  347

Query  181  CATGGACGTTTTCCTAGTAGTTAACATGCGCCCGACAAGACCCAATCGTTGCTTCAAATT  240
            ||||||||||||||||||||||||||||||||  ||  |||||  ||   | ||||||||||
Sbjct  348  CATGGACGTTTTCCTAGTAGTTAACATGCGCCCTACTAGACCTAACAGATGCTTCAAATT  407

Query  241  CTTAGCCCAACACGCTTTACGTTGCGACCCCGATTATGTTCCTCACGAGGTGATTAGAAT  300
            | | ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  408  CCTTGCCCAACACGCTTTACGTTGCGACCCCGATTATGTTCCTCACGAGGTGATTAGAAT  467

Query  301  CGTAGAGCCGTCTTGGGTAGGCAGCAACAACGAATACAGAATTAGTCTGGCCAAGAAAGG  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  468  CGTAGAGCCGTCTTGGGTAGGCAGCAACAACGAATACAGAATTAGTCTGGCCAAGAAAGG  527

Query  361  CGGTGGCTGCCCAATCATGAACCTTCACTCTGAGTACACCAACTCGTTTGAAGAGTTTAT  420
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Sbjct  528  CGGTGGCTGCCCAATCATGAACCTTCACTCTGAGTACACCAACTCGTTTGAAGAGTTTAT  587

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Sbjct  648  CGAGGAAGAGGAGATTCTTCTTGAAGTGTCTTTAGTCTTTAAAATTAAGGAATTCGCTCC  707

Query  541  C  541
            |
Sbjct  708  C  708
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B)

Chrysodeixis chalcites nucleopolyhedrovirus, complete genome

Sequence ID: gb|AY864330.1| Length: 149622 Number of Matches: 1

Range 1: 35043 to 35763 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1317 bits(713)	0.0	721/724(99%)	3/724(0%)	Plus/Plus

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Sbjct  35283  CAAAGTAATAATATTGCACCAGCTTTGACATGAGGCTAGTGACGTCATCGTTTTCCTCTA  35342

Query  301    TCTTACCGATTTTTGGGTATTTCTTCAAGATCGAATAAGAATTATGATATTCGAAAGGCG  360
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35343  TCTTACCGATTTTTGGGTATTTCTTCAAGATCGAATAAGAATTATGATATTCGAAAGGCG  35402

Query  361    TCAGCAGAGTCGTAAttttttGCCCGCGATACTAATCACTCGCTTGATACACACCATGC  420
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  35403  TCAGCAGAGTCGTAATTTTTTGCCCGCGATACTAATCACTCGCTTGATACACACCATGC  35462

Query  421    CTTCGTGGTGATTTACAAAGAGAATGCGGTTGGCCAGCAGCTTGAGTTCAATGGGACTGT  480
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Sbjct  35463  CTTCGTGGTGATTTACAAAGAGAATGCGGTTGG-C--CAGCTTGAGTTCAATGGGACTGT  35519

Query  481    ATTGCCGTTTGAGTTGATAAAAAATCAATTGTAAATCGTCAAAACGGCAGGCGTATATGG  540
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C)

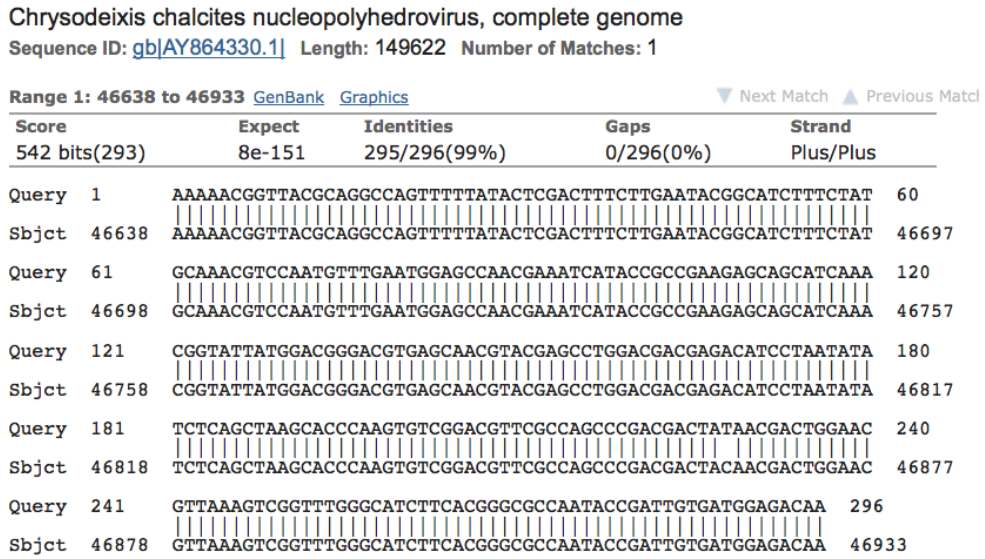


Figure. 1. Sequence alignment of the PCR products of the A) *polyhedrin*, B) *lef-8* and C) *lef-9* genes obtained from ChchSNPV-TF1 DNA using the degenerate oligonucleotides with those of ChchNPV from Netherlands.

3.2. Susceptibility of *C. chalcites* instars to ChchSNPV-TF1 OBs

Susceptibility to infection decreased with increasing larval instar. LC_{50} values were $1.45x10^3$ OBs/ml for L_2, $1.48x10^4$ OBs/ml for L_3, $1.95x10^5$ OBs/ml for L_4, $1.80x10^6$ OBs/ml for L_5 and $2.20x10^7$ OBs/ml for L_6 (Table 2). Speed of kill decreased with increasing larval instar (Weibull hazard function: α =7.0698). Mean time to death (MTD) values for L_2, L_3, L_4, L_5 and L_6 instars were 126 h, 137 h, 142 h, 150 h and 168 h, respectively (Table 2). However, MTD values for L_3, L_4 and L_5 did not differ significantly from one another. Mean OB production values also increased significantly with larval instar ($F_{(2, 6)}$=226.553, P<0.001), with an average of $4.83x10^9$ OBs/larva in L_4, $2.27x10^{10}$ OBs/larva in L_5 and $1.10x10^{11}$ OBs/larva in L_6 (Table 2).

Table 2. LC_{50} values, relative potencies, mean time to death (MTD) values and median OB yield values of ChchSNPV-TF1 in different instars of *C. chalcites*.

Instar	LC_{50}	Relative	Fid. lim. (95%)		MTD	Fid. lim (95%)		Mean OB yield ($x10^9$	Fid. lim. (95%)	
	(OBs/ml)	Potency	Low	High	(h)	Low	High	OBs/larva)	Low	High
L_2	$1.45x10^3$	1	-	-	126a	123	132	-	-	-
L_3	$1.48x10^4$	0.098	0.06	0.18	137ab	129	142	-	-	-
L_4	$1.95x10^5$	0.007	0.004	0.014	142b	134	147	4.83a	3.60	6.05
L_5	$1.80x10^6$	0.001	0.0009	0.002	150bc	140	154	22.74b	10.54	34.93
L_6	$2.20x10^7$	0.0001	0.00009	0.0002	168c	153	170	110.38c	60.22	160.54

Logit regressions were fitted in POLO Plus (LeOra Software, 1987). Relative potencies were calculated as the ratio of effective concentrations relative to L_2 instars. A test for non-parallelism was not significant for all larval stages (χ^2=9.12; d.f=4; *P*=0.058). Mean time to death (MTD) values were estimated by Weibull survival analysis (α=7.0698) (LeOra Software, 1987). The OB productivity was analyzed by ANOVA using the SPSS v12 program, ($F_{(2, 6)}$=226.5, *P*<0.001).

3.3. Tinopal UNPA-GX and Leucophor UO were selected as the most effective enhancers of OB pathogenicity

Inoculation of L_2 and L_4 *C. chalcites* with OBs alone resulted in 38 and 39% mortality, respectively (Table 3). Among the different optical brighteners tested, only three optical brighteners resulted in a significant increase in the insecticidal activity of ChchSNPV-TF1 OBs against L_2 (χ^2=9.266; d.f.=8; *P*<0.001). Inoculation of OBs in mixtures with Tinopal UNPA-GX, Tinopal UNPA-GX (free acid), and Leucophor UO resulted in 64, 62 and 59% mortality, respectively (Table 3). In L_4, all optical brighteners significantly enhanced OB activity with mortalities of 51 to 97%, with the exception of Blankophor ER. The most active optical brighteners in L_4 were the same as those that enhanced OB activity in L_2: Tinopal UNPA-GX (97% mortality), Tinopal UNPA-GX (free acid) (96% mortality) and Leucophor UO (89% mortality). As Tinopal UNPA-GX and Tinopal UNPA-GX free acid were different forms of the same compound and produced the same enhancement, Tinopal UNPA-GX was selected with Leucophor UO (a cheaper compound) to study the potentiation effect on *C. chalcites* at two different concentrations; 1 and 10 mg/ml for Tinopal UNPA-GX or 1 and 10 µl/ml for Leucophor UO.

Table 3. Mortality percentage in L_2 and L_4 *C. chalcites* instars following treatment with ChchSNPV-TF1 OBs alone or in mixtures with 10 mg/ml of powered optical brighteners or 10 µl/ml of liquid optical brighteners.

Treatment	L_2 Mortality (%)	*P*	L_4 Mortality (%)	*P*
ChchSNPV-TF1 OBs alone	38	-	39	-
ChchSNPV-TF1+Blankophor ER	38	>0.05	51	>0.05
ChchSNPV-TF1+Tinopal UNPA-GX	64	<0.05	97	<0.001
ChchSNPV-TF1+Tinopal UNPA-GX (free acid)	62	<0.01	96	<0.001
ChchSNPV-TF1+Leucophor SAC	36	>0.05	79	<0.01
ChchSNPV-TF1+Leucophor UO	59	<0.05	89	<0.001
ChchSNPV-TF1 Hostalux PN	43	>0.05	83	<0.001
ChchSNPV-TF1+Leucophor AP	39	>0.05	79	<0.001
ChchSNPV-TF1+Blankophor CLE	36	>0.05	67	<0.05*

OB concentrations used for L_2 and L_4 instars were $1.45x10^3$ OBs/ml and $1.95x10^5$ OBs/ml, respectively. The percentage of mortality of OBs alone was compared with that observed in mixtures with optical brightener in each instar. The results were analyzed by generalized linear model with binomial error specified. No evidence of overdispersion was observed in the results (GLIM).

3.4. Tinopal UNPA-GX and Leucophor UO in mixtures with ChchSNPV-TF1 OBs increased OB pathogenicity and speed of kill but reduced OB production

The addition of 1 mg/ml Tinopal UNPA-GX or 1 µl/ml Leucophor UO to OB suspensions enhanced OB pathogenicity in all instars except L_2. In contrast, mixtures of OBs and optical brighteners at 10 mg/ml for Tinopal UNPA-GX or 10 µl/ml for Leucophor UO enhanced OB pathogenicity in all instars tested. Mixtures of OBs and 10 mg/ml Tinopal UNPA-GX enhanced OB pathogenicity from 4.43 to 397-fold for L_2 to L_6 instars, respectively, whereas 10 µl/ml Leucophor UO had a lower potentiation effect, from 1.46 to 143-fold for L_2 to L_6 instars, respectively. The potentiation effect was of a greater magnitude in the three later instars, L_4, L_5 and L_6 (Table 4).

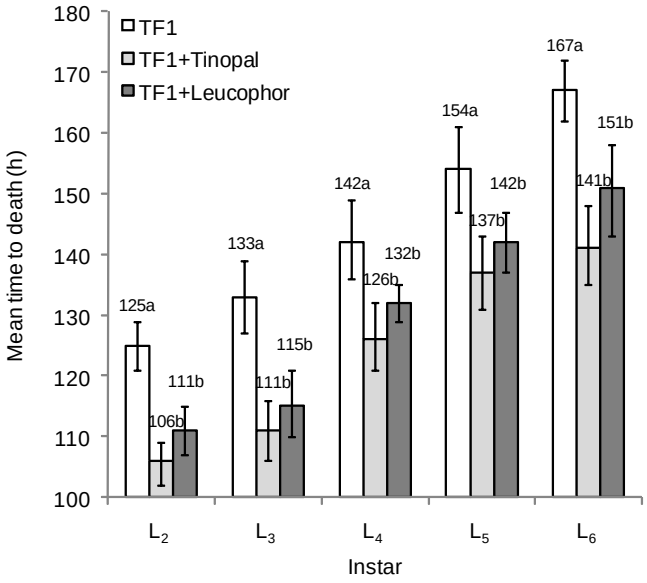
Table 4. LC_{50} values, relative potencies and slope of different treatments; virus alone (TF1) and virus with Tinopal UNPA-GX sodium salt (TF1+Tinopal) and Leucophor UO (TF1+UO) among the different *C. chalcites* instars.

Instar	Treatment	LC_{50} (OBs/ ml)	Relative potency	Fiducial limits (95%)		Slope	Stand. error
				Low	High		
L_2	TF1 alone	$1.45x10^3$	1.00	-	-	0.83	0.09
	TF1+1 mg/ml Tinopal	$2.71x10^3$	0.54	0.26	1.12	0.74	0.09
	TF1+1 µl/ml UO	$4.26x10^3$	0.34	0.16	0.72	0.79	0.09
	TF1+10 mg/ml Tinopal	$3.27x10^2$	4.43	2.36	8.34	0.94	0.09
	TF1+10 µl/ml UO	$9.95x10^2$	1.46	0.76	2.80	0.84	0.09
L_3	TF1 alone	$1.48x10^4$	1.00	-	-	1.07	0.09
	TF1+1 mg/ml Tinopal	$2.53x10^3$	5.85	3.30	10.38	0.80	0.09
	TF1+1 µl/ml UO	$6.82x10^3$	2.17	1.16	4.07	.83	0.09
	TF1+10 mg/ml Tinopal	$6.39\ x10^2$	23.19	13.75	39.12	0.99	0.10
	TF1+10 µl/ml UO	$2.48x10^3$	5.96	3.35	10.66	0.78	0.08
L_4	TF1 alone	$1.95x10^5$	1.00	-	-	0.88	0.09
	TF1+1 mg/ml Tinopal	$2.65x10^4$	7.35	9.66	14.96	0.56	0.08
	TF1+1 µl/ml UO	$5.04x10^4$	3.87	4.27	9.89	0.60	0.08
	TF1+10 mg/ml Tinopal	$2.98x10^3$	65.43	52.58	110.27	0.85	0.09
	TF1+10 µl/ml UO	$9.72x10^3$	20.08	10.71	45.40	0.73	0.08
L_5	TF1 alone	$1.80x10^6$	1.00	-	-	0.89	0.09
	TF1+1 mg/ml Tinopal	$1.29x10^5$	13.98	8.12	24.08	0.94	0.09
	TF1+1 µl/ml UO	$2.70x10^5$	6.67	3.64	9.22	0.79	0.08
	TF1+10 mg/ml Tinopal	$2.55x10^4$	70.41	60.75	121.66	0.98	0.09
	TF1+10 µl/ml UO	$4.47x10^4$	40.21	26.39	50.12	0.96	0.09
L_6	TF1 alone	$2.20x10^7$	1.00	-	-	0.80	0.08
	TF1+1 mg/ml Tinopal	$2.38x10^5$	89.16	71.06	93.59	0.63	0.08
	TF1+1 µl/ml UO	$3.61x10^5$	59.84	26.87	68.88	0.60	0.08
	TF1+10 mg/ml Tinopal	$5.36x10^4$	397.13	283.32	750.54	0.70	0.08
	TF1+10 µl/ml UO	$1.49x10^5$	142.56	111.71	210.13	0.62	0.08

Logit regressions were fitted in POLO Plus (LeOra Software, 1987). Relative potencies were calculated as the ratio of effective concentrations relative to ChchSNPV-TF1 OBs alone.

MTD values were reduced in mixtures of OB and either of the optical brighteners. Specifically, OBs in mixtures with 10 mg/ml Tinopal UNPA-GX resulted in a reduction of 19 to 26 h for L_2 to L_6, respectively. Similarly OBs in mixtures with 10 µl/ml Leucophor UO resulted in a reduction of 14 to 16 h for L_2 to L_6, respectively

A)



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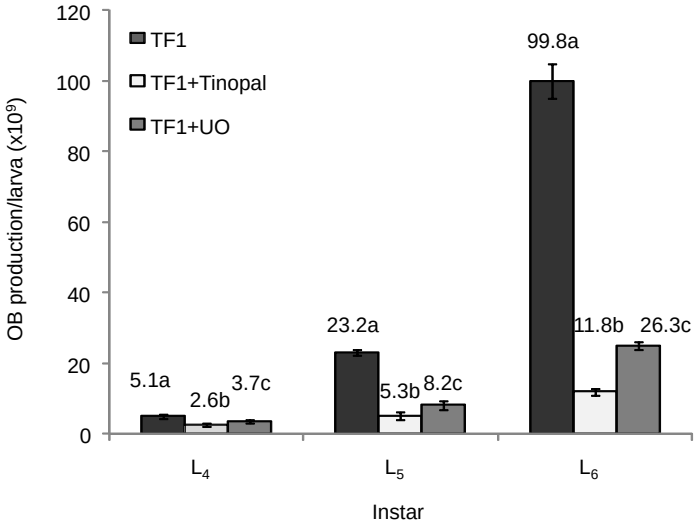

Figure 1. (A) Mean time to death of ChchSNPV-TF1 OBs alone (TF1), or in mixtures with 10 mg/ml Tinopal UNPA-GX (TF1+Tinopal) or 10 µ/ml Leucophor UO (Tf1+UO) across C. chalcites instars. Values were estimated by Weibull analysis and are indicated above the bars. Values followed by identical letters did not differ significantly for treatment comparisons within each instar (t-test, *P*> 0.005). (B) Mean OB yield obtained after infection of C. chalcites larvae inoculated with ChchSNPV-TF1 OBs alone (TF1), or in mixtures with 10 mg/ml Tinopal UNPA-GX (TF1+Tinopal) or 10 µ/ml Leucophor UO (Tf1+UO) in all *C. chalcites* instars. Values above bars indicate menas. Values followed by identical letters did not differ significantly (ANOVA, Tuckey *P*> 0.005).

compared to that of the OBs alone (Weibull hazard function α=6.4883) (Fig. 1 A).

The addition of 10 mg/ml Tinopal UNPA-GX or 10 µl/ml Leucophor UO significantly reduced the median number of OBs per larva compared with OBs alone ($F_{(2,6)}$=134.966, *P*<0.001; $F_{(2,6)}$=221.249, *P*<0.001; and $F_{(2,6)}$=106.897, *P*<0.001 for L_4, L_5 and L_6, respectively). Median OB yield was reduced by 1.95 to 8.45-fold for L_4 and L_6, respectively, with the addition of Tinopal UNPA-GX, whereas Leucophor UO reduced the total OB production 1.38 to 3.79-fold for L_4 to L_6, respectively, compared with OBs alone treatments. Tinopal UNPA-GX, which reduced MTD values more drastically, resulted in lower OB yields than Leucophor UO (ANOVA, Tukey *P*<0.001) (Fig. 1 B). The decrease in OB production was correlated with the increased speed of kill of the virus when inoculated in mixtures with optical brighteners.

4. DISCUSSION

Strategies for the biological control of *C. chalcites* in banana crops have been poorly explored to date. The control of *C. chalcites* currently presents a number of difficulties due to the low number of plant protection products authorized for this crop, the difficulty in the correct application of these compounds and an absence of commercial biological control products (Del Pino et al., 2011). In the present study, larval susceptibility to ChchSNPV-TF1 OBs was determined across different instars and the effect of OB and optical brightener mixtures on the insecticide properties of this virus were determined.

Larvae were markedly less susceptible to infection with increasing instars as has been reported for other species of Lepidoptera (Asser-Kaisser et al., 2011; Kouassi et al., 2009). This stage-related resistance to infection increases steadily with larval body weight in some species (Myers et al., 2011). In many cases, the physiological basis for this process remains uncertain. Larvae are able to rid themselves of primary infection by sloughing off infected gut cells during the molt (Rohrmann, 2008; Washburn et al., 1998). The peritrophic membrane (PM) is also a key barrier against infection by baculoviruses (Levy et al., 2007; Plymale et al., 2008; Wang and Granados, 2000). Larvae can resist infection by increasing the thickness of the PM, indicating that the PM is not only a passive physical barrier but can also be remodeled in response to gut infection (Levy et al., 2007; Zhu et al.,

2007). Susceptibility to viral infection decreased through successive instars as the PM became progressively less permeable (Lehane et al., 2007).

In field conditions, effective crop protection is favored when all pest instars can be controlled following a single application of a viral insecticide, since in natural populations, overlap of larval generations may be common. Due to the lower susceptibility of late instars, that are the principal cause of feeding damage in crops, the search for enhancer substances that improve OB insecticidal activity has attracted interest (Shapiro, 2000). Certain substances, including optical brighteners, granulovirus enhancins, plant extracts and chitin-synthesis inhibitors can be effective at increasing the insecticidal efficacy of OBs (Guo et al., 2007; Shapiro et al., 2010; Toprak et al., 2012; Wang and Granados, 2000). Optical brighteners were first identified as viral protectants against inactivation by UV radiation (Dougherty et al., 1996; Shapiro, 1992). Apart from their UV-protective activity, optical brighteners enhance OB potency and also allow the virus to replicate normally in semi-permissive species, and in resistant insect biotypes (Adams et al., 1994; Greene et al., 1976; Morales et al., 2001). The efficacy of optical brighteners appears to involve a combination of different effects in the intestinal tract. Brighteners inhibit the sloughing of infected midgut cells (Washburn et al., 1998) and inhibit the apoptotic response of midgut cells (Dougherty et al., 2006), both of which increase the probability of establishment of primary infection in the midgut. Those compounds also inhibit chitin synthesis and dramatically increase PM porosity which facilitates movement of occlusion derived virions from the gut lumen to epithelial cells (Wang and Granados, 2000; Zhu et al., 2007).

Laboratory bioassays in L_2-L_6 confirmed previous observations that optical brighteners can enhance the potency of OBs and reduced larval survival time and OB production. Tinopal UNPA-GX and Leucophor UO were selected for detailed testing because both brighteners were effective in increasing OB potency in preliminary bioassays as observed in previous studies using other alphabaculoviruses (Lasa et al., 2007; Okuno et al., 2003; Shapiro and Shepard, 2008). Leucophor UO is chemically related to Tinopal UNPA-GX and was included for its lower cost. The degree of enhancement of OB potency observed in the present study in L_6 instars treated with Tinopal UNPA-GX was 397-fold, which is of similar magnitude to the 583-fold increase in potency reported in *S. exigua* L_5

treated with mixtures of *S. exigua* nucleopolyhedrovirus OBs and Tinopal UNPA-GX (Murillo et al., 2003), or the 360-fold increase in potency in *Lymantria dispar* L_2 treated with mixtures of nucleopolyhedrovirus (LdMNPV) and Tinopal LPW.[15] Wang and Granados (Wang and Granados, 2000) observed that treatment of *Trichoplusia ni* L_5 inoculated with 10 mg/ml Calcofluor resulted in complete disruption of PM formation in 2 h suggesting that optical brighteners require only a short time in the gut of the host insect to produce enhancing activity. However, this effect is quickly reversed, after 2 h of feeding on fresh diet a fully developed peritrophic membrane could be observed. However, following application of a brightener in the field, larvae will be feeding continuously on contaminated foliage, so the addition of optical brighteners to baculovirus formulations would likely prove very effective in potentiation of OB activity under field conditions. Formulation of OBs with brighteners could be particularly valuable in situations where it is necessary to control different larval stages simultaneously following application of a virus-based insecticide.

Increased speed of kill among the different larval instars exposed to an OB suspension in mixtures with optical brighteners has been reported in studies on homologous and heterologous alphabaculoviruses (Morales et al., 1994; Shapiro and Shepard, 2008; Washburn et al., 1998), although other studies have reported no significant differences on speed of kill (Boughton et al., 2001; Martinez et al., 2003; Murillo et al., 2003), probably as a result of differences in the host-pathogen system under study and the chemical composition of the optical brightener (Okuno et al., 2003).

The reduction in OB yield observed in larvae inoculated with mixtures of ChchSNPV-TF1 OBs and optical brighteners is consistent with previous studies on SeMNPV (Murillo et al., 2003) and reflects the trade-off between MTD and OB yield (Cory and Myers, 2004). However this effect is likely to be little consequence in the performance of a virus pesticide for which a high prevalence of lethal infection and rapid death resulting in improved crop protection are the main objectives.

Viral formulations with optical brighteners appear to offer a valuable means of improving the efficacy of ChchSNPV-TF1 as a potential biological insecticide. However, field trials will be required to support these laboratory results and to justify the additional cost of incorporating an optical brightener to the OB formulation

(Martínez et al., 2000). However, the possible negative effects of optical brighteners on pollinators or crop-growth in the field should also be considered (Goulson et al., 2003, 2000) before employing these substances in virus insecticide formulations on a large scale.

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CHAPTER 7

Efficacy of an alphabaculovirus for the control of *Chrysodeixis chalcites* (Lepidoptera: Noctuidae) on tomato and banana crops

ABSTRACT

A *Chrysodeixis chalcites* single nucleopolyhedrovirus (genus *Alphabaculovirus*, family Baculoviridae) isolate from the Canary Islands (ChchSNPV-TF1) is highly pathogenic against larvae of this pest in laboratory assays. To determine the efficiency of this virus as a biological insecticide, trials were conducted on tomato and banana plants, in plant growth-chambers and greenhouses, respectively. Treatments of $2x10^6$, $1x10^7$ and $5x10^7$ viral occlusion bodies (OBs)/l applied to tomato plants in a growth-chamber all resulted in ~80-90% lethal infection in larvae collected at 2 days post-application and increased to ~100% lethal infection in larvae collected at 7 days post-application. In a subsequent greenhouse experiment, the efficacy of applications of $1x10^8$ OBs/l and $1x10^9$ OBs/l to young banana plants in greenhouses in Tenerife (Canary Islands, Spain) were compared to that of product label recommended rates of indoxacarb and *Bacillus thuringiensis* (Bt). Average mortality of larvae collected from plants at different intervals post-application varied from 54 to 96% in virus treatments, whereas indoxacarb and Bt treatments resulted in 22-32% mortality, depending on time point. All treatments significantly reduced defoliation by this pest, compared to control plants. Notably, the $1x10^9$ OBs/l treatment was 3 to 4-fold more effective in reducing larval infestations than the chemical or Bt treatments. *C. chalcites* acquired viral infection more rapidly in tomato plants than in banana plants during the first hour, probably due to plant characteristics. However, by 9 hours of exposition, almost 50% mortality is obtained in both crops, suggesting that ChchSNPV-TF1 treatment in tomato crops might be as effective as in banana crops. This information should prove useful in the registration of ChchSNPV-TF1 as the basis for a biological insecticide for use in the Canary Islands.

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1. INTRODUCTION

The golden twin spot tomato looper, *Chrysodeixis chalcites* (Esper) (Lepidoptera; Noctuidae), is regarded as a major pest of greenhouse-grown crops in many countries (Shepard et al., 2009). In Spain, major damage has been reported in banana crops in the Canary Islands (Del Pino et al., 2011), where it is considered an emerging pest, and in horticultural crops in Almería (Amate et al., 1998; Cabello and Belda, 1994). In banana plants, injury mainly occurs on the young unfolding leaves, which are cut or perforated by *C. chalcites* larvae of all instars (Domínguez et al., 2012a). More importantly, late instar larvae often feed on banana fruits, producing skin injuries that result in >30% losses in bananas grown under greenhouse conditions in the Canary Islands (Del Pino et al., 2011; Domínguez et al., 2012a).

Chemical-based control measures usually involve applications of azadirachtin, chlorpyrifos, fenamiphos or indoxacarb, among others, but require multiple applications that increase production costs, hamper the commercialization of products that can occasionally contain pesticide residues and if improperly used, can generate the development of insect resistance (Horowitz et al., 1998; Perera and Molina, 2007). However, the low number of active substances authorized by the European Union for use in banana (Magrama, 2013) limits the possible use of alternative active substances, thus increasing the risks associated with resistance to chemical control measures.

A number of insect baculoviruses have an established record as the basis for highly effective biological insecticides against lepidopteran pests (Caballero et al., 2009; Cherry et al., 2000; Lasa et al., 2007a; Moscardi, 1999;). Previous studies indicated that an indigenous nucleopolyhedrovirus (genus *Alphabaculovirus*, family Baculoviridae) isolate of *C. chalcites*, named ChchSNPV-TF1, from Tenerife, Canary Islands, Spain, was highly pathogenic to *C. chalcites* larvae under laboratory conditions (Bernal et al., 2013a, 2013b). The pathogenicity and speed of kill of ChchSNPV-TF1 was comparable to that of other baculovirus-based insecticides (Caballero et al., 2009; Moscardi, 1999).

The performance of a virus-based insecticide as a pest control agent is likely to be influenced by a number of environmental factors, that difficult control in the field (Cory and Hoover, 2006, Ignoffo, 1992; Lasa et al., 2007b), particularly because

virus occlusion bodies (OBs) are consumed by pest insects over an extended period in the field and, consequently, are exposed to harsh environmental conditions during an equally extended period (Bianchi et al., 2000). The aim of the present study was to evaluate the efficacy of ChchSNPV OBs to control *C. chalcites* infestations on tomato and banana plants in comparison with insecticidal products currently in use in the Canary Islands.

2. MATERIAL AND METHODS

2.1. Insects and viruses

A laboratory colony of *C. chalcites* was started using pupae received from the Instituto Canario de Investigaciones Agrarias (ICIA), Tenerife, Spain, in 2007. This colony was refreshed periodically with pupae from the Canary Islands. Insects were reared at 25ºC, 70±5% humidity, and a photoperiod of 16h:8h (light:dark), on a semisynthetic diet (Greene et al., 1976). Adults were fed 30% w/v diluted honey. The ChchSNPV-TF1 strain was originally isolated from a single infected larva collected during a natural epizootic in a banana crop in Tenerife, Spain.

ChchSNPV-TF1 OBs were produced by infecting sixth instar laboratory-reared larvae with $9.02x10^8$ OBs/ml, suspended in 10% sucrose solution and 0.001% Fluorella blue food dye, using the droplet feeding method (Hughes and Wood, 1981). Larvae that drank inoculum within 10 min were placed in 1.5 l plastic containers with diet. Larvae were checked daily for signs of polyhedrosis disease. Dead and moribund insects were collected and stored at -20ºC. OBs were extracted by thawing and homogenizing the cadavers in water, and purified by filtration and centrifugation. Purified OBs were resuspended in sterile water and their concentration was determined by counting triplicate samples using an improved Neubauer hemocytometer (Hawksley, Lancing, UK) under phase contrast microscopy at x400. Purified OBs were stored at 4ºC for up to 1 month prior to use in laboratory and greenhouse assays. The identity of these OBs was confirmed by restriction endonuclease analysis using *Bgl*II, and their biological activity was checked in second instar *C. chalcites* using the droplet feeding bioassay technique and compared to established OB activity values (Hughes and Wood, 1981). Twenty-five newly molted second instar *C. chalcites* from the laboratory colony were starved for 8 to 12 h at 26°C and then allowed to drink from an aqueous

suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs at the following concentrations: $1x10^5$, $2x10^4$, $4x10^3$, $8x10^2$ and $1.6x10^2$ OBs/ml, calculated in previous studies (Bernal et al., 2013b). A virus-free solution was also fed to a group of insects as a control. Results were subjected to logit analysis using the POLO-PC program (LeOra Software, 1987).

2.2. Growth-chamber trial on tomato plants

A trial was performed using bloom tomato plants (*Solanum lycopersicum* L., Solanaceae) maintained in a walk-in plant growth room (3.38 m W x 4.95 m L x 2.40 m H) illuminated using 8 fluorescent tubes (60 W). OB suspensions ($2x10^6$, $1x10^7$ and $5x10^7$ OBs/l) or water, as control, were applied until the run-off using a compressed-air hand sprayer (Matabi 5, Antzuola, Guipuzcoa, Spain). All treatments included 0.1% (v/v) Agral (Syngenta Agro S.A., Madrid, Spain) as wetter-sticker.

At 1 h post-application, when plants were completely dry, tomato branches with seven leaves were removed, each stem was placed in a 25 ml glass jar containing a hydroponic nutrient solution (Hoagland's solution), and these branches were then placed in 2 l glass containers. Finally, each container/branch was artificially infested with 200 *C. chalcites* second instars. Prior to analysis, 25 larvae from the laboratory colony were individualized as controls to determine whether the laboratory colony harboured an inapparent nucleopolyhedrovirus infection. The conditions of the growth chamber were controlled at 25±1 ºC, 70±5% humidity, and a 16:8 h (light:dark) photoperiod.

Groups of 25 larvae were collected from each container/branch at four different intervals post-application (p.a.): 2 h and 2, 5 and 7 days. Collected larvae were individually placed in 25 ml plastic cups with artificial diet and checked daily until death or pupation. Bioassays were performed on four occasions. Percentage mortality was calculated for each treatment and subject to repeat measures analysis of variance (ANOVA) in SPSS v12 (SPSS Inc., IL), as data were distributed normally. The characteristics of the variance-covariance matrix were examined for this analysis by applying Maunchly's sphericity test (Mitchell, 2003). The significance of treatment effects at each sample time were determined by within-subject comparisons among the estimated means with Tukey test ($P \leq 0.05$).

2.3. Greenhouse assays on banana plants

Experiments on the efficacy of ChchSNPV-TF1 on young banana plants (*Musa acuminata*, var. Dwarf Cavendish) under mesh greenhouses in Güimar, Tenerife, Canary Islands, Spain. The experiments involved five treatments: (i) 0.004% (w/v) indoxacarb (Steward 30% WG, DuPont); (ii) 0.05% *B. thuringiensis var. kurstaki* (Biobit 16% WP, Novo) as a bioinsecticide; (iii) ChchSNPV-TF1 $1x10^8$ OBs/l (iv) ChchSNPV-TF1 $1x10^9$ OBs/l, (v) water control. Indoxacarb and Bt treatments were applied at the product label-recommended rates in banana crops in the Canary Islands (Agrocabildo, 2013). All treatments included 0.1% (v/v) Agral wetter-sticker and were applied using a compressed-air hand sprayer (Solo® 402, Hamilton, New Zealand). Experimental plots comprised four rows with seven plants per row, totaling 28 young banana plants, of which 18 were border plants and 10 were central plants. Plants were present at 1 m intervals with a 1 m space between rows, giving each plot an area of 28 m^2. The first trial was performed using eight replicate plots in October-November 2011 (2011-assay). The second trial was performed using three replicated plots in September 2012 (2012-assay). Both trials involved a fully randomized design. Plants were artificially infested with 80-160 *C. chalcites* eggs placed in groups of ~20-40 on each of the four youngest leaves of each plant. Five days after the infestation, when larvae had reached the second instar, plots were sprayed with a 1 l volume of each treatment, equivalent to 357 l/ha. Applications were made from 7.00–10.00 h.

In the first trial the percentage of virus mortality, was determined by collecting 25 *C. chalcites* larvae from the border plants in each plot at time 0, and at 1, 3, 5 and 7 days p.a.. Larvae were reared individually in the laboratory in 25 ml plastic cups with artificial diet until death or pupation. Numbers of surviving larvae and foliar feeding injury were estimated for the 10 central plants of each plot, by direct counting of the number of larvae and the foliar holes immediately before the application (time 0) and at 7 days p.a.. Percentages of surviving larvae and foliar feeding injury were calculated based on the initial number of larvae or foliar holes counted, representing these initial values 100%. In the second trial, only percentage of larval mortality was evaluated, using the same methods as described for the first trial. Virus-induced mortality percentages for each treatment were subjected to repeat measures analysis of variance (ANOVA) in SPSS v12 (SPSS Inc., IL). The

characteristics of the variance-covariance matrix were examined for this analysis by applying Maunchly's sphericity test (Mitchell, 2003). Within-subject comparisons among the estimated means were determined by Tukey test (SPSS v12, SPSS Inc., IL).

2.4. Rate of acquisition of viral infection

In a similar trial as that used in tomato, the rate of acquisition of a lethal infection was evaluated using bloom tomato branches and banana leaves. Virus concentrations of $2x10^6$ OBs/l or water as control were applied until the run-off using a compressed-air hand sprayer. All treatments included 0.1% (v/v) Agral as wetter-sticker. When plants were completely dry, each stem was placed in a 25 ml glass jar containing Hoagland's solution, and these branches were then placed in 2 l glass containers. Finally, each container was artificially infested with 500 *C. chalcites* second instars. The containers were maintained in a walk-in plant growth room (3.38 m W x 4.95 m L x 2.40 m H) illuminated using 8 fluorescent tubes (60 W). Prior to analysis, 25 larvae from the laboratory colony were individualized as controls to determine whether the laboratory colony harboured an unapparent nucleopolyhedrovirus infection. Groups of 25-30 larvae were randomly collected from treated plants at 5, 10, 15, 20, 40, 60, 80, 100, 120 min. and 4, 6, 9, 12 and 24 h. intervals. Collected larvae were individually placed in 25 ml plastic cups with artificial diet and checked daily until death or pupation. Bioassays were performed on five occasions. Percentage mortality was calculated for each treatment and subject to repeat measures analysis of variance (ANOVA) in SPSS v12 (SPSS Inc., IL), as data were distributed normally. The significance of treatment effects at each sample time was determined by within-subject comparisons among the estimated means with Tukey test ($P \leq 0.05$).

3. RESULTS

3.1. Efficacy of ChchSNPV on tomato plants grown in phytotron

No virus mortality was registered in control larvae reared in the laboratory to determine the prevalence of infection, indicating that the *C. chalcites* population used to infest plants was healthy. The number of *C. chalcites* larvae that acquired a lethal virus infection increased significantly over time ($F_{(3,9)}=54.203$; $P<0.001$). The $2x10^6$ OBs/l concentration resulted in 55% lethal infection in larvae at 2 h p.a.

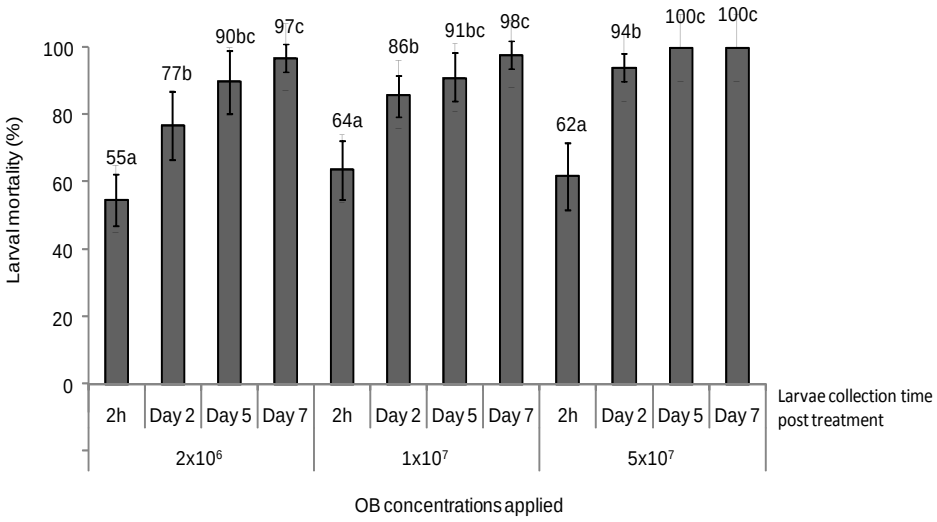

Figure. 1. Percentage of mortality in larvae collected from tomato plants at different times post-application and reared in the laboratory until death or pupation. Experimental treatments involved one of three different OB concentrations. Values above bars represent means and those followed by identical letters did not differ significantly among each larvae collection time (*P*>0.05).

indicating rapid acquisition of infection, and reached 97% mortality in larvae collected at day 7 p. a. (Fig. 1). Similar results were obtained with OB concentrations of $1x10^7$ and $5x10^7$ OBs/l (Fig. 1), showing no interaction between viral concentration and collection time (concentration*time interaction $F_{(6,18)}$=1.581; *P*=0.210).

3.2. Efficacy of ChchSNPV-TF1 in banana plants grown in greenhouses

Lethal polyhedrosis disease was not observed in larvae collected at time point zero in the first or second trial. However, following OB treatment, virus mortality varied from 1 to 3% in control larvae in both trials, depending on the sample time, probably due to cross-contamination from virus treatments.

In the first trial, all treatments resulted in a significant increase in mortality compared to the control treatment ($F_{(4,24)}$=121.845; *P*<0.001). Application of $1x10^8$ OBs/ml ChchSNPV-TF1 resulted in 54% lethal infection (day 1 p.a.) to 81% lethal infection (day 5 p.a.) (Fig. 2A). Whereas application of $1x10^9$ OBs/ml resulted in 86% lethal infection (day 1 p.a.) to 93% lethal infection (day 3 p.a.). In contrast, indoxacarb and Bt treatments resulted in 22-33% mortality and 13-32% mortality,

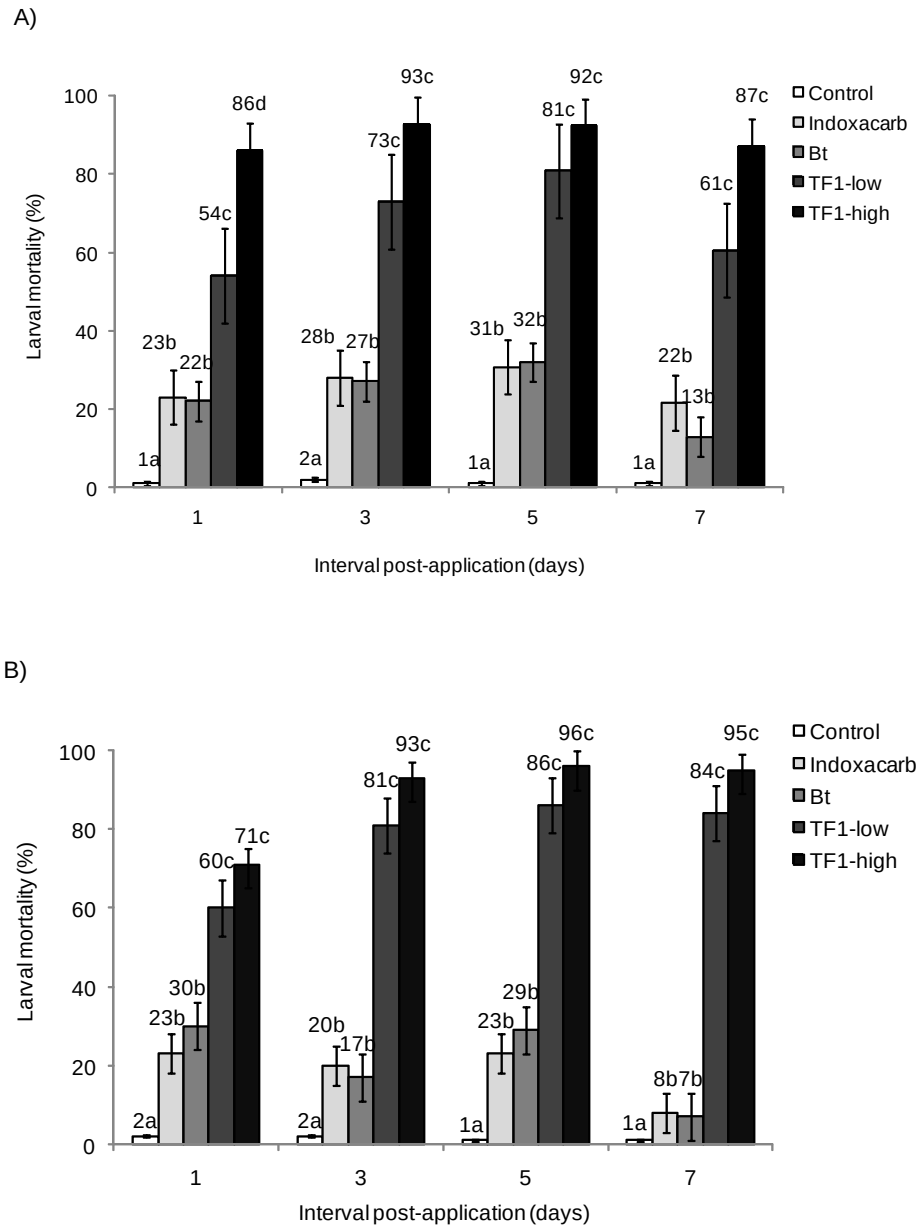

Figure. 2. Percentage mortality in larvae collected from banana plants at four different intervals after treatment and reared in the laboratory until death or pupation. Experimental treatments involved application of water (control), indoxacarb, *Bacillus thuringiensis var. kurstaki* (Bt) and two ChchSNPV concentrations, $1x10^8$ OBs/l (TF1-low) and $1x10^9$ OBs/l (TF1-high), in 2011 (A), and in 2012 (B) in greenhouse trials. Values above bars represent means and those followed by identical letters did not differ significantly ($P>0.05$).

respectively, depending on sample time. A clear interaction was found between the different treatments and time (treatment*time interaction $F_{(12,72)}$=2.306; *P*=0.015).

Significant reductions in larval densities on experimental plants in terms of larval percentages were also observed in all treatments compared to the control ($F_{(4,35)}$=41.323; *P*<0.001). Before the application (time 0), the mean number of larvae registered in control, indoxicarb, Bt, ChchSNPV-TF1 1x10^8 OBs/l and 1x10^9 OBs/l plot treatments was 30.15, 18.70, 26.40, 28.61 and 33.51 larvae/plant, respectively, while at 7 days p.a. this number was reduced to 20.77, 4.65, 7.87, 6.97 and 3.10 larvae/plant, respectively, surviving the 69%, 25%, 30%, 24% and 9% of the larvae, respectively (Fig. 3A). The greatest reduction was observed in the treatment involving 1x10^9 OBs/ml while the treatments involving indoxacarb, Bt, and ChchSNPV-TF1 at 1x10^9 OBs/ml had similar densities of larvae ($F_{(2,21)}$=1.630; *P*=0.220) (Fig. 3A).

Foliar feeding injury was significantly correlated with numbers of surviving larvae ($F_{(4,35)}$=11.118; *P*<0.001) (Fig. 3B). The highest levels of defoliation were registered in control plants whereas the lowest levels of defoliation were observed in plants treated with 1x10^9 OBs/ml of ChchSNPV-TF1. Levels of defoliation in treatments involving indoxacarb, Bt and 1x10^8 OBs/ml of ChchSNPV-TF1 were similar among these treatments ($F_{(2,21)}$=5.273; *P*=0.140) (Fig. 3B).

In the second trial, all treatments resulted in a significant increase in mortality compared to the control treatment ($F_{(4,8)}$=668.185; *P*<0.001) (Fig. 2B). An interactions was also found in this second trial between treatments and time (treatment*time interaction $F_{(12,24)}$=9.254; *P*<0.000). Application of 1x10^8 OBs/ml ChchSNPV-TF1 resulted in 60% lethal infection (day 1 p.a.) to 86% lethal infection (day 5 p.a.) (Fig. 2B), whereas application of 1x10^9 OBs/ml resulted in 71% lethal infection (day 1 p.a.) to 96% lethal infection (day 5 p.a.). In the indoxacarb treatment pest mortality fell from 23 to 8% during the trial, whereas mortality in the Bt treatment fell from 30 to 32% during the same period.

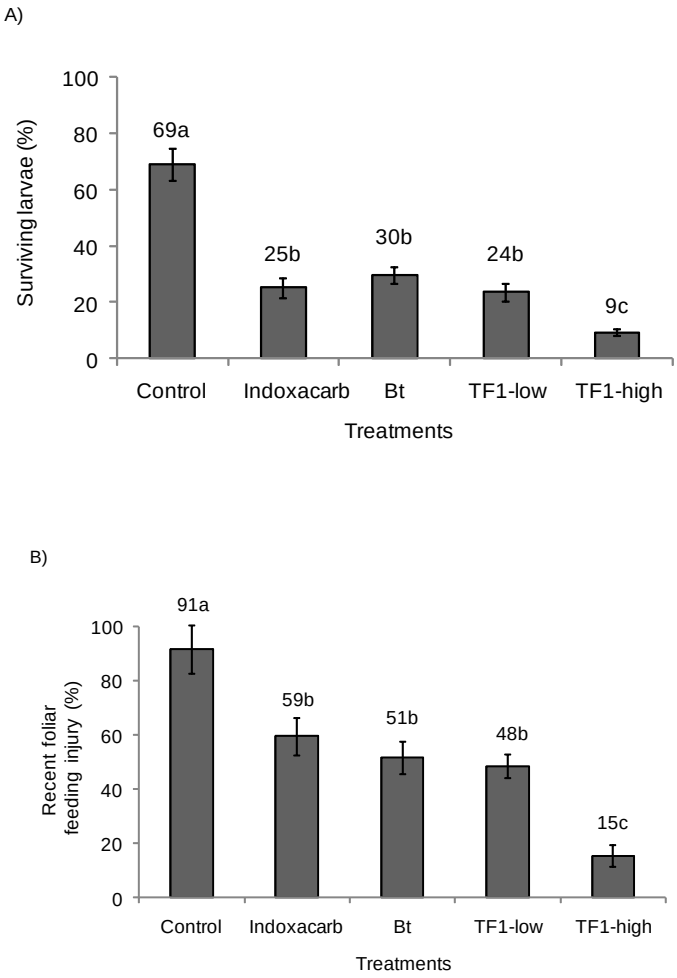

Figure. 3. Percentage surviving larvae (A), and recent foliar feeding injury (B) in banana plants treated with water (control), chemical (indoxacarb), Bt (*Bacillus thuringiensis var. kurstaki*) and two ChchSNPV-TF1 concentrations, TF1-low ($1x10^8$ OBs/l) and TF1-high ($1x10^9$ OBs/l) at day 7 p.a.. Values above bars represent means and those followed by identical letters did not differ significantly.

3.3. Rate of acquisition of viral infection

No lethal viral disease was observed in control larvae reared in the laboratory to determine the prevalence of infection, indicating that the *C. chalcites* population was healthy. The percentage of *C. chalcites* larvae that acquired a lethal infection increased .significantly .over .time ($F_{1,13}$=17,654; .*P*<0.000) .(Fig. 4). During the .first

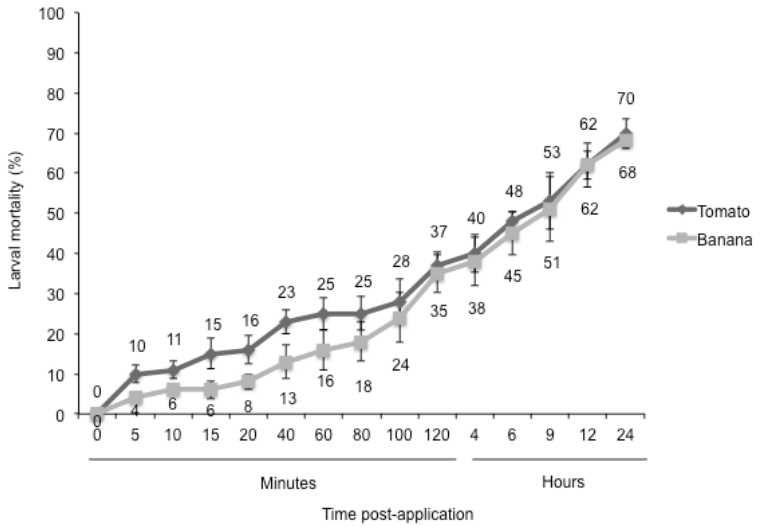

Figure. 4. Percentage of larva mortality of collected at different time points and reared in the laboratory after application of $2x10^6$ OBs/ml of ChchSNPV-TF1 to tomato and tomato branches and maintained in a walk-in plant growth room. Values above bars represent means.

hour of exposition, *C. chalcites* acquired lethal infection more rapidly in tomato plants. After 40 min., 23 and 13% mortality ($P<0.01$) was obtained in larvae from tomato and banana plants, respectively. However, larvae from subsequent sample times showed no significant differences in viral mortality regardless of their plant host ($P>0.05$). By 9 h exposure, 50% larvae from either tomato or banana died due to NPV infection and ~70% by 24 h.

4. **DISCUSSION**

The present study aimed to determine the efficacy of different concentrations of ChchSNPV-TF1 OBs for the control of *C. chalcites* larvae on tomato plants in growth chambers and on banana plants cultivated in greenhouses. Application of $2x10^6$ OBs/l of ChchSNPV-TF1 controlled *C. chalcites* on growth chamber grown tomato plants as efficiently as OB applications that were five-fold or 25-fold higher. As pesticide applications to tomato plants usually involve volumes of 600-1000 l/ha, depending on plant phenology, the growth-chamber rates that we used would be equivalent to $1.2x10^8$-$2x10^9$ OBs/ha of ChchSNPV-TF1. This appears to be a useful starting point to define application rates for *C. chalcites* control in field or greenhouse-grown tomatoes. Crucially, this rate is approximately 1000-fold lower

than that OB application rates determined for commercially available virus-based insecticides, such as *Spodoptera exigua* multiple nucleopolyhedrovirus (SeMNPV) or *Helicoverpa armigera* nucleopolyhedrovirus (HearNPV) (Cherry et al., 2000; Gupta et al., 2010; Lasa et al, 2007a).

In the greenhouse trials on young banana plants the low rate virus treatment ($1x10^8$ OBs/ml) resulted in larval mortality that was significantly higher than that of chemical and Bt treatments, whereas larval survival on treated plants and defoliation levels were similar to those chemical and Bt treatments. Similarly, application of the higher rate of $1x10^9$ OBs/ml resulted in exceptionally high levels of pest mortality (~90%) in larvae collected at various intervals post-application.

Surprisingly, larvae treated with indoxacarb or the Bt survived in high numbers during the first days of the first trial and the efficacy of these treatments appeared to decrease over time, perhaps as a result of the growth and development of the pest (Gupta et al., 2010). Although larvae also become increasingly resistant to baculovirus infection as they age, the lower susceptibility of later larval stages (Asser-Kaiser et al., 2011; Kouassi et al., 2009) can be compensated for increased consumption of OB-contaminated leaf surfaces leading to a higher ingested dose of OBs (Goulson et al., 1995). The apparently low efficacy of indoxacarb or the Bt insecticide currently used in banana crops in the Canary Islands also suggests the possible existence of resistance to these products in the pest population (Horowitz et al., 1998; Perera and Molina, 2007), although this was not determined in the present study. Cherry et al. (2000) and Gupta et al. (2010) demonstrated that certain chemical insecticides were faster at killing *H. armigera* larvae than HearNPV, but the number of surviving larvae on treated plants did not differ significantly between virus and chemical treatments.

The high potential of ChchSNPV-TF1 OBs as a bioinsecticide is likely to be affected by numerous factors, including the timing of applications. In this study, OBs were applied in banana when the majority of the pest population was in the second instar. At this stage larvae are highly susceptible to infection and cause little injury to plants compared to that of later instars. Indeed, foliar feeding by *C. chalcites* early instars in banana plants constitutes indirect injury that does not usually translate into yield losses or cosmetic damage to banana fruits. In contrast

late instars often feed on fruits, resulting in economically significant losses when infestation levels are high (Del Pino et al., 2011; Perera and Molina, 2007).

Effective spray targeting of the feeding sites of the pest is also likely to greatly influence the performance of baculoviruses insecticides, the effectiveness of which depends on larval feeding behavior and probability of consuming a lethal dose of OBs (Hunter-Fujita et al., 1998). Early instars of *C. chalcites* feed on the undersides and internal parts of young leaves. This behavior theoretically favors the persistence of OBs applied to the underside of banana leaves, especially under greenhouse conditions, where protection conferred by the leaves in addition to the UV filtering activity of the greenhouse structure is likely to markedly improve the persistence of OBs (Smits et al., 1987). Nonetheless, the persistence of ChchSNPV OBs on greenhouse-gown banana crops in the Canary Islands needs to be confirmed experimentally.

The highest concentration of ChchSNPV-TF1 OBs was more effective in reducing plant injury than the chemical and Bt insecticides in banana crops. In many instances, nucleopolyhedrovirus treatments can be as effective as chemical insecticides, and sometimes even better (Cherry et al., 2000; Gupta et al., 2010; Lasa et al., 2007b; Moore et al., 2004; Moscardi, 1999).

In the present study just one liter at $1x10^9$ OBs/l was necessary to protect efficiently banana young plants present in a 28 m^2 plot, therefore, in one ha just $3.57x10^{11}$ OBs would be necessary. However, given that pesticides are usually applied to banana crops at volumes of 1600-2000 l/ha, depending on plant phenology (Domínguez et al., 2012b), the rate of $1x10^9$ OBs/l would be equivalent to approximately $1.6\text{-}2.0x10^{12}$ OBs/ha although higher dose rates may be necessary to protect fully grown banana plants efficiently, given the high volume applications need to effectively cover large sized plants.

It seems that ChchSNPV-TF1 was more effective in controlling *C. chalcites* larvae in tomato plants than in banana plants, as a lower rate of OBs/l were needed for an efficient protection. In banana field trials, environmental factors such as temperature, leaf surface exudates, sunlight, wind and rain probably reduced treatment efficacy (Moscardi, 1999), and may explain the higher virus rates needed with respect to those for banana plants grown in greenhouses. Rate acquisition experiments revealed that larvae fed on tomato plants acquired viral infection

approximately 2 times more rapidly, but only during the first hour of virus exposure while no differences were found at other sample times regardless of their feeding source. It is likely that the hardness of banana leaves compared to those of tomato does not let as many larvae ingest a lethal dose during the first hour of exposure. The first 48 h of feeding are considered crucial in the acquisition of a viral infection (Cory and Hoover, 2006; Lasa et al., 2007b). Previous studies with SeMNPV recorded 14.9 and 30.9% mortality in larvae collected 6 h post-application (Lasa et al., 2007a). However, larvae that feed in plants contaminated with ChchSNPV-TF1 acquired viral infection more rapidly. After 20 min. 8-16% *C. chalcites* larvae from banana and tomato pants died, respectively; and by 6 h, mortality reached almost 45%. When both plants were grown under the same greenhouse conditions, similar results were obtained, suggesting that ChchSNPV-TF1 treatment in tomato crops might be as effective as in banana crops.

In conclusion, ChchSNPV-TF1 appears to have a remarkable potential as a biological control agents against *C. chalcites* larvae on tomato or banana plants, which merits registration as a crop protection product for use in banana or tomato crops in the Canary Islands.

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CHAPTER 8

General discussion

This study arises from the need to control *Chrysodeixis chalcites* (Esper) (Lepidoptera: Noctuidae) in banana crops in the Canary Islands, where infestations of this pest cause up to 30% fruit losses (Bernal et al., 2013a; Del Pino et al., 2011). Chemical-based control measures against *C. chalcites* require multiple applications that increase production costs and may hamper the commercialization of a produce with pesticide residues (Horowitz et al., 1998; Perera and Molina, 2007). Moreover, the low number of active ingredients authorized in this crop has increased the frequency in which they are used and, hence the risk of pest resistance. All this has prompted the need to find alternative, more sustainable and environmentally friendly methods. Insect-infecting baculoviruses have been reported worldwide (Jehle et al., 2006), including those from *C. chalcites*, and many have proved useful as effective biological insecticides against different crop and forest pests (Caballero et al., 2009; Cherry and Williams, 2001; Moscardi, 1999). However, no baculovirus-based control programs have been devised for *C. chalcites* to date. The aim of this thesis was to improve the basic understanding of different biotic and abiotic factors that play key roles in the biotechnological development of a new bioinsecticide based on a Canarian baculovirus isolate of *C. chalcites* single nucleopolyhedrovirus (ChchSNPV) (Baculoviridae: *Alphabaculovirus*).

Until this thesis was initiated, two ChchSNPV isolates had been described: one from dead *C. chalcites* larvae infesting tomato and sweet pepper crops in greenhouses in the Netherlands (ChchSNPV-NL) (van Oers et al., 2005, 2004) and the other from *C. chalcites* larvae obtained in horticultural crops in El Ejido, Almería, Spain, (ChchNPV-SP1) (Murillo et al., 2000). However, local isolates are generally more appreciated for both practical and technical reasons, and isolation of Canarian strains had not been performed before. From a practical point of view, indigenous isolates are much easier to register (Muñoz et al, 1998). Technically, they tend to be more active against their local host populations (Erlandson, 2009; Erlandson et

al., 2007; Kouassi et al., 2009). For these reasons and given the high intraspecific diversity of lepidopteran-infecting baculoviruses (Barrera et al., 2011, 2013; Erlandson, 2009, Williams et al., 2011), the finding of active indigenous isolates from the Canaries became a matter of time. Indeed, during a period of high infestation of *C. chalcites* in banana crops in these islands, high mortality rates owing to ChchSNPV local strain/s were found (Chapter 2). Since then, identification and selection of an isolate that could be used in control programs against *C. chalcites* in the Canary Islands was set off.

One of the first objectives of the thesis was to evaluate the natural diversity of the 97 ChchSNPV isolates collected in banana crops and compare it with that of the two previously described ChchSNPV isolates (Chapter 2). Five genetic variants or strains were identified. They were closely related between them and also similar to previously characterized ChchSNPV isolates, as observed in the restriction endonuclease (REN) profiles of their genomes. Genetic diversity was limited to minor differences, reflected in the presence and distribution of restriction sites, as usually occurs between geographical isolates of many other baculoviruses (Caballero et al., 1992; Chen et al., 2002; Escribano et al., 1999; Figueiredo et al., 2009; Zhang et al., 2005). However, biological characterization of the different strains revealed that ChchSNPV-TF1 (ChchTF1), the most widespread (collected in all samples areas in all islands) and prevalent (most abundant in all samples areas) isolate, was also the most pathogenic against a local insect population. These differences are common among virus isolates from distinct geographical regions (Alexandre et al., 2010; Escribano et al., 1999; Rowley et al., 2011; Takatsuka et al., 2003) or even from the same region (Barrera et al., 2011; Figueiredo, et al., 2009; Milks et al., 1997) and add up more evidence that native isolates tend to be more pathogenic against their indigenous insect populations than homologous isolates from elsewhere (Erlandson et al., 2007). Clearly, there is an adaptive advantage for the virus to retain high infectivity toward local host populations, presumably as a result of continuous host-pathogen coevolution, which favors the widespread of indigenous isolates.

Field isolates themselves are often a mixture of genotypes (Cory et al., 2005; Redman et al., 2010; Rowley et al., 2011). This genetic diversity seems to allow a more rapid adaptation to varying environmental conditions and thus contributes to

virus survival (Hitchman et al., 2007; Hodgson et al., 2004; Simón et al., 2004). The next step of this thesis was therefore to determine the genotypic structure of ChchTF1 (Chapter 3). Eight different genotypes were indentified within ChchTF1, as revealed by *Bgl*II-REN profiles. ChchTF1-A presented a profile identical to that of the wild-type population, indicating its high prevalence, which was confirmed by qPCR (36% of the genotypes). Prevalence of a particular genotypic variant is very common in NPV isolates (Barrera et al., 2013; Muñoz et al., 1999; Muñoz and Caballero, 2000; Simón et al., 2004), although the mechanisms underlying this dominance are still unknown. The fact that genotypes present in low proportions are not eliminated suggests that heterogeneity is important for virus survival (López-Ferber et al., 2003; Possee and Rorhmann, 1997; Simón et al., 2005). Three biological parameters were then analysed. The dose-response (pathogenicity) of the eight cloned genotypes, determined by insect bioassay, revealed that none was as pathogenic as the wild-type population, ChchTF1, and that all the genotypes presented similar pathogenicity levels. However, speed of kill bioassays showed that ChchTF1-B and –C were the most virulent genotypes and killed larvae 15 h more rapidly than the wild-type. This was correlated with the lower productivity of these genotypes, although other less virulent ones such as ChchTF1-F, -G or –H were as productive as ChchTF1-B or –C. A trade-off between virulence and productivity is common in baculoviruses (Barrera et al., 2011; Escribano et al., 1999; Muñoz and Caballero, 2000; Simón et al., 2004, 2008). Fast-killing genotypes tend to produce fewer OBs, since a shorter infection period decreases larval growth and thus production of viral progeny (Wilson et al., 2000). As virus productivity is a crucial component of virus fitness, increases in yield should be highly beneficial to the virus for transmission and persistence (Cory and Myers, 2003). In conclusion, ChchTF1 is genotypically structured to exploit host resources maximally and, in turn, increase the likelihood of transmission under natural conditions.

Interactions between genotypes have been described to have positive (Hodgson et al., 2004; López-Ferber et al., 2003; Simón et al., 2005), negative (Muñoz et al., 1998) or neutral (Milks et al., 2001) influence on virus transmissibility. The reduced relative potency of cloned variants compared with the wild-type population could be due to interactions among genotypes resulting in altered pathogenicity, as observed in other host-pathogen systems (Hodgson et al., 2004;

Simón et al., 2004). To examine this, OB mixtures and co-occluded mixtures containing only the most abundant genotypes (ChchTF1-ABC) were constructed. They displayed an increased pathogenicity and virulence in comparison with ChchTF1 (Chapter 4), both desirable characteristics for a bioinsecticide. Since baculovirus-based bioinsecticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest, an increased pathogenicity and virulence are desirable features for a baculovirus. This makes the ChchTF1-ABC mixture the most suitable as the basis for a biological insecticide.

The higher virulence of ChchTF1-ABC was particularly interesting since one of the most outlined drawbacks of baculoviruses as biological control agents is their low speed of kill in relation with chemical insecticides. Increased virulence can be achieved with recombinant baculoviruses expressing insecticidal toxins or by deletion of genes affecting host physiology (Bonning et al., 1995; Chang et al., 2003; O'Reilly and Miller, 1991; Shim et al., 2013). However, public concern with genetically manipulated organisms make this strategy impractical. In this thesis, co-occlusion of the most prevalent genotypes represents a novel approach to increase ChchSNPV virulence. This strategy reduced the virus speed of kill by 33%, a similar rate to that obtained with recombinant baculoviruses.

Genome sequencing of phenotypically different genotypes provides genetic data relevant to the genotypic and phenotypic diversity of this virus (Cory et al., 2005; Kamiya et al., 2004), important aspects for virus adaptation and survival under different conditions (Cory et al., 2005; Hodgson et al., 2001). With this aim, part of this thesis was devoted to sequence and analyse the most (ChchTF1-A, -B, and -C) and the less (ChchTF1-G and -H) frequent genotypes (Chapter 4). The genomes of ChchTF1-A, -B, -C, -G, and -H were very similar in size to that of the ChchSNPV-type isolate, ChchSNPV-NL (149,622 bp) (Acc. no. AY864330), from The Netherlands (van Oers et al., 2005). A whole genome sequence alignment between ChchTF1A, -B, -C, -G and -H and ChchSNPV-NL showed 98-99% similarity at the nucleotide level. This analysis also demonstrated that variable genomic regions were located principally in the *hoar* and *bro-d* genes, which represent a major source of intra-specific variability among genotypes in many baculoviruses (Bideshi et al., 2003; Erlandson, 2009; Le et al., 1997). These

variable regions may be involved in the phenotypic differences observed between pure genotypes, although experiments with knock-out viral constructs are necessary to corroborate this hypothesis. In addition, nucleotide substitutions will be analysed by selection pressure analysis (Rubinstein et al., 2011; Yang, 2007), which has contributed to detect genes undergoing positive selection in other baculoviruses (Harrison and Bonning, 2004).

In the development of baculoviruses as biocontrol agents, one of the greatest limitations is an efficient and economically viable mass production system, which represents one the major costs of a biopesticidal product. Therefore, the foremost objective in any viral mass production program is to develop efficient production systems that yield baculovirus OBs at a competitive cost (Claus and Sciocco de Cap, 2001; Szewczyk et al., 2006). As obligate pathogens viruses need to be produced either in cell culture (*in vitro*) or in host larvae (*in vivo*). However, to date, baculoviruses have been successfully produced at a commercial scale only in living host insects (Caballero et al., 2009; Cherry et al. 2000; Claus and Sciocco de Cap, 2001; Lasa et al., 2007a; Shapiro, 1986). In Chapter 5, an effective ChchTF1 *C. chalcites* larvae mass production method is described. Parameters such as larval instar, timing of inoculation, concentration of virus inoculum, and larval density per container were optimized to obtain as much as $8.07x10^{13}$ OBs/container (with each container holding 150 larvae). Such a high production has not been achieved so far in any other insect-baculovirus system (Caballero et al., 2009; Sun et al., 2005). The key aspect to achieve the greatly increased production with *C. chalcites* was inoculation of the host insect in the later sixth instar. Infected larvae died with higher weights than those infected earlier and produced significantly greater OB yields compared with other lepidopteran species that only reach the fifth instar in laboratory rearing, like *S. exigua* (Lasa et al., 2007a). The exponential increase in OB yield in increased larval instars has been described in other host-pathogen systems (Kalia et al., 2001; Gupta et al., 2007; Monobrullah and Nagata, 2000). Direct correlation exists between insect host weight at death and OB production, as larger susceptible tissues can be infected and thus produce higher OB amounts. The physiological larval time at viral inoculation also influenced final OB yield. Larvae 24 h post L_6 molting were markedly bigger than recently molted L_6, and produced six times more OBs. However the ability of ChchSNPV to kill larvae at

that stage does not exist in other host-pathogen systems since advanced instars, become highly resistant to the virus (Escribano et al., 1999; Arrizubieta et al., pers. comm.).

The inoculum concentration is another crucial aspect in optimizing mass-production systems. When the viral inoculum is too high, the infection time gets reduced and larvae die earlier whereas if it is too low, not all the insects acquire a lethal disease. In chapter 5, five different viral concentrations where tested, but no differences were observed on OB production per larva or mg of larva or on mean time to death (MTD). As a result, the higher concentration (LC_{90}), which killed the highest number of larvae while producing the greatest OB yields *per* number of inoculated larvae was selected for further studies. The proportion of larval deaths from NPV infection is also higher with inocula concentrations close to CL_{95} in other insect-baculovirus systems (Grzywacz et al., 1998; Gupta et al., 2007; Moscardi et al., 1997).

The ethology of the host is an important issue to decide rearing larvae in isolation or in groups, in particular the degree of larval cannibalism. In *C. chalcites*, 24 h post L_6 molting larvae could be reared at a density of 150 larvae per container (1.5 l), to yield the record $8.07x10^{13}$ OBs/container, a significantly higher figure than in containers with 1, 25, 50 and 100 larvae. Cannibalism was not as evident as in other species like *H. armigera* (Kakimoto et al., 2003), *S. exigua* (Elvira et al., 2010; Lasa et al., 2007a) and *S. frugiperda* (Chapman et al., 1999). This was especially true, for sixth instars compared with lower instars, probably because the latest instar is less active and aggressive that the earliest instars of *C. chalcites*. Yields as high as $8.07x10^{13}$ OBs have not been obtained in any other host-virus system before by far (Lasa et al., 2007a; Sun et al., 2005). Taking into account that field applications generally require $2x10^{11}$-$1x10^{12}$ OBs/ha for effective insect mortality (depending on crops, caterpillar species, virus strain and formulation) (Moscardi, 1999), a single 150-larva container, would allow treatment of as many as 80-400 ha. Results of field and greenhouse assays in tomato and banana crops infested with *C. chalcites* and treated with this virus support this argument (Chapter 7). In conclusion, optimization of inoculum doses, the instar and physiological stage of larvae at inoculation and the rearing density, significantly increases NPV

productivity and represent key factors in making the commercial production of this virus economically feasible.

An important part of this thesis was dedicated to determine the efficacy of ChchTF1 as the basis for a bioinsecticide product. Firstly, the susceptibility of the different pest instars to the virus strain was determined. The host stage usually affects the concentration-mortality response and survival time of virus infected hosts with important consequences on timing viral insecticide applications (Erlandson, 2009; Milks, 1997). *C. chalcites* larvae were markedly less susceptible to infection with increasing age (Chapter 6), as reported for most other lepidopteran species (Asser-Kaiser et al., 2011; Kouassi et al., 2009). This stage-related resistance to infection increases steadily with larval body weight (Myers et al., 2011). This is in part attributed to the peritrophic membrane, the first barrier against NPV infection (Levy et al., 2007; Plymale et al., 2008). Susceptibility to viral infection decreases through successive instars, which bear a more developed and increasingly less permeable peritrophic membrane (Lehane, 1997).

Another part of this thesis consisted in the search for synergistic substances of ChchTF1 in the field. Optical brighteners, firstly identified as UV protectants, have demonstrated to increase the insecticidal activity of many baculoviruses (Dougherty et al., 1996; Guo et al., 2007; Shapiro, 1992; Washburn et al., 1998). In the present thesis, the effectiveness of several optical brighteners was tested, and two of them increased pathogenicity and virulence more effectively than other brighteners (Chapter 6). Addition of 1% Tinopal enhanced virus pathogenicity by 4.43 in L_2 and 397-fold in L_6, and 1% Leucophor by 1.46 in L_2 and 143-fold in L_6. Virulence was also improved and larvae died 14-26 h earlier. OB yields were reduced by up to 3.8 fold, but this trait does not affect efficacy in inundative control programs. The use of optical brighteners could thus greatly improve the insecticidal efficiency in applications targeted at late instars (Bernal et al., 2013b; Murillo et al., 2003). Formulation of OBs with brighteners could be particularly valuable in situations in which *C. chalcites* infestations consist in overlapping larval generations. In these instances with single-dose applications might control all larval ages. However, addition of chemicals to bioinsecticide products is not well accepted and natural products, like plant extracts, should be tested (Shapiro et al., 2010).

The efficiency of ChchTF1 in controlling *C. chalcites* L_2 was assessed under laboratory conditions. However, validation under environmental conditions, similar to those in which the product is to be used, is required for product registration. This work was done in Chapter 7. Application of ChchTF1 concentrations as low as $2x10^6$ OBs/l controlled *C. chalcites* efficiently in tomatoes grown in growth-chambers. As pesticide applications on tomato plants usually involve volumes of 600-1000 l/ha, depending on plant phenology, the growth-chamber rates were equivalent to $1.2x10^8$-$2x10^9$ OBs/ha. Remarkably, this rate is approximately 1000-fold lower than those determined for commercially available baculovirus-based insecticides, such as SeMNPV or HearSNPV (Cherry et al., 2000; Gupta et al., 2010; Lasa et al, 2007a, 2007b). In field trials with banana crops, two different viral concentrations were evaluated; $1x10^8$ and $1x10^9$ OBs/l. The latter is normally used on field applications with other baculoviruses (Moscardi, 1999; Caballero et al., 2009). Surprisingly, the low-rate virus treatment killed significantly more larvae than the chemical and Bt treatments that were also administered for comparison. Larval survival on treated plants and defoliation levels were similar to those of chemical or Bt treatments. The higher rate, in turn, resulted in exceptionally high levels of pest mortality (~90%) in larvae collected at various intervals post-application. In many instances, NPV treatments have proven as effective as chemical insecticides, and sometimes significantly better (Cherry et al., 2000; Gupta et al., 2010; Moscardi, 1999; Lasa et al., 2007b; Moore et al., 2004; Gómez-Bonilla et al., 2013). Although larvae also become increasingly resistant to baculovirus infection as they age, the lower susceptibility of later larval stages (Asser-Kaiser et al., 2011; Kouassi et al., 2009) can be compensated by increased consumption of OB-contaminated leaf surfaces, leading to a higher ingested OB dose (Goulson et al., 1995). Only one liter of the product, at $1x10^9$ OBs/l, was necessary to protect young banana plants in a 28 m^2 plot efficiently, which would make $3.57x10^{11}$ OBs/ha. Since pesticides are usually applied to banana crops at volumes of 1600-2000 l/ha, depending on plant phenology (Domínguez et al., 2012), the $1x10^9$ OBs/l rate would be equivalent to approximately 1.6-2.0$x10^{12}$ OBs/ha. Yet, higher dose rates may be necessary to protect fully grown banana plants efficiently, given the high volume applications needed to effectively cover large sized plants. According to the mass production system described previously, a single production unit with 150 larvae would yield

enough virus concentration to protect ($8.07x10^{13}$ OB) 40-50 hectares of banana crops efficiently against *C. chalcites*.

Rate acquisition of deadly virus doses is an important parameter to assess virus efficiency in the field. *C. chalcites* larvae fed on tomato plants acquired viral infection approximately twice as rapidly during the first hour of exposure than those fed on banana plants. Probably, the hardness of banana leaves when compared with softer tomato leaves may explain this difference. However, longer exposures did not result in differences between rate acquisition values in the two types of crops. Previous studies concluded that the first 48 h of feeding are considered crucial in the acquisition of the viral infection (Cory and Hoover, 2006; Lasa et al., 2007a). For instance, Lasa et al (2007a), recorded 14.9-30.9% mortality in larvae collected 6 h post-application (Lasa et al., 2007a). *C. chalcites*, larvae fed on ChchTF1-contaminated plants acquired viral infection more rapidly. After only 20 min exposure, eight and 16% mortality was registered in banana and tomato pants, respectively; and after six hours, mortality values reached almost 45%. These differences may be related with the insect-baculovirus system and with the virulence showed by ChchTF1. One more piece of evidence of the exceptional properties of ChchTF1 as a crop protection agent against *C. chalcites*.

Still, to implement correct control programs with ChchNPV as a microbial biocontrol agent (MBA), several features need to be explored for each plant-pest-virus complex, including type of spraying that allows a better virus intake, compatibility with biological and chemical control measures, field persistence rates of OBs on plant surfaces, storage stability, and appropriate application rates and timing.

Strategies for the biological control of *C. chalcites* in banana crops had been poorly explored until now. In the present thesis, the exceptional properties of ChchTF1 as a pest control agent and its low-production costs have been revealed in a context in which chemical control of *C. chalcites* continues facing important difficulties (few active ingredients, incorrect applications, etc.). For this reason, the ChchTF1-ABC mixture of genotypes has been subject of a patent application (P201330487) and we believe it merits registration as a microbial biocontrol agent.

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CONCLUSIONES

1. Entre los 97 aislados caracterizados de ChchSNPV obtenidos en cultivos de platanera en las Islas Canarias, se identificaron 5 variantes genotípicas mediante perfiles de restricción. Todas ellas fueron similares entre sí y a los aislados previamente caracterizados de Holanda ChchSNPV-NL y Almería, ChchSNPV-SP1.

2. Aunque muy similares a nivel genómico, los distintos aislados de ChchSNPV difieren significativamente en la patogenicidad de los OBs. El aislado más frecuente y con mayor dispersión en las Islas Canarias, ChchSNPV-TF1, a su vez fue el más patogénico y virulento para una población autóctona de *C. chalcites*. Su elevada patogenicidad y virulencia es comparable a la de otros baculovirus actualmente comercializados como bioinsecticidas.

3. La caracterización genotípica demostró que ChchTF1 está compuesto por al menos ocho genotipos diferentes, siendo ChchTF1-A el genotipo más frecuente en la población silvestre del virus. Ninguno de los genotipos puros fue más patogénico que ChchTF1 lo que sugiere interacciones entre genotipos que incrementan la patogenicidad del aislado silvestre. Sin embargo, ChchTF1 presentó una menor virulencia que los genotipos puros, que dio lugar a una mayor productividad viral. ChchTF1 está genotípicamente estructurado para maximizar la transmisibilidad del virus en la naturaleza.

4. Los ensayos con mezclas experimentales de genotipos puros del virus pusieron de manifiesto que la mezcla co-ocluida compuesta por los tres genotipos más frecuentes mejora las propiedades insecticidas del aislado silvestres ChchTF1. La mezcla co-ocluida ChchTF1-ABC tiene un gran potencial como ingrediente activo para el desarrollo de un bioinsecticida para el control de *C. chalcites* en las Islas Canarias.

5. La secuencia completa de los genotipos más frecuentes, ChchTF1-A, -B y –C, y los menos frecuentes, ChchTF1-G y –H, puso de manifiesto una gran

similitud (del orden del 99%) entre los mismos, siendo los genes *hoar* y *bro-d* las regiones que presentan mayor variabilidad.

6. Los ensayos de producción masiva demostraron que se pueden obtener producciones tan altas como $8.07x10^{13}$ OBs cuando se infectan lotes de 150 larvas con la LC_{90}, 24 horas después de haber mudado al sexto estadio, lo que representa un incremento de 59.645 veces sobre la concentración de inoculación.

7. La susceptibilidad de C. chalcites a ChchTF1 disminuye conforme incrementa el estadio larvario, larvas de segundo estadio fueron 10.000 veces más susceptibles que las de sexto estadio. El tiempo medio de mortalidad fue 42 h mayor en larvas L_6 que en larvas L_2. Paralelamente, la producción de OBs incrementó en estadios más avanzados; larvas L_6 produjeron 23 veces más OBs que las larvas L_4.

8. La formulación de ChchTF1 con abrillantadores ópticos mejora sus propiedades insecticidas. La adición de 1% de Tinopal UNPA-GX mejora la patogenicidad entre 4,43 y 397 veces dependiendo del estadio. La virulencia por su parte se vio también mejorada entre 14 y 26 horas; sin embargo, la producción de OB se redujo del orden de 8,5 veces por efecto del Tinopal. El Leucophor UO también mejoró las propiedades insecticidas del virus, pero su actividad sinérgica fue significativamente menor.

9. Una concentración de $2x10^6$ OBs/l de ChchTF1 es suficiente para controlar de manera efectiva las larvas de *C. chalcites* en plantas de tomate en condiciones controladas. Los ensayos de invernadero en cultivo de platanera evidenciaron que el tratamiento con ChchTF1 ($1x10^9$ OBs/l) fue entre 3 y 4 veces más efectivo que los tratamientos habituales con un insecticida químico y otro biológico (Bt). En tratamientos donde se usan volúmenes de 1600-2000 l/ha, para mantener la concentración de $1x10^9$ OBs/l, sería necesario aplicar $1.6\text{-}2.0x10^{12}$ OBs/ha. Teniendo en cuenta que con 150 larvas se obtienen producciones del orden de $8.07x10^{13}$, esta

cantidad de inóculo viral sería suficiente para proteger eficientemente entre 40 y 50 ha de cultivo de platanera.

10. ChchTF1 presenta un gran potencial como agente de control biológico contra *C. chalcites* tanto en plantas de tomate como de platanera. Por tanto, reúne todas las condiciones para ser registrado como agente de control para su uso en cultivos de platanera o tomate en las Islas Canarias.

11. Finalmente, una buena parte de los resultados de esta tesis forman parte del contenido de una solicitud de patente para el desarrollo de un nuevo bioinsecticida (P201330487).

CONCLUSIONS

1. Among the 97 field-collected ChchSNPV isolates characterized, five genotypic variants were identified by restriction endonuclease analysis, all of which were closely related to one another and to the previously characterized ChchSNPV-NL from the Netherlands and ChchNPV-SP1 from Almería, Spain.

2. Although genetically very similar, ChchSNPV isolates differed significantly in OB pathogenicity. The most prevalent and widespread strain in the Canary Islands, ChchSNPV-TF1 (ChchTF1), was the most pathogenic and virulent against a local insect population. The high pathogenicity and rapid speed of kill of this virus is comparable to other baculoviruses currently commercialized as bioinsecticide products.

3. Genotypic characterization revealed that ChcHTF1 is composed at least of eight different genotypes, being ChchTF1-A the most prevalent in the wild-type population. None of the pure genotypes was as pathogenic as the ChchTF1 indicating that interactions between genotypes increased the pathogenicity of the wild-type. However, ChchTF1 presented lower virulence than pure genotypes, reflected in an increased OB yield. ChchTF1 is genotypically structured to maximize the likelihood of transmission.

4. Experimental mixtures assays revealed that the co-occluded mixture composed by the most prevalent genotypes, in a similar proportion as in the wild-type population, presented an increased pathogenicity and virulence compared with ChchTF1. ChchTF1-ABC co-occluded shows great potential as the active ingredient of a bioinsecticide to control *C. chalcites* in the Canary Islands.

5. The complete genome sequences of the most prevalent genotypes, ChchSNPV-TF1-A, -B and -C, and the lowest ones, ChchTF1-G and –H, revealed a high degree of similarity (around 99%), being the *hoar* and *bro-d* genes the genomic variable regions.

6. Massive pordution assays revelaed that yields as high as $8.07x10^{13}$ OBs can be obtained by infecting lots of 150 larvae 24 h post L_6 molting in 1.5 l container with the LC_{90}, which represents a 59,645-fold increased over the inoculated concentration.

7. Susceptibility to ChchTF1 OBs decreased as larval stage increased; second instars (L_2) were 10,000-fold more susceptible than sixth instars (L_6). Virus speed of kill was 42 h faster in L_2 than in L_6. OB production increased in late instars; L_6 larvae produced 23-fold more OBs than L_4.

8. Formulation of ChchTF1 with optical brighteners improves the viral efficacy. Addition of 1% of Tinopal UNPA-GX decreased the pathogenicity between 4.43 to 397-fold depending the instar. The virulence was also improved by 14 to 26 h, however that OB yield was reduced by 8.5-fold. The degree of enhancement by Leucophor UO was significantly lower.

9. $2x10^6$ OBs/l of ChchTF1 controlled *C. chalcites* on growth chamber grown tomato plants efficiently. Greenhouse trials in the Canary Islands revealed that the $1x10^9$ OBs/l treatment was 3 to 4-fold more effective in reducing larval infestations than the chemical or Bt treatments. Given that pesticides are usually applied to banana crops at volumes of 1600-2000 l/ha, depending on plant phenology, the rate of $1x10^9$ OBs/l would be equivalent to approximately $1.6\text{-}2.0x10^{12}$ OBs/ha. Taking into account that $8.07x10^{13}$ OBs are obtained with 150-infected larvae between 40 and 50 ha would be efficiently tretaed.

10. ChchTF1 appears to have a remarkab*le potential* as a biological control agents against *C. chalcites* larvae on tomato or banana plants, which merits its registrat[i]on as a crop protection product for use in banana or tomato crops in the Canary Islands.

11. Finally, the results of this thesis have contributed to a Spanish patent application (P201330487).

LIST OF PUBLICATIONS

Bernal, A., Carnero, A., Hernández-Suárez, E., Williams, T., Caballero, P., Simón, O., 2013. A native variant of *Chrysodeixis chalcites* nucleopolyhedrovirus: The basis for a promising bioinsecticide for control of *C. chalcites* in Canary Islands' banana crops. Biological Control 67, 101-110.

Bernal, A., Simón, O., Williams, T., Muñoz, D., Caballero, P., 2013. A *Chrysodeixis chalcites* single nucleopolyhedrovirus population from the Canary Islands is genotypically structured to maximize survival. Applied and Environmental Microbiology. DOI: 10.1128/AEM.02409-13.

Bernal, A., Williams, T., Muñoz, D., Caballero, P., Simón, O., 2013. Complete genome sequence of five *Chrysodeixis chalcites* nucleopolyhedrovirus genotypes from a Canary Islands isolate. Genome Announcement. DOI: 10.1128/genomeA.00873-13.

Bernal, A., Williams, T., Muñoz, D., Simón, O., Caballero, P. , 2013. Boosting *in vivo* mass-production of *Chrysodeixis chalcites* nucleopolyhedrovirus in its homologous host. Pest Management Science.

Bernal, A., Williams, T., Caballero, P., Simón, O., 2013. Stage-specific insecticidal characteristics of a nucleopolyhedrovirus isolate from *Chrysodeixis chalcites* enhanced by optical brightener. Pest Management Science. DOI: 10.1002/ps.3617.

Bernal, A., Simón, O., Williams, T., Carnero, A., Hernández-Suárez, E., Muñoz, D., Caballero, P., 2013. Efficacy of an alphabaculovirus for the control of *Chrysodeixis chalcites* (Lepidoptera: Noctuidae) on tomato and banana crops. Biological Control.

Para la realización de esta tesis Alexandra Bernal obtuvo una beca de postgrado I+D+I de Formación de Tecnólogos del Gobierno de Navarra y posteriormente obtuvo contratos como ayudante de proyecto a cargo de los proyectos AGL2008-05456-C03-01 concedido a Primitivo Caballero Murillo y RTA2010-00016-C2-02 concedido a Oihane Simón de Goñi