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Spodoptera frugiperda nucleopolyhedrovirus: the basis for a biopesticide product in Colombia

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Pamplona, 2013

Departamento de Producción Agraria
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Spodoptera frugiperda nucleopolyhedrovirus: the basis for a biopesticide product in Colombia

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Y para que así conste, firman la presente en Pamplona a 18 de noviembre de 2013,

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RESUMEN

Spodoptera frugiperda (Lepidoptera: Noctuidae) es una plaga que puede causar pérdidas de hasta un 60% en las producciones de maíz en Colombia. Por lo tanto, hay una reconocida demanda para desarrollar métodos sostenibles de control contra esta plaga. El nucleopoliedrovirus múltiple de *S. frugiperda* (SfMNPV: Baculoviridae) es una prometedora alternativa al uso de insecticidas químicos. El objetivo del presente trabajo fue establecer las bases científicas para el desarrollo de un nuevo biopesticida a partir de un SfMNPV autóctono de Colombia.

Inicialmente se evaluó la diversidad intraespecífica del SfMNPV a nivel interpoblacional. Durante un muestreo larvario en diferentes regiones de Colombia se obtuvieron 38 aislados y se seleccionó SfCOL por su alta prevalencia (92%). El tamaño del genoma de SfCOL se estimó en 133.9 Kb, con diferencias mínimas respecto al genoma de un aislado de Nicaragua (SfNIC) en cuanto al número y posición de los sitios de restricción. La región variable de SfCOL fue colinear con el genoma de otros aislados de SfMNPV (Pautas de lectura abierta, ORFs *sf20* a *sf27*). Sin embargo, dos ORFs no presentaron homología con SfMNPV, pero fueron altamente similares a los ORFs *splt20* y *splt21* del NPV de *S. litura.* Los ensayos de actividad biológica con dos poblaciones de *S. frugiperda* de regiones diferentes pusieron de manifiesto que SfCOL fue igual de potente que SfNIC frente a la población Mexicana (en términos de concentración-mortalidad). Sin embargo, SfCOL fue doce veces más potente que SfNIC frente a la población Colombiana, y tres veces más potente para la población de Colombia que para la población Mexicana.

El análisis de la diversidad intrapoblacional mostró diez variantes genotípicas diferentes dentro de SfCOL (SfCOL-A a SfCOL-J). El genotipo SfCOL-A fue el más frecuente en la población (71±2%), y su perfil de restricción con *Pst*I fue igual al de SfCOL. Además, SfCOL-A presentó el genoma más largo, mientras que el resto de variantes genotípicas mostraron deleciones de diferentes tamaños (3.8–21.8 Kb) afectando la misma región variable del genoma localizada entre los ORFs *sf20* a *sf33.* SfCOL-A fue 4,4 veces más potente que el aislado silvestre SfCOL, mientras que su velocidad de acción fue similar. Los genotipos con deleciones presentaron potencias insecticidas similares o menores que SfCOL y seis de ellos

presentaron una velocidad de acción mayor que la del SfCOL. La potencia de las distintas mezclas co-ocluidas de dos genotipos, obtenidas con SfCOL-A y uno de los tres genotipos deletados (SfCOL-C, -D ó –F), fue menor que la potencia del SfCOL-A. Esto sugiere que la presencia de genotipos con deleciones disminuyen la potencia pero incrementan la productividad del aislado silvestre SfCOL, el cual está estructurado para maximizar capacidad de transmisión en condiciones naturales.

Finalmente, se evaluó la eficacia de una formulación basada en la obtención de microencapsulados de SfCOL y SfCOL-A. El análisis de control de calidad puso de manifiesto que parámetros tales como concentración viral, humedad, contenido de bacterias y eficacia, se encontraban dentro de los límites aceptables. La formulación no aumentó la patogenicidad de SfCOL y SfCOL-A. Sin embargo, fue efectiva para mantener la infectividad de los OBs durante al menos tres meses de almacenamiento acelerado. La pérdida de actividad insecticida de los OBs microencapsulados fue inferior al 12%, mientras que los OBs sin formular perdieron entre el 36 y 46% de su actividad. Adicionalmente, los formulados de SfCOL y SfCOL-A mostraron un buen grado de fotoprotección, tras seis horas de exposición a la radiación UV-B; en cambio, los no formulados perdieron entre un 50 y 90% de su actividad, respectivamente. En condiciones de invernadero, las eficacias de los formulados SfCOL y SfCOL-A y SfCOL-A sin formular fueron superiores al 80%, en plantas de maíz, y similares a la del tratamiento químico convencional. Las aplicaciones en campo abierto de una concentración de $8x10^{11}$ OBs/Ha (800g/Ha), tanto si los SfCOL y SfCOL-A están formulados o no, permiten proteger eficazmente el cultivo del en las condiciones agro-climáticas de Colombia. A esta concentración de OBs el nivel de daño fresco en las plantas de maíz se mantiene por debajo del umbral económico de daño (35% de plantas dañadas en las condiciones agro-climáticas de Colombia).

Este estudio constituye la base para el desarrollo tecnológico de un biopesticida a base del SfMNPV y se propone como un componente para los futuros diseños de programas de control integrado de plagas del maíz en Colombia.

SUMMARY

Spodoptera frugiperda (Lepidoptera: Noctuidae) can cause up to 60% of losses of maize in Colombia. Therefore, there is a well-recognized demand for the development of sustainable control measures against this pest. The multiple nucleopolyhedrovirus of *S. frugiperda* (Baculoviridae; SfMNPV) has been considered as promising alternative to chemical insecticides. The aim of the present work was to establish the scientific basis for the development of a new biopesticide based on a local SfMNPV isolate from Colombia.

First the intra-specific diversity of SfMNPV in Colombia was evaluated at inter-population level. Thirty-eight isolates from three different geographical regions of Colombia were obtained, and SfCOL was selected by its high prevalence (92%). The SfCOL genome size was estimated to be 133.9 Kb, with few differences in terms of number and position of restriction sites respect a Nicaraguan isolate (SfNIC). The variable region of SfCOL was collinear with that of other SfMNPV isolates (Open reading frames, ORFs *sf20* to *sf27*). However, two ORFs had no homologies with SfMNPV, but presented a high similarity with the *splt20* and *splt21* ORFs of *S. litura* NPV. The bioassays with two different colonies of *S. frugiperda*, revealed that SfCOL was as potent (in terms of concentration-mortality metrics) as SfNIC towards a Mexican insect colony. However, SfCOL was twelve times more potent for a Colombian colony than SfNIC and three times more potent for the Colombian colony than for the Mexican colony.

The analysis of the intra-population diversity of SfCOL revealed ten different genotypic variants (SfCOL-A to SfCOL-J). SfCOL-A was the most prevalent (71±2%) showing a *Pst*I restriction profile indistinguishable from that of SfCOL. Additionally, SfCOL-A presented the largest genome while the other genotypic variants showed deletions of different sizes (3.8–21.8 Kb) affecting the same genomic region between ORFs *sf20* to *sf33*. The SfCOL-A was 4.4-fold more potent than SfCOL wild type, whereas speed of kill of SfCOL-A was similar to that of SfCOL. Deletion genotypes OBs were similarly or less potent than SfCOL but six deletion genotypes were faster killing than SfCOL. The potency of different genotypic co-occluded mixtures consisting of two genotypes, involving equal proportions of SfCOL-A and one of three deletion genotypes (SfCOL-C, -D or –F), was lower than SfCOL-A potency This

suggests that the presence of deleted genotypes reduces the OB potency but increases OB production of the SfCOL wild type isolate, which is structured to maximize the transmission of the virus in natural conditions.

Finally, the efficacy of a formulation based on microencapsulation of SfCOL and SfCOL-A was evaluated. Quality control analysis revealed that the viral concentration, moisture, bacteria content and viral efficacy were under acceptable limits. The formulation did not increase the pathogenicity of SfCOL and SfCOL-A. However, it was effective to maintain the OBs infectivity at least after three months of accelerated conditions of storage. The rate of inactivation was not greater than 12% for microencapsulated viruses, while unformulated OBs lost between 36 and 46% of insecticidal activity. Additionally, the formulated viruses showed a good level of photoprotection after six hours of UV-B radiation, being the inactivation of unformulated SfCOL and SfCOL-A between 50 and 90%, respectively. In greenhouse conditions in maize plants, the efficacies of formulated SfCOL and SfCOL-A as well as unformulated SfCOL-A were higher than 80% and similar to conventional chemical treatment. In field, applications of either unformulated or formulated SfCOL and SfCOL-A at dose $8x10^{11}$ OBs/Ha (800g/Ha) protect the maize crops under environmental conditions of Colombia. At this OBs concentration, the level of fresh damage in maize plants is maintained below the economic injury level (35% of plants with fresh damage under environmental conditions of Colombia).

This study constitutes the basis for the technological development of an SfMNPV-based biopesticide and we propose it as a component for future designs of Integrated Pest Management programs for maize crop protection in Colombia.

CHAPTER I

General Introduction

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GENERAL INTRODUCTION AND SCOPE OF INVESTIGATION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) is an important pest of the Americas, including Colombia (Clark et al., 2007). This insect has a very wide host range and has been able to attack several crops, however the most frequently hosts are maize and sorghum (Sparks, 1979). The pest affects the crop during the entire growing cycle and can produce yield reductions of up to 34% (Carnevalli and

Florcovski, 1995). The economic impact of the pest in Colombia is considerable, given that corn is the principal cereal produced in the country (Quintero et al., 2004).

Control of *S. frugiperda* in maize crops includes the integration of cultural, physical, biological and chemical control (Williams et al., 1999). However, the control with broad spectrum and highly toxic (categories I and II) synthetic chemical insecticides is the main method for reducing the effects of the pest (Andrews, 1988; Ashley et al., 1983; Molina-Ochoa et al., 2003). Toxicity categories are assigned to insecticides based on their potency (ability to cause harm at low doses) by various routes of exposure (oral, inhalation or dermal). Toxicity increases as mean lethal concentration decreases, thus category I insecticide are more toxic than category IV insecticide (WHO, 2009). Several problems are associate with the use of chemical pesticides, including a high toxicity with high risk of farmer poisonings and residuality in human and animal food (Lapied et al., 2009), besides interfering with the beneficial action of natural control agents and causing deleterious effects on non-target species (Chandler and Summer, 1991). Furthermore, chemical insecticides require several applications to be effective and some products have become ineffective due to the development of insect resistance (Chandler and Summer, 1991).

Several natural enemies such as predators, parasitoids and pathogens may be considered as control agents. Entomopathogens such as viruses, fungi, nematodes and bacteria could be used to control the *S. frugiperda* but only a few of them have shown potential for the control of this pest (Gardner and Fuxa, 1980). Baculoviruses are an interesting alternative for control *S. frugiperda* keeping the larval population density below the economic injury level. They have a high insecticidal activity, besides they are considered environmentally safe, due to its narrow host range (Lapied et al., 2009; Szewczyk et al., 2006).

Alphabaculovirus (Nucleopolyhedrovirus; *Baculoviridae*) have been successfully used in various countries as control agents against insect pests (Moscardi 1999). For instance, *Helicoverpa armigera* NPV (HearNPV) has been produced for use against the cotton bollworm in China (Sun and Peng, 2007). In Brazil it is extensive the use of *Anticarsia gemmatalis* NPV (AgNPV) to control the velvetbean caterpillar, the principal pest of soya (Moscardi, 1999; Szewczyk et al., 2006). Furthermore, in Spain the *S. exigua* NPV(SeMNPV) has been developed as a biopesticide to use in Almería greenhouses against *S. exigua* (Caballero et al., 2009).

S. frugiperda multiple NPV (SfMNPV) has been isolated from fall armyworm populations in North, Central and South America (Barreto et al., 2005; Berretta et al., 1998; Escribano et al., 1999; Loh et al., 1981; Loh et al., 1982; Maruniak et al., 1984; Shapiro et al., 1991). Some isolates have been evaluated under field conditions as potential biopesticides to control *S. frugiperda* on maize with significant levels of *S. frugiperda* larvae mortality in maize crops (Armenta et al., 2003; Barreto et al., 2005; Behle and Popham, 2012; Cisneros et al., 2002; Moscardi, 1999; Valicente and Costa, 1995; Williams et al., 1999).

The growing interest in developing a biopesticide based SfMNPV has led to the study of its diversity (Escribano et al., 1999; Figueiredo et al., 2009; Harrison et al., 2008; López-Ferber et al., 2003; Simón et al., 2004; 2011). Thus heterogeneity has been demonstrated between populations by restriction profiles (Escribano et al., 1999; Knell and Summers, 1981; Loh et al., 1982; Maruniak et al., 1984) and PCR technique (Rowley et al., 2010). The SfMNPV genetic differences and differential fitness have been observed among geographic isolates (Escribano et al., 1999; Rowley et al., 2010) and among genotypic variants recovered from one field isolate using *in vitro* techniques in culture cells (Harrison et al., 2008; Simón et al., 2004). The SfMNPV genotypic variants involved larger genomic deletions with the loss

of *egt* and *pif* genes function, resulting in fast killing and deficient for oral infectivity strains, respectively (Harrison et al., 2008; Simón et al., 2005b).

A limitation of baculoviruses as biocontrol agents is, among others, their susceptibility to environmental conditions such as temperature, pH, moisture, exposure to UV light, and by the action of some plant metabolites such as peroxidases, that generate free radicals (Ignoffo and García, 1992). In this sense, microencapsulation of baculoviruses has been used in recent years as a promising alternative for the formulation of baculoviruses. This technique allows protect the virus from environmental conditions and improving the storage stability (Behle and Popham, 2012; Villamizar et al., 2010).

Despite the high potential of this virus as a biopesticide, there is no information about diversity of Colombian isolates of SfMNPV for control of *S. frugiperda* larvae and not product based on SfMNPV has so far been registered in this country. The aim of this study was to study the diversity of Colombian SfMNPV and select the most appropriate for use against *S. frugiperda* in a control program designed in Colombia. To achieve this goal, both the inter-population and intra-population diversity of Colombian SfMNPV isolates were evaluated by molecular and biological characterization. The selected SfMNPV isolate was formulated and its efficacy was evaluated in greenhouse and open field assays. The results of this thesis will be the basis of a biopesticide against fall armyworm to use in maize crops in Colombia.

FALL ARMYWORM *Spodoptera frugiperda*

Taxonomy and morphology

Spodoptera frugiperda (J. E. Smith, 1797) is an important pest widely distributed in American continent. This pest was described first in the United States, causing damage in western Florida. The taxonomy of the insect is:

Orden: Lepidoptera

Family: Noctuidae

Genus: *Spodoptera*

Specie: *Spodoptera frugiperda*

S. frugiperda is an insect that has complex metamorphosis (holometabolous) and, as such, in its life cycle passes through four stages of development: egg, larva, pupa and adult (Sparks, 1979). The eggs are deposited in layers attached to foliage and a layer of scales imparting a furry or moldy appearance covers them. The color of the larvae can vary from light to dark green depending on the instars and plant substrate on which it feeds. In fourth to sixth instars the head is brown or black with a prominent white line between ocelli (simple eyes). The head capsule widths are about 0.35 mm to 2.6 mm since first to sixth instars, respectively, and the lengths are about 1.7 mm to 34.2 mm during the complete larval stage. The pupa is reddish brown in color, and measures 14 to 18 mm in length and about 4.5 mm in width. The pupae may be naked or unprotected, or protected by a cocoon made by the larva before pupation. The moths have a wingspan of 32 to 40 mm. In the male moth, the forewing generally is shaded gray and brown, with triangular white spots at the tip and near the center of the wing, while females are less distinctly marked. The hind wing is iridescent silver-white with a narrow dark border in both sexes (Sparks, 1979).

Distribution and host plants

The fall armyworm (FAW), *Spodoptera frugiperda* is a tropical insect considered as the most important pest of maize crops in the Western Hemisphere (Lopez-Edwards et al., 1999). This pest is distributed in tropical areas of South and Central América (from Argentina and Chile to Mexico) and in subtropical environments of North America, including south Florida

and Texas (Lopez-Edwards et al., 1999; Murua et al., 2008; Prowell et al., 2004) (Figure 1).

The FAW known like maize leafworm or southern grassworm is a polyphagous pest with 186 host plants described in 42 families, with preference for the *Poaceae* family (35.5 %) including maize (*Zea mays),* forage grasses (*Panicum spp*), sugar cane *(Saccharum officinarum)*, rice (*Oryza* sativa) and sorghum (*Sorghum sp*). This pest it is also recorded on soybean (*Glycine max*), peanuts (*Arachis* hypogaea) and cotton (*Gossypium hirsutum*) (Cazmus et al., 2010). This polyphagous condition together with the ability to migrate when weather conditions are not favorable facilitates the wide distribution of the pest in different geographic regions of the west hemisferio.

Figure 1. Geographical distribution map of the fall armyworm *Spodoptera frugiperda* (Ashley, 1986).

Despite *S. frugiperda* has been described in diverse crops, the insect is considered the primary pest in maize due to economic impact in several countries that are major producers of maize in America like United

States (Nagoshi et al., 2007), Brazil (Busato et al., 2004), México (Lopez-Edwards et al., 1999), Argentina (Murua et al., 2008) and Colombia (ICA, 2008).

The fall armyworm has been genetically differentiated in two strains associated to its main host plants: the maize (*Zea mays* L.) and the rice (*Oryza sativa* L.) strains (Prowell, 1986 ; Prowell et al., 2004). These two strains are identical morphologically but differ in their genetics at several markers at the nuclear and mitochondrial level (Nagoshi and Meagher, 2003; Nagoshi et al., 2007; Saldamando and Vélez-Arango, 2010).

In Colombia, the population differentiation was assessed by using a PCR-RFLP of the mitochondrial cytochrome oxidase subunit I (COI) gene and a PCR for the tandem repeated unit FR (FAW rice strain) (Saldamando and Vélez-Arango, 2010). The maize strain was found on 42% in maize, 34% in cotton, 19% in sorghum and 0.04 % in rice crops. In contrast, the rice strain was found on 53% in rice and 35% in maize, 0.06% in cotton, 0.06% in sorghum. These results suggest that maize strain specificity is superior to rice strain (Saldamando and Vélez-Arango, 2010).

Biology and ecology

In the tropical and subtropical regions, the insect breeding can be continuous with four to six generations per year. However, in cold zones the insect behaves like a seasonal pest, due to FAW have no diapause mechanisms. Changes in population density of *S. frugiperda* are strongly influenced by weather conditions and migratory flights. Infestation and migration to southeastern Canada and northern into the eastern and central United States (Figure 1) occurs by the populations of insects that spend the winter in the South and Texas (Mitchell, 1979). In Colombia, the presence of FAW was first detected in 1914 in Antioquia department and was widespread to the Caldas, Valle and Tolima departments (Vélez-Arango et al., 2008).

The FAW has four life stages: egg, larva, pupa and adult (Figure 2). The duration of complete life cycle of the insect depends of environmental conditions. Life cycle occurs in ~30 days during the summer, but 60 days in the spring and autumn, and 80 to 90 days during the winter. When temperatures decrease, the life cycle of the armyworm slows (Morrill and Greene, 1973). Therefore the time duration of each stage is described in a range of time.

Adults' moths have nocturnal habits for feeding, oviposition and mating. Around of 1500 eggs per female are laid on the leaves of the plant host, predominantly in the lower surface of the lower leaves and after hatching 2-10 days the larvae emerge. The larval development through six instars and usually takes 14-21 days. Larval densities are usually reduced to one to two per plant when larvae feed in close proximity to one another, due to cannibalistic behavior. The larvae feed on the young leaves causing skeletonizing effect and can attack the growing point of the plant (bud, whorl, etc.) destroying the growth potential of plants, or clipping the leaves. Pupation normally takes place in the soil, at a depth 2 to 8 cm and occurs inside a loose cocoon, during 9-13 days for development. Adults emerge at night and live for 7-21 days. The moths are most active during warm, humid evening. The females deposit the eggs in first 4-5 days of life.

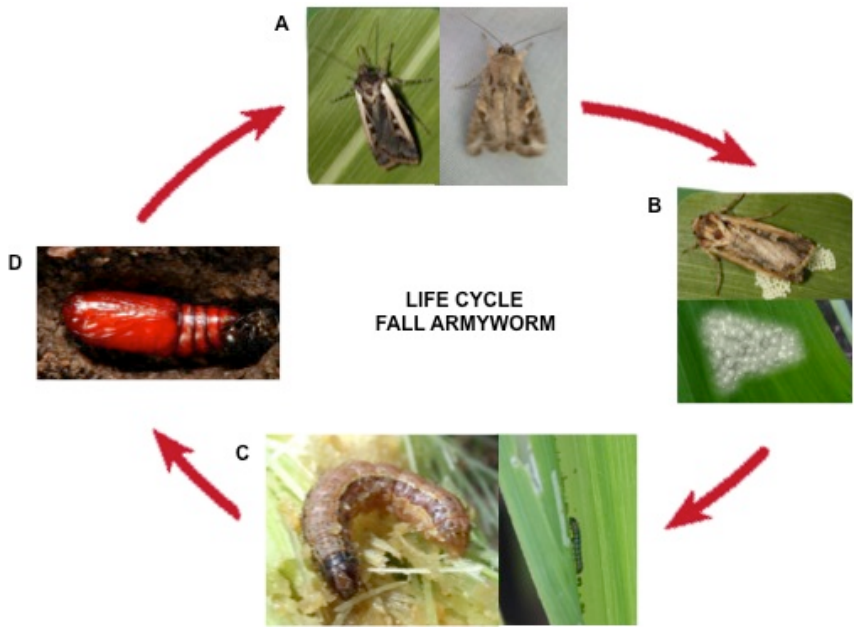

Figure 2. Stages of the life cycle of *Spodoptera frugiperda.* A) Sexually dimorphic female and male moths. B) Female oviposition and eggs. C) Different instars larvae feeding on foliage. D) Pupation in soil.

Damages and control

Larvae cause damage by consuming foliage (Figure 3). Young larvae initially consume leaf tissue from one side, leaving the opposite epidermal layer intact. By the second or third instar, larvae begin to make holes in leaves, and eat from the edge of the leaves inward reducing the photosynthetic area. Feeding in the whorl of maize often produces a characteristic row of perforations in the leaves. Older larvae cause extensive defoliation (Willink et al., 1993). Depending the phenological stages of the plant the larvae cause economical impact, when it affects the young plants the losses may be total, whereas if affects the plants in advanced stage, these can be restored to the defoliation reaching a normal production. In Colombia, FAW causes losses of up 60% in maize (ICA, 2008). Integration of cultural, physical, biological and chemical control, can reduce the insect populations

below economic injury level (Garcia, 1996), which ranged between 35-40% for maize crops in Cuba (Fernández, 2002).

The cultural measures used to help reduce damage include good fertilization to ensure rapid development of the plant and minimize the exposure of young plants to pest attack and rotation of the crops to avoid re-infestation. Additionally, the good elimination of weeds can help to avoid pest use alternative hosts (Garcia, 1996).

Figure 3. *Spodoptera frugiperda* damages to maize plants.

The continuous rains, particularly in the first three weeks of planting maize, which occurs time greater oviposition and larval birth, contribute to the reduction of damage due to high mortality of small larvae. Activities such as irrigation, especially when you spray on at night, interfere with oviposition females and contribute to mortality of small larvae (Garcia, 1996)

Chemical pesticides are the most commonly practice used to reduce the feeding effects of *S. frugiperda* larvae (Chandler and Summer, 1991). Since the 70s and 80s decades Lindane, Carbaryl, and Endosulfan occupied a prominent place to control this pest (Andrews, 1988). More recently, the use of pyrethroids has increased significantly due to its lower persistence in the environment and good efficacy for controlling Lepidoptera including *S. frugiperda*. The agrochemicals have high toxicity and interfere and interfere

with the persistence and effectiveness of the natural control agents causing a negative ecological impact. Additionally, there is a high risk of poisoning and residual effect in the human food by chemical insecticides. Additionally, this control method can be ineffective because the agrochemicals have generated resistance in natural populations of *S. frugiperda* repeatedly treated with chemical insecticides (Chandler and Summer, 1991). These problems have stimulated the developments of more environmental friendly control methods.

S. frugiperda has a complex of natural enemies represented by parasitoids, predators, and pathogens. Numerous species of parasitoids affect fall armyworm and its value in reducing larval populations has long been recognized (Molina-Ochoa et al., 2004; Pair et al., 1986). Several studies have been focused to describe the parasitoids of *S. frugiperda* in America and some had been evaluated to control the pest, like *Telenomus* sp. (Hymenoptera: Scelelionidae), *Euplectrus plathypenae* (Hymenoptera: Eulophidae), *Chelonus insularis* (Hymenoptera: Braconidae), *Rogas* sp. (Hymenoptera: Braconidae), *Archytas marmoratus* (Diptera: Tachinidae), etc. (Andrews, 1988). Some studies has been evaluated the control of *S. frugiperda* using predators like *Podisus nigrispinus* (Heteroptera: Pentatomidae) or *Eriopis connexa* (Coleoptera: Coccinellidae) (Tavares et al., 2010; Zanuncio et al., 2008). Pathogens such as viruses can be considered as control agents due to its high specificity (= biosecurity) and virulence, as for example the *Alphabaculovirus* of *S. frugiperda* (SfMNPV; *Baculoviridae*), some of them with great potential for the development of formulated products (Behle and Popham, 2012; Williams et al., 1999).

BACULOVIRUS TAXONOMY AND MORPHOLOGY

Baculoviruses are arthropod-specific viruses described in around 700 insect species (Martignoni and Iwai, 1981). Despite they have been described infecting several insect orders most of these viruses infect insects of the

orders Lepidoptera, Hymenoptera and Diptera. The presence of these viruses can be traced historically to the development of the silk industry, where they were described affecting the silk worm (Benz, 1986). The first direct observations of the virus as refractile structures were made in mid-1930 and in 1940 with the development of the electron microscopy, the morphology was described as polyhedral structures containing rod shaped structures packing the DNA. The rod shaped gave the name of baculovirus to this group of viruses, proposed by Mauro Martignoni in 1973 (Rohrmann, 2010). Additionally, the same internal structures were observed in viruses with ovoid or granular form. The morphological structures were used to taxonomic classification of the baculovirus in a single family (*Baculoviridae*) and two genera: *Nucleopolyhedrovirus* (NPVs) and *Granulovirus* (GVs) (Bilimoria, 1986). With the analysis of molecular biology, since 2006, the *Baculoviridae* family was divided into four genera based on phylogenetic analysis and common biological and structural characteristics (ICTV, 2012; Jehle et al., 2006), which has been adopted by the International Committee on Taxonomy of Viruses since 2008. Baculoviruses infecting Lepidoptera are divided into *Alphabaculovirus* and *Betabaculovirus* corresponding to the OB morphology of NPVs and GVs, respectively. Baculoviruses that infect Hymenoptera and Diptera insects corresponds to *Gammabaculovirus* and *Deltabaculovirus* genera, respectively. Besides, the *Alphabaculovirus* genera is subdivided into Group I and Group II based on the use of GP64 as their BV fusion protein (Group I) or lack of GP64 and use of protein F (Group II) (Westenberg et al., 2004).

A characteristic feature of baculoviruses is the presence of occlusion or inclusion bodies (OBs) that contains the virions into large proteinaceous capsules which protects the virions from environmental factors and against post-mortem decay in the host (Rohrmann, 2010). The *Baculoviridae* family has two different morphological occlusion bodies, the small ellipsoidal shaped about 0.25 to 0.5 μm called granules characteristic of the genus

Betabaculovirus and polyhedral to irregular shaped with size 0.15 to 15 µm in diameter called polyhedral characteristic of the genera *Alphabaculovirus, Gammabaculovirus,* and *Deltabaculovirus* (Figure 4).

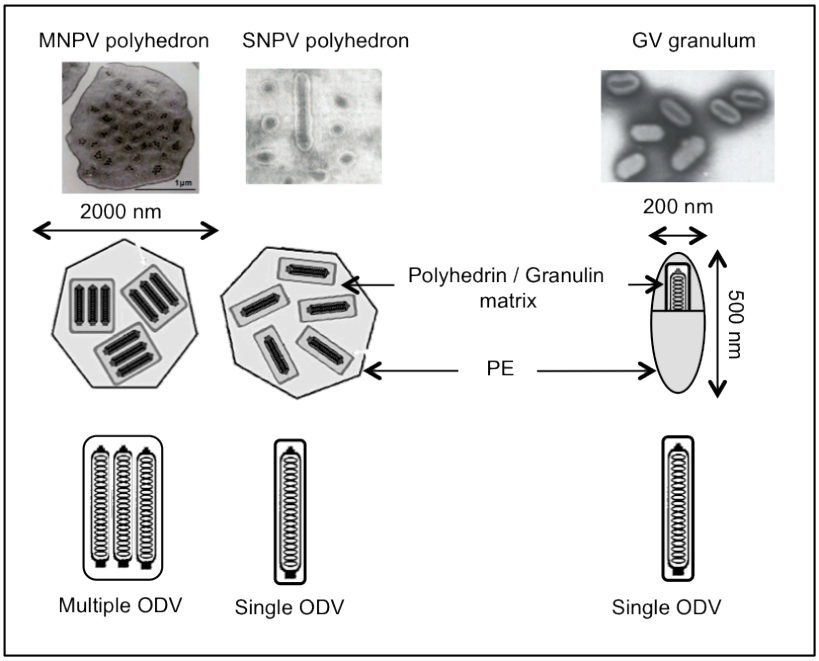

Figure 4. Schematic representations and micrographs of occlusion bodies morphology in the genera of the family *Baculoviridae*

The OBs are composed of matrix protein (30-40% of total viral protein) being polyhedrin the major protein component of polyhedral and granulin the major protein component of granules. These proteins form a crystalline lattice that occludes dozen of virions randomly oriented in a polyhedron or a single virion in a granulum (Miller, 1986). The OBs are surrounded by a polyhedral envelop (PE) composed by a glycoprotein multilayered lattice that forms a net with hexagonal pores ranging from 6 to 15 nm which protein is denominated PP34 (Gross and Rohrmann, 1993). The PE resists the digestion from viral and non-host enzymes but permit

permeation by anions from alkaline host midgut (Gross and Rohrmann, 1993). In the life cycle of the baculoviruses, there are two divergent virion morphotypes: budded virus (BVs) produce during the initial stage of the multiplication cycle (non lytic phase) and occlusion-derived viruses (ODVs) at the final stages of replication (lytic phase) (Blissard and Rohrmann, 1990). Both morphotypes encapsulate nucleocapsids with identical structure and genetic information (Slack and Arif, 2007), however, lipid and protein composition of the envelopes of BVs and ODVs are different due to differences in their assembly pathways (Braunagel and Summers, 1994) (Figure 5).

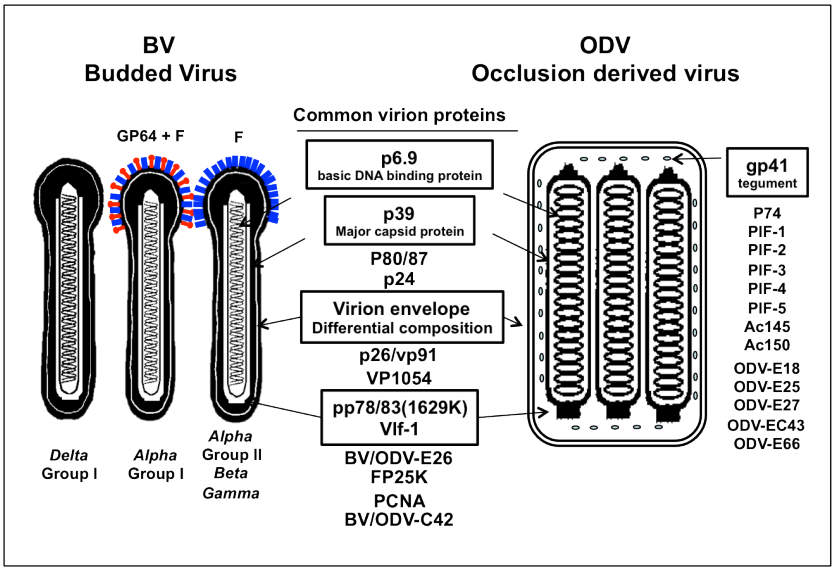

Figure 5. Virions forms produced during the infection cycle of the baculoviruses: budded virus (BV) and occlusion derived virus (ODV).

The ODVs are formed in the nucleus, where the nucleocapsids obtain a lipid bilayer envelope from the inner nuclear membrane (INM) (Braunagel and Summers, 2007) and subsequently are occluded into OBs. The ODVs can contain one (Granulovirus and Nucleopolyhedrovirus simple) or many nucleocapsids (Nucleopolyhedrovirus multiple). They are

responsible for horizontal transmission among susceptible individuals in a population as well as to initiate primary infection (Granados and Williams, 1986), where the ODV envelope fuse directly with membranes of midgut cells of the host (Haas-Stapleton et al., 2004). It has been identified approximately 14 different virus-encoded membrane proteins residing in the ODV membrane (Slack and Arif, 2007). Several of these proteins were shown to be essential for oral infectivity and were named *per os* infectivity factors (PIF), which play important roles in initiating virus entry and infection, and in host range determination (Peng et al., 2012; Slack and Arif, 2007).

BVs only contain a single nucleocapsid (for GVs and NPVs) and obtain its envelope from the plasma membrane of infected cells, when nucleocapsids bud out of the cells. The BVs envelopes are bulbous at one end and have notches in the surface called peplomers (Adams et al., 1977).

The BVs are broadly specialized to infect many internal tissues of the host including tracheoblasts, hemocytes and fat body and occurs in the secondary infection of the host. BVs entry occurs by receptor mediated adsorptive endocytosis (Volkman and Goldsmith, 1985). The major viral proteins of BV membranes are mediators of binding and fusion with endosomal membranes (Blissard and Wenz, 1992). GP64 and its homologous Fusion (F) protein F are the major membrane proteins in BV. GP64 is a member of class III fusion proteins, which are characterized by the fact that they do not require proteolytic cleavage to be activated (Rohrmann, 2010). In contrast, F protein belongs to class I fusion proteins and its activation needs a cleavage event mediated by a furin-like protease or proprotein convertase (IJkel et al., 2000). F protein mediates both receptor binding and virus fusion (IJkel et al., 2000).

The morphology of the nucleocapsids (NC) is similar in all baculoviruses, rod shaped are 40-70 nm in diameter and 250-400nm in length and contain the complete viral genome (Boucias and Pendland,

1998). Nucleocapsids have a base on one end and a apical cap on the other end (Federici, 1986). They are composed principally by structural protein VP39 (Blissard et al., 1989) forming a ring-like structure around the nucleoprotein core (Slack and Arif, 2007). The viral DNA is highly condensed inside of the nucleocapsids by the basic protein P6.9 or VP12 (Slack and Arif, 2007). This polypeptide is characterized by having a high content of arginine, and is recognized as a type of protamine protein (Wilson et al., 1987). Furthermore, p6.9 is a phosphoprotein whose phosphorylation is inhibited by the presence of Zn^{2+} (Funk and Consigli, 1993; Wilson and Consigli, 1985).Thanks to this feature, p6.9 is phosphorylated by kinases at the nuclear pore or in the nucleus of infected cells, which alters the p6.9 protein to allow the release of the viral genome (Wilson and Consigli, 1985). At the base of nucleocapsids other proteins are located such as p24 or p80 and the phosphoprotein PP78/83 (Russell et al., 1997). Both VP39 as PP78/83 intervene in the movement of the nucleocapsid through the cytoplasm of the host cell due to their association with the actin filaments (Charlton and Volkman, 1993). Additionally, nucleocapsids possess other proteins associated to DNA packaging, as VP1054, desmoplakin, alkaline VLF1 nuclease, which collaborate with the proper structuring of the viral DNA (Slack and Arif, 2007).

INFECTION CYCLE

The baculovirus infection occurs exclusively in the larval stages of insects, which consumes OBs from plants or soil contaminated (Figure 6). The OBs are dissolved due to the alkaline pH (10 to 11) of the larval midgut juice and the ODVs are released in the gut lumen. The peritrophic membrane (PM) is the first barrier protecting midgut cells that the ODV must breach. The ODV bypass the PM through lesions or by increment of permeability of PM due to a viral enhancing factor “enhancins” (Rohrmann, 2010; Slack and Arif, 2007).

The infection of the intestinal cells (columnar cells) occurs by fusion of the lipid bilayer envelopes of ODVs with midgut cell membranes, resulting in the release of nucleocapsids into the cytosol. The fusion is mediated by a complex formed by different structural proteins on the ODV surface; p74 (PIF0), PIF1, PIF2, PIF3, PIF4, PIF5 and ODV-E56 (Peng et al., 2010; 2012; Simón et al., 2012). Recently a new *per os* infectivity factor was identified in SfMNPV genome, *sf58*, specific of *Alphabaculovirus* (Simón et al., 2012). The nucleocapsids are transported to the nucleus, most likely under involvement of the cellular microtubular structures. NC enters the nucleus through nuclear pores (Granados and Williams, 1986) and then viral DNA transcription and replication occurs. The nucleocapsids are then transported in the cytosol and "budded" into the extracellular space where pass the basal lamina and travel via the haemolymph and/or tracheal system to infect other cells types to establish a secondary infection (Engelhard et al., 1994). *Alphabaculovirus* possess a broad tropism cells, however *Betabaculovirus* is divided into three groups according to their tropism cells. The first group is characterized by invade the host through the intestinal epithelium and then infect only fatty tissue. The second group infect different cells similar to *Alphabaculovirus* and third group only infects the intestinal epithelium, which has so far only one member, *Harrisina brillians* GV (Mukawa and Goto, 2008). *Gammabaculovirus* and *Deltabaculovirus* viral infection is limited to intestinal cells (Moser et al., 2001; Rohrmann, 2010)

The budded virus (BV) acquires its membrane from the basal side of the cell which consist of plasma membrane and glycoprotein coded by the virus called GP64 (in group I NPVs) and F protein (group II NPVs). Budded virus is responsible for cell-to-cell transmission within the infected insect. GP64 or F protein interacts with a cell membrane receptor molecule and has a role in the fusion with the endosomal structure (Blissard, 1996). The nucleocapsids are released to cytoplasmic space by the acidification of the endosome (Kingsley et al., 1999; Markovic et al., 1998). The BV are

involved in the secondary infection which systematically spread the virus infection via haemolymph among susceptible cells and tissues including tracheal cells, fat body, muscle cells, hypodermis, nerve cells, as well as reproductive and glandular tissues (Federici, 1997; Granados and Lawker, 1981). In these tissues occurs the production of occlusion bodies (OBs) corresponding to the secondary infection, where large amounts of polyhedrin or granulin are synthesized for embedded the nucleocapsids to form OBs to complete the cycle (Hamblin et al., 1990; Wood et al., 1994).

In the final stage of baculovirus infection, two viral genes *chitinase* and *cathepsin* have an important role in the liquefaction of the larval cuticles, releasing millions of OBs to environment to spread and infect other insects. These genes code two lytic enzymes, *chitinase* that is a chitin-degrading enzyme and *cathepsin* that has cysteine protease activity (Slack et al., 1995). The formed OBs protect the virus for many years on the environment, and allow the spread within insect population by horizontally infection to initiate again the cycle.

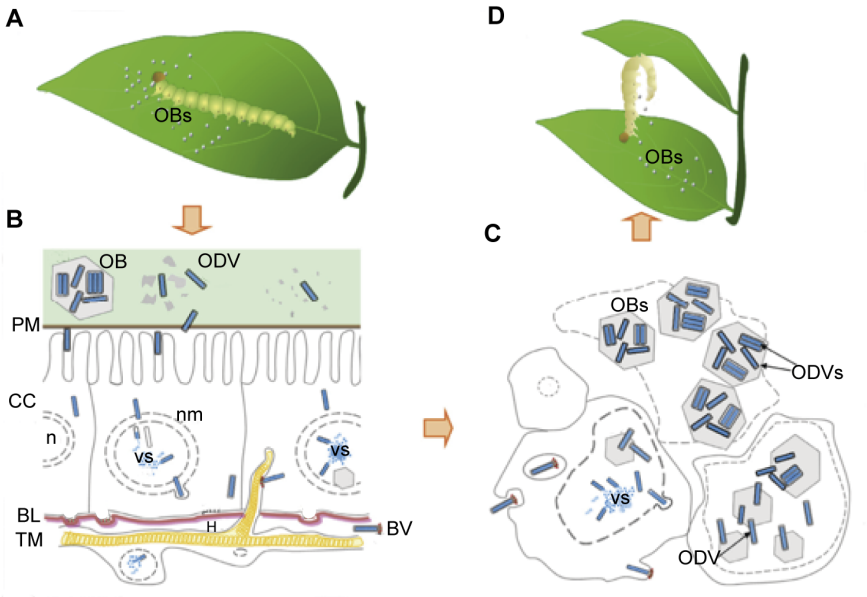

Figure 6. Schematic representation of the baculovirus (*Alphabaculovirus*) infection cycle. A) Larva ingests food contaminated with OBs. B) OBs are solubilised in the midgut releasing ODVs which, upon overcoming the PM, infect midgut epithelial cells. Newly formed nucleocapsids disseminate inside the larval body. C) In the late stage of infection nucleocapsids acquire their envelope from the nuclear membrane forming ODVs (arrows), which may contain one or several nucleocapsids, and are occluded within a polyhedrin matrix forming the OBs. D) Dead larva full of OBs typically appears hanging in a the upper part of the plant. OB (Occlusion Body); ODV (Occlusion Derived Virion); PM (Peritrophic Membrane); CC (Columnar Cell); n (nucleus); nm (nuclear membrane); vs (virogenic stroma); BL (Basal Lamina); TM (Tracheal Matrix); H (Haemolymph) (Ferrelli et al., 2012)..

Genome and gene structure

The baculoviruses have large circular double-stranded DNA genomes ranging in size from 80 to 180 kbp, depending on the species, being the *Neodiprion lecontei* NPV the smallest genome (81,755 Kbp) and *Xestia c-nigrum* GV the largest genome (178,733 Kbp) reported in the Genbank (Hayakawa et al., 1999; Lauzon et al., 2004). The representation of the whole circular genome of the baculovirus includes the *polyhedrin*/*granulin* as ORF number 1 and the following ORFs are numbered sequentially in a

clockwise direction. The orientation of the ORFs is bidirectional, and the clockwise orientation ORFs as *polyhedrin* gen varies between 38 to 56% in the different genomes (van Oers and Vlak, 2007) (Figure 7).

Between 79 and 181 open reading frames (ORFs) have been reported within the genomes, some of which are considered orthologs according to the analysis of complete sequenced genomes and these genes are considered crucial for life cycle of the baculovirus. The similarities among them suggest that these genome regions were present in the common ancestor of baculoviruses (Garavaglia et al., 2012). Until 2011, thirty-three core genes have been described (Miele et al., 2011), but recently with the development of new algorithms for analysis included four additional genes (Garavaglia et al., 2012). The core genes have been grouped into five functional categories that include replication, transcription, packaging and assembly, cell cycle arrest/interaction with host proteins and oral infectivity (Miele et al., 2011) (Table 1). Other non-essential genes could possibly provide adaptative advantages of virus against host (Jehle et al., 2006).

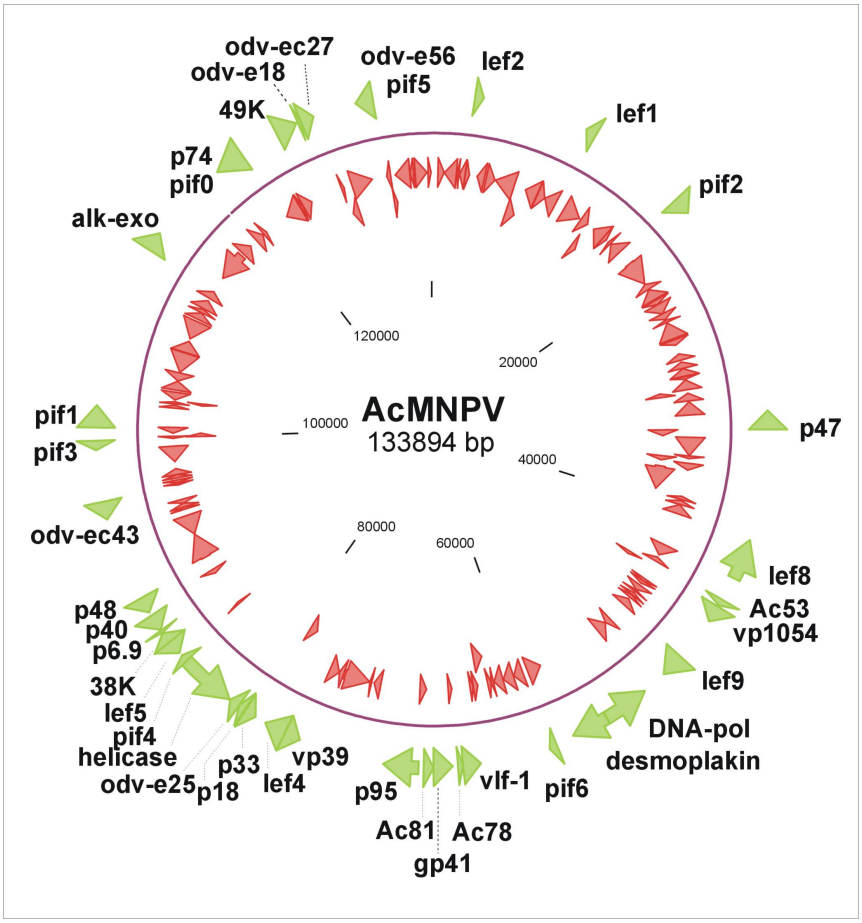

Figure 7. Genomic organization of *Autographa californica* MNPV (ICTV recognized as representantive specie of *Alphabaculovirus*. In green are indicate 37 core genes.

The baculovirus genome presents very short non coding intergenic regions (less to 10%). The transcription termination signals and promoter regions may be included inside other genes ORFs (Ayres et al., 1994; Durantel et al., 1998).

Most of genes of baculoviruses have a single copy, but there are some that have multiples copies, like *bro* genes (*Baculovirus repeated orf*) and *hrs* (homologous regions). In the case of the *bro* genes, there is widely variation in terms of copy numbers between baculovirus species, depending

of viral specie may be up to 16 copies like *Lymantria dispar* MNPV (Kuzio et al., 1999) or variable number of copies, five in BmNPV (Gomi et al., 1999) or a single copy in AcMNPV or SfMNPV (Harrison et al., 2008). Although their function is still unclear, it has been shown that BRO proteins location are viral cycle dependent (nucleus or cytoplasm) and its presence respond to frequent duplication events that would provide advantages regarding adaptive genomes with fewer copies of *bro* (Kang et al., 2006).

Homologous repeat regions (*hrs*) are AT rich regions, composed of repeated sequences which include direct repeats as well as imperfect palindromes motifs. *Hrs* have been reported implicated in functions such as origins of DNA replication and transcriptional enhancement for early gene expression (Pearson and Rohrmann, 1995) and have been found also near to regions of variability in baculovirus genomes (de Jong et al., 2005; Hayakawa et al., 2000; Li et al., 2002). It has been suggested that *hrs* constitute factors of genome plasticity as mediators of intra- and inter-molecular recombination events during baculovirus evolution (van Oers and Vlak, 2007). AcMNPV has nine *hrs* with a copy number of repeats ranging from one to eight (Ayres et al., 1994) and SfMNPV has eight *hrs* with varied number of repeats (1-7) in each *hr* (Harrison et al., 2008). Non-*hr* origins are characterized by direct or inverted repeats, palindromes and AT rich regions, but not present in multiples copies. Non-*hr* origins elements confer strong replication capacity to DNA, which has been confirmed for AcMNPV, SeMNPV, SpltMNPV (van Oers and Vlak, 2007).

Table 1. Core genes shared by 57 baculoviruses

AcMNPV ORF	Gene designation	Description
Replication		
6	*lef-2*	DNA replication/primase-associated factor
14	*lef-1*	DNA primase
65	*dnapol*	DNA replication
95	*helicase*	Unwinding DNA
Transcription		
40	*p47*	RNA polymerase subunit
50	*lef-8*	RNA polymerase subunit
62	*lef-9*	RNA polymerase subunit
90	*lef-4*	RNA polymerase subunit/capping enzyme
99	*lef-5*	Transcription initiation factor
Packaging, assembly, and release		
53	*ac53*	Likely involved in nc assembly
54	*vp1054*	Nucleocapsid protein
66	*Desmop*	Present in nucleocapsid
77	*Vlf1*	Involved in expression of the *p10* and *polh* genes
78	*ac78*	Unknown function/transmembrane domain
80	gp41	Tegument protein
83	*p95*	Viral capsid-associated protein
89	*vp39*	Major capsid protein
92	*p33*	Sulfhydryl oxidase
93	*p18*	Egress of nucleocapsids
94	*odv-e25*	ODV envelope protein
98	*38k*	Required for nucleocapsid assembly
100	*p6.9*	Nucleocapsid protein
101	*p40*	Subunit of protein complex
103	*p48*	BV production and ODV envelopment
109	*odv-ec43*	Associated with ODV
133	*alk exo*	Involved in DNA recombination and replication
142	*49k*	Required for BV production
143	*odv-e18*	ODV envelope protein
Cell cycle arrest and/or interaction with host proteins		
81	*ac81*	Unknown function
144	*odv-e27*	ODV envelope protein
Oral infectivity		
22	*pif-2*	Required for *per os* infection (PIF-2)
68	*ac68*	Required for *per os* infection (PIF-6)
96	*ac96*	Required for *per os* infection (PIF-4)
115	*pif-3*	Required for *per os* infection (PIF-3)
119	*pif-1*	Mediates binding of ODV to midgut (PIF-1)
138	*p74*	Mediates binding of ODV to midgut (PIF-0)
148	*odv-e56*	ODV envelope protein (PIF-5)

Gene expression and DNA replication

Baculovirus genes are expressed in a temporarily regulated cascade of transcriptional events that include four stages: immediate early, delayed early, late and very late (Rohrmann, 2010) (Figure 8).

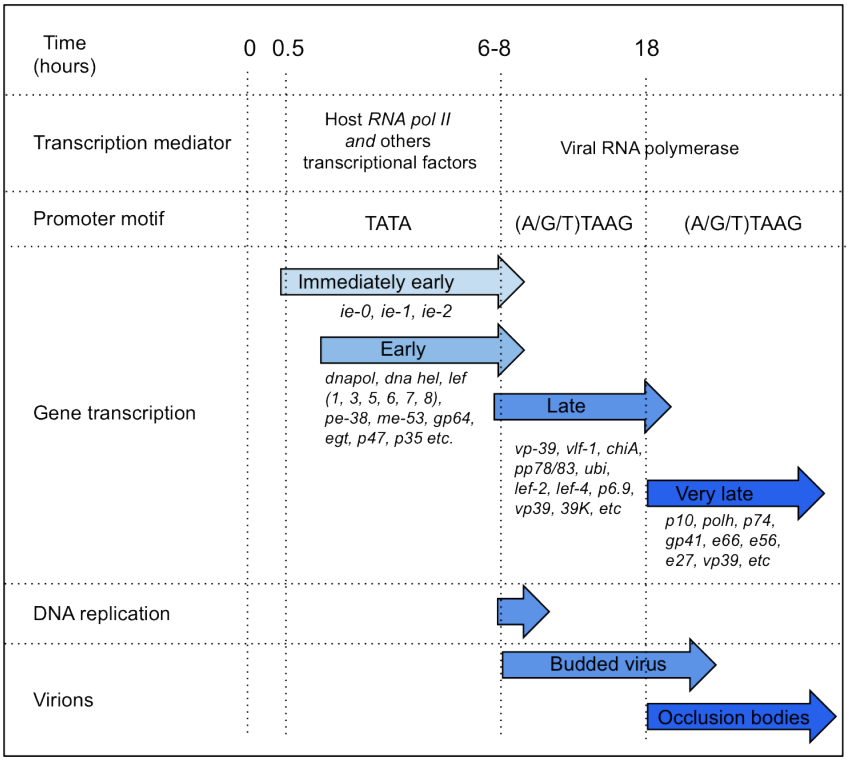

Figure 8. Baculovirus gene expression.

Small DNA viruses use host RNA polymerases to transcribe their genes, in contrast baculovirus use host and viral RNA polymerases (Passarelli, 2007). Thus, the cell host RNA polymerase II transcribes genes of the early stages (Fuchs et al., 1983) and occurs over a period spanning between 0 to 6 hours post infection (h p.i.). The immediate early genes do not require viral factors synthetized of *novo* for transcription (*ie-0*, *ie-1*, *ie-2*, *pe38*) or use of transcription enhancers elements, while delayed early require synthesis of viral factor IE-1 and use enhancers to continue with the

infection (Friesen, 1997; Guarino and Smith, 1992). The late stage require of products expressed at early stage called LEFs (late expression factors) that play important role in DNA replication and late gene transcriptions (Hefferon and Miller, 2002). In the late stage begin the ADN viral replication within the nucleus of the infected cells and occur between 6 and 24 h p.i. Replication origins (*oris*) had been associated to homologous regions (*hrs)* (Cochran and Faulkner, 1983) and to non-*hr* regions (Kool et al., 1994). It had been suggested that DNA replication could include rolling circle mechanism, recombination-depended mechanism, or by a combination of both (Ferrelli et al., 2012). The very late genes expression occurs between 18 and 76 h p.i. and is characterized by a marked increase of transcription of very late genes like *p6.9*, *polyhedrin* and *p10* (Lu and Miller, 1995). The majority of late and very late genes encode virus structural proteins or proteins involved in ODV or polyhedron assembly (Ferrelli et al., 2012).

In the baculovirus genomes there are promoters motifs in upstream regions of early and late genes. Most baculovirus early genes are transcribed by host RNA polymerase II and these genes contain a conserved CAGT motif preceded by a TATA-box (Rohrmann, 2010). There are others early promoters like GATA motif, which recognizes zinc-finger transcription and CGT motif (A(A/T)CGT(G/T) upstream of the TATA box (van Oers and Vlak, 2007). Late gene promoters are characterized by the presence of a canonical (A/T/G)TAAG motif includes the mRNA initiation site. Very late baculovirus genes also have TAAG motifs (van Oers and Vlak, 2007).

BACULOVIRUS DIVERSITY

Inter-specific diversity

Baculoviridae family is characterized by the high degree of inter- and intraspecific diversity. The diversity between the baculoviruses is represented by the large number of insect species that are infected by these

viruses, most of which are included within the orders Lepidoptera, Diptera and Hymenoptera (Martignoni e Iwai, 1987).

The speciation process of the viruses is associated with a host in a co-evolutionary duet, this is reflected by the major lineages clustered into clades based on the host insect that they infect (Rohrmann, 2010). This is supported in the fact that the phylogeny of baculoviruses follows the phylogeny of the different hosts within an order reflecting the pattern of insect families. Thus, the baculoviruses have evolved from non- occluded viruses infecting midgut tissue, to occluded viruses infecting midguts (gamma- and deltabaculoviruses) and finally to occluded viruses with the ability to spread the infection to other tissues (alpha- and betabaculoviruses) (Herniou and Jehle, 2007). In co-evolution process, the viruses have more chances to adapt than the hosts, due to a shorter replication time, higher offspring numbers and a high natural heterogeneity (Herniou and Jehle, 2007)

The actual classification of baculoviruses based on molecular characteristics proposed in the 9th ICTV report (Jehle et al., 2006) and approved by the ICTV in 2008, includes 33 species in *Alphabaculovirus* (lepidopteran NPVs) 14 species in *Betabaculovirus* (lepidopteran GVs), two species in *Gammabaculovirus* (hymenopteran NPVs) and only one specie in *Deltabaculovirus* (dipteran NPVs) (ICTV, 2012) (Table 1).

Based on sequencing studies a criterion for demarcating baculovirus species has been depicted. Jehle et al. (Jehle et al., 2006) suggested that when the distance measured by the Kimura 2-parameter between single or concatenated genes (*polh, lef-8* and *lef-9*) is larger than 0.050, two (or more) viruses are distant enough to be considered as different virus species, however when this distance is smaller than 0.015 belong to the same species. For the pair of viruses with the distance between 0.015 and 0.050, complementary information such as biological characteristics and host range

should be provided for species demarcation (Jehle et al., 2006). According to this criterion, eight new species were proposed and accepted recently (ICTV, 2012).

The first baculovirus genome to be completely sequenced was that of the AcMNPV C6 strain (Ayres et al., 1994). To date genome sequences of more than 60 baculoviruses have been determined, including sequencing of geographical isolates from same specie or even genotypic variants from one isolate.

Intra-specific diversity

The intra-specific heterogeneity is present among isolates belonging to the same species from distant geographic regions (inter-population diversity) (Escribano et al., 1999; Muñoz and Caballero, 2001; Rowley et al., 2010) or even within a single isolate, which exist as mixtures of genotypes (intra-population diversity) that differ in gene content (Cory et al., 2005; Figueiredo et al., 2009; Hodgson et al., 2001; Muñoz et al., 2000; Redman et al., 2010; Simón et al., 2004). The natural conspecific genotypes had been separated by *in vivo* (Cory et al., 2005; Muñoz et al., 1999; Redman et al., 2010) and *in vitro* (Kikhno et al., 2002; Ogembo et al., 2007; Simón et al., 2004; Yanase et al., 2000) cloning techniques. The genetic variation among genotypic variants and geographical isolates has been assessed using DNA restriction endonuclease profiles (Barrera et al., 2011; Escribano et al., 1999; Simón et al., 2004) and DNA sequence variation by PCR approach (Rowley et al., 2010).

Nucleopolyhedrovirus genotypic variants can be co-occluded in a single OB, even within the same virion (Clavijo et al., 2010). It has been estimated that MNPV ODV may contain until 29 genomes (Bull et al., 2001) and this condition seems to favor the infection of later instars and less permissive host species (Zwart et al., 2009).

The genotypic diversity can be the result of natural recombination (Hajós et al., 2000) and the ubiquitous presence of transposon-like elements (Jehle et al., 1998) after simultaneous infections of multiples genotypes. Recombination occurs *in vivo* with high frequency (up to 50%) between closely related virus genotypes (Hajós et al., 2000).

Table 2. Recognized ICTV and sequenced baculoviruses species.

Genus	Name	Abbreviation	Accesion number	Genome (bp)
α-I	*Antheraea pernyi* NPV-Z ***	AnpeNPV-Z	NC_008035	126629
	Antheraea pernyi NPV-L2 ***	AnpeNPV-L2	EF207986	126246
	Anticarsia gemmatalis NPV	AgMNPV-2D	NC_008520	132239
	Autographa californica multiple NPV *	AcMNPV-C6	NC 001623	133894
	Bombyx mandarina NPV **	BomaNPV	NC_012672	126770
	Bombyx mori NPV	BmNPV	NC_001962	128413
	Choristoneura fumiferana DEF multiple NPV	CfDEFMNPV	NC_005137	131160
	Choristoneura fumiferana multiple NPV	CfMNPV	NC_004778	129593
	Choristoneura rosaceana NPV	ChroNPV	NC_021924	129052
	Epiphyas postvittana NPV	EppoNPV	NC_003083	118584
	Hyphantria cunea NPV ***	HycuNPV	NC_007767	132959
	Maruca vitrata NPV ***	MaviMNPV	NC_008725	111953
	Orgyia pseudotsugata multiple NPV	OpMNPV	NC_001875	131995
	Plutella xylostella multiple NPV **	PlxyMNPV	NC_008349	134417
	Rachiplusia ou MNPV **	RoMNPV	NC_004323	131526
α-II	*Adoxophyes honmai* NPV	AdhoNPV	NC_004690	113220
	Adoxophyes orana NPV **	AdorNPV	NC_011423	111724
	Agrotis ipsilon multiple NPV	AgipNPV	NC_011345	155122
	Agrotis segetum NPV ***	AgseNPV	NC_007921	147544
	Apocheima cinerarium NPV **	ApciNPV	NC_018504	123876
	Chrysodeixis chalcites NPV ***	ChChNPV	NC_007151	149622
	Clanis bilineata NPV ***	ClbiNPV	NC_008293	135454
	Ectropis obliqua NPV	EcobNPV	NC_008586	131204
	Euproctis pseudoconspersa NPV ***	EupsNPV	NC_012639	141291
	Helicoverpa armigera multiple NPV	HearMNPV	NC_011615	154196
	Helicoverpa armigera NPV NNg1	HearSNPV	NC_011354	132425
	Helicoverpa armigera NPV	HearNPV-C1	NC_003094	130759
	Helicoverpa armigera NPV G4	HearNPV-G4	NC_002654	131405
	Helicoverpa zea single NPV	HzSNPV	NC_003349	130869
	Leucania separata NPV ***	LeseNPV-AH1	NC_008348	168041
	Lymantria dispar multiple NPV	LdMNPV	NC_001973	161046
	Lymantria xylina MNPV **	LyxyMNPV	NC_013953	156344
	Mamestra configurata NPV A	MacoNPV90-2	NC_003529	155060
	Mamestra configurata NPV B	MacoNPV-B	NC_004117	158482
	Mamestra configurata NPV-90-4	MacoNPV-	AF539999	153656

Genus	Name	Abbreviation	Accesion number	Genome (bp)
	Orgyia leucostigma NPV **	OrleNPV	NC_010276	156179
	Spodoptera exigua multiple NPV	SeMNPV	NC_002169	135611
	Spodoptera frugiperda multiple NPV	SfMNPV-3AP2	NC_009011	131331
	Spodoptera litura NPV	SpliNPV-G2	NC_003102	139342
	Spodoptera litura NPV II	SpliNPV-II	NC_011616	148634
	Thysanoplusia orichalcea NPV		NC_019945	132978
	Trichoplusia ni single NPV	TnSNPV	NC_007383	134394
	Wiseana signata NPV	WisiNPV		
	Buzura suppressaria NPV	BusuNPV		
	Choristoneura occidentalis NPV **	ChocNPV	NC_021925	128446
	Mamestra brassicae multiple NPV	MabrMNPV		
	Spodoptera littoralis NPV	SplitNPV		
	Adoxophyes orana GV	AdorGV	NC_005038	99657
	Agrotis segetum GV **	AgseGV	NC_005839	131680
	Artogeia rapae GV	ArraGV		
	Choristoneura fumiferana GV	ChfuGV		
	Choristoneura occidentalis GV **	ChocGV	NC_008168	104710
	Clostera anachoreta GV **	ClanGV	NC_015398	101487
	Cryptophlebia leucotreta GV	CrleGV	NC_005068	110907
	Cydia pomonella GV *	CypoGV	NC_002816	123500
	Epinotia aporema GV **	EpapGV	NC_018875	119082
	Harrisina brillians GV	HabrGV		
β	*Helicoverpa armigera* GV	HearGV	NC_010240	169794
	Lacanobia oleracea GV	LaolGV		
	Phthorimaea operculella GV	PhopGV	NC_004062	119217
	Pieris rapae GV **	PiraGV	NC_013797	108592
	Plodia interpunctella GV	PlinGV		
	Plutella xylostella GV	PlxyGV	NC_002593	100999
	Pseudaletia unipuncta GV	PsunGV	NC_013772	176677
	Spodoptera litura GV **	SpliGV	NC_009503	124121
	Trichoplusia ni GV	TrniGV		
	Xesti c-nigrum GV	XecniGV		
	Neodiprion lecontei NPV *	NeleNPV	NC_005906	81755
γ	*Neodiprion sertifer* NPV	NeseNPV	NC_005905	86462
	Hemileuca sp. NPV **	He NPV	NC_021923	140633
	Neodiprion abietis NPV**	NeabNPV	NC_008252	84264
δ	*Culex nigripalpus* NPV *	CuniNPV	NC_003084	108252

This table contains all recognized by ICTV and complete genome sequenced baculovirus species, sorted by genus (αI-A*lphabaculovirus* group I, αII-*Alphabaculovirus* group II, β-*Betabaculovirus*, γ-*Gammabaculovirus*, δ-*Deltabaculovirus)*. NPV is the abbreviation of nucleopolyhedrovirus; GV is the abbreviation of Granulovirus. The accession numbers are from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and correspond to the sequences of complete genomes. (*) ICTV recognized species as genus representant. (**) sequenced virus but not in ICTV list. (***) recently included virus in ICTV list.

The genetic variation has been observed in hypervariable regions in the genome rather than evenly spread (Cory et al., 2005; Harrison et al., 2008; Muñoz et al., 1999; Stiles and Himmerich, 1998) and some of the variable regions include homologous regions and *bro* genes, indicating the presence of recombinational hot spots (Erlandson, 2009).

The high intraspecific diversity in the baculoviruses has been demonstrated in several nucleopolyhedrovirus species, e.g. *Pannolis flammea* NPV (Cory et al., 2005), *S. exempta* NPV (Redman et al., 2010) and *S. exigua* NPV (Muñoz et al., 1999) were cloned in 24, 17 and 7 genetically-distinct genotypes respectively using *in vivo* cloning technique. Additionally, *in vitro* cloning technique in insect cells has been used to recover 7 and 25 genotypic variants from *Autographa californica* MNPV (Stiles and Himmerich, 1998) and *Helicoverpa armigera* NPV (Ogembo et al., 2007), respectively. The same technique has been used to separate genotypes from *Spodoptera frugiperda* MNPV (SfMNPV) from Nicaragua and United States field isolates (Harrison et al., 2008; Simón et al., 2004).

The intra-population diversity is a source for natural selection, since genomic variability has important effects in the baculovirus fitness parameters related to pathogenicity, virulence and the OB production (yield). Several of the variants isolated from wild-type NPV showed differences in virulence and pathogenicity (Cory et al., 2005; Harrison et al., 2008; Kikhno et al., 2002; Muñoz et al., 1999; Simón et al., 2004).

The conspecific genotypes can act independently (Milks et al., 2001), synergistically (Hodgson et al., 2004; López-Ferber et al., 2003; Simón et al., 2005c) or antagonistically (Muñoz and Caballero, 2000) depending on various factors that influence the response (environmental conditions, order to infection, insect response, etc). The positive interaction in synergistic interactions could be due to defective genotypes use the transcription products of the complete genotype in co-infected cells (Simón

et al., 2005c; 2006). In the case of the SfMNPV from Nicaragua (SfNIC), all single genotypes were less pathogenic than the wild type or mixture of genotypes, and certain combinations were found to have positive effects (López-Ferber et al., 2003), indicating that the mechanism of this interaction could be due to complementation between genomes or to dilution of an essential factor that would be deleterious if produced by all the genotypes (Simón et al., 2006). The antagonist interaction has been observed in *Spodoptera exigua* NPV genotypic variants where parasitic mutants harbor large deletions with replicative advantage (Muñoz et al., 1998). Defective genomic variants can be maintained within a heterogeneous baculovirus population by the co-occlusion process (Simón et al., 2005c), this can be the basis for viral lineages adaptation to new environmental conditions or new host species (Erlandson, 2009).

Maintenance of diversity

The mechanisms of maintenance of genetic variation could be related to the pathogen like trade-offs between virus fitness components, interactions among genotypes and differential selection for genotypes (Hodgson et al., 2003) and related with the ecology of the host. The trade-offs occur when negative genetic correlations exist among beneficial traits, for instance the correlation between the duration of infection and the production of infective OBs, such that the longer a virus takes to kill its host, the more OBs will be produced (Cory and Myers, 2003). Differential selection occurs when particular genotypes perform better under different ecological conditions, for example during infection of different host species, where alternative hosts present a very plausible option for the generation of diversity (Cory, 2010; Hitchman et al., 2007).

The ecology of the host can be a factor of selection of genotypic variants, promoting the persistence of selected genotypes (Cory, 2010). The tritrophic interactions between plant, insect, and virus genotypes can favor the differential selection of variants. Differential pathogenicity was observed

when two *P. flammea* NPV variants were used to infect *P. flammea* insects reared on two pine species (Hodgson et al., 2002) indicating that the viruses adapt to different host plants to increase their transmissibility (Cory, 2010).

The influence of host plant on virus interactions could be due to many factors including plant architecture that affects virus persistence, palatability that modifies host mobility and virus acquisition, plant chemistry that modulates infection in the gut and nutrient content that determines host survival (Cory and Myers, 2003). Additional environmental factors that affect the virus performance and selection could be sensitivity to ultraviolet irradiation (UV), temperature host life span (Cory, 2010).

NUCLEOPOLYHEDROVIRUS MULTIPLE OF *Spodoptera frugiperda*

Spodoptera frugiperda multiple nucleopolyhedrovirus (SfMNPV) is classified into *Baculoviridae* family, specifically inside the *Alphabaculoviruses* group II (ICTV, 2012; Jehle et al., 2006). SfMNPV has been studied intensively since first report at the beginning of 70s decade (Knudson and Tinsley, 1974; Summers and Anderson, 1973), initially the morphology, structure, physical and serological properties of the virus particle were described (Bud and Kelly, 1980) (Figure 9). Different isolates of SfMNPV were identified from larvae collected from various regions of America (Barreto et al., 2005; Escribano et al., 1999; Loh et al., 1981; Loh et al., 1982; Maruniak et al., 1984; Shapiro et al., 1991) to date appear to be variants of the same virus species.

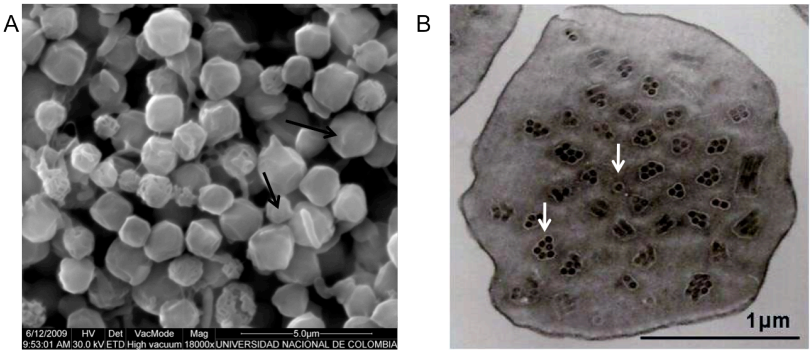

Figure.9. A) Scanning Electron Microscopy micrograph showing OBs of different sizes (black arrows). B) Transmission Electron Microscopy micrograph of an OB. Several virions are found within the OBs. Note that virions may co-envelope several or single nucleocapsids (white arrows).

Due to the special interest in the capacity of the virus as biological control agent, several studies had been focused in the inter (Barreto et al., 2005; Escribano et al., 1999; Loh et al., 1981; Rowley et al., 2010) and intra-population diversity (Harrison et al., 2008; Maruniak et al., 1984; Simón et al., 2004) and studies on NPV ecology have used SfMNPV as a model virus (Simón et al., 2005c; 2006). Genetic variation among SfMNPV isolates with variable fitness has been previously assessed by restriction endonuclease digestion and the size of complete genome of SfMNPV was calculated by construction of physical maps (Loh et al., 1981; Simón et al., 2005a). Several genotypic variants have been recovered from field isolates using *in vitro* plaque assay techniques in insect's cells (Harrison et al., 2008; Maruniak et al., 1984; Simón et al., 2004) and the general characteristic of variants is the presence of deletion genotypes located within a specific hypervariable region (ranging from 1.4 to 16.2 Kb) around the *egt* gene. Harrison *et al*. (2008) purified different genotypic variants from a Missouri field isolate and the SfMNPV-3AP2 pure genotype showed a rapid speed of kill phenotype correlated with a deletion that removed parts of gene encoding ecdysteroid UDP-glucosyltransferase (*egt*). Similarly, deletions were observed in eight of nine genotypes cloned from a Nicaraguan field

isolate (SfNIC) and in some not infective *per os* genotypes, the deletions affected genes such as *pif-1* and *pif-2* (Simón et al., 2004, 2005c). Single genotypes including the complete and deleted genotypes presented lower pathogenicity than wild-type isolates or experimental mixtures of genotypes (López-Ferber et al., 2003; Simón et al., 2005c; 2008), suggesting that interactions between genotypes increase the transmissibility of the wild-type population. The analysis of partial sequence of virus was first made including partial genes and variable regions (Gonzales et al., 1989; Liu and Maruniak, 1995; Simón et al., 2005b; Tumilasci et al., 2003), then complete genome sequence of a Brazilian SfMNPV field isolate (Wolff et al., 2008) and complete genomes of SfMNPV genotypic variants (Harrison et al., 2008; Simón et al., 2011) were published. The comparison of these SfMNPV genomes showed high similarity among them (Harrison et al., 2008; Simón et al., 2011; Wolff et al., 2008).

The SfMNPV Nicaraguan field isolate has been evaluated as a potential biopesticide to control maize armyworm (Armenta et al., 2003; Cisneros et al., 2002; Moscardi, 1999; Williams et al., 1999). Applications of the virus cause significant levels of mortality larvae on maize crop without affecting natural enemy populations (Armenta et al., 2003; Gómez et al., 2013; Williams et al., 1999). It has been observed that these isolates have different effectiveness toward different populations of *S. frugiperda*, being more susceptible to native isolates (Escribano et al., 1999).

BACULOVIRUSES AS BIOLOGICAL CONTROL AGENTS

In recent decades, interest in the use of entomopathogens for the control of insect populations has intensified, especially against those populations that cause economic losses in crops. In this sense, baculoviruses play a major role in the suppression of a variety of different insects, causing epidemics that reduce the size of insect populations of agricultural and forest pest insects (Rohrmann, 2010). Over 50 baculovirus products have been used

against different insect pests worldwide (Moscardi et al., 2011) (Table 3). The baculoviruses have received special attention as microbial insecticides due to their narrow host range, which make them safe for other insects and organisms in the environment (Szewczyk et al., 2006) and their high pathogenicity and virulence.

Table 3. Main examples of baculoviruses developed as microbial insecticides (Moscardi et al., 2011).

Host insect	Baculovirus	Product name (s)	Target crop (s)
Adoxophyes honmai	GV	-	Tea
Adoxophyes orana	GV	Capex 2	Apple
Anticarsia gemmatalis	NPV	Baculovirus Nitral, Coopervirus, Coopervirus, Baculovirus AEE	Soybean
Buzura suppressaria	NPV	-	Tea, tung oil tree
Cydia pomonella	GV	Madex, Virosoft, capex, Carpovirusine, Granupon, Virin Cyap, Cyd-X	Apple, pears
Helicoverpa zea	NPV	Elcar, Gemstar	Cotton, vegetables
Homona magnanima	GV	-	Tea
Lymantria dispar	NPV	Gypcheck	Forestry
Neodiprion abietis	NPV	Abietiv	Balsam fir
Neodiprion lecontei	NPV	Lecontvirus	Pine
Neodiprion serifer	NPV	Neocheck-S, Virox	Forestry
Orgyia pseudotsugata	NPV	TM Biocontrol	Douglas fir
Phthorimaea operculella	GV	PTM Baculovirus, Matapol	Field and stored potatoes
Spodoptera exigua	NPV	Spod-X, Ness-A, Ness-E, Virex	Horticulture, glasshouse and field crops

The insects belonging to Lepidoptera are the main pests that cause significant economic losses to forest and agricultural crops, for that *Alpha-* and *Betabaculoviruses* have been used for the development of biopesticides. The use of baculoviruses as pest control agents started as

early as 1930 with the protection of pine trees with *Diprion hercyniae* NPV (Bird and Burk, 1961). Subsequently, the use of baculoviruses spread to diverse agricultural crops including alfalfa, cabbage, corn, cotton, lettuce, soybean, tobacco and tomato (Granados and Federici, 1986) to control different insects populations as *Anticarsia gemmatalis*, *Helicoverpa* y *Heliothis spp*, *Spodoptera littoralis*, *Spodoptera exigua*, *Cydia pomonella*, *Lymantria dispar*, *Orgyia pseudotsugata*, *Neodiprion sertifer, Neodiprion lecontei* y *Plodia interpunctella*. The firsts commercial baculovirus pesticides were developed for control pests in cotton crops (Sun and Peng, 2007), for instance *Helicoverpa zea* simple nucleopolyhedrovirus (HzSNPV) was registered under the name Elcar^{TM} and was used against the cotton bollworm *Helicoverpa zea,* GemStar was used for control of *Helicoverpa armigera* in different crops including soybean, sorghum, maize, tomatoes and beans (Mettenmeyer, 2002). *S. exigua* MNPV formulations are used to protect sweet peppers in Spain (Caballero et al., 2009; Lasa et al., 2007). In south America, the most recognized experience with the use of baculoviruses for control pest, is localized in Brazil, where the *Anticarsia gemmatalis* multiple nucleopolyhedrovirus (AgMNPV) is used extensively in soybean crops (Moscardi, 1999; Szewczyk et al., 2006).

Some products on basis of SfMNPV have been developed for control of FAW. A wettable powder formulation was evaluated under field conditions with high larvae mortality in Brazil (Valicente and Costa, 1995) and an aqueous formulation was evaluated in maize crops in Honduras and México (Williams et al., 1999). Recently, a fast killing genotypic variant was formulated by encapsulation to provide UV protection and evaluated in field grown cabbage with improved half-life for efficacy (Behle and Popham, 2012).

Despite the baculoviruses are efficient pathogens to control different insect pests; there are some limitations for their use, including the high costs of *in vivo* production and low speed of kill among others. To avoid costs

limitation, approaches that involve *in vitro* production processes in insect cell cultures at low cost (Chakraborty et al., 1996; Nguyen et al., 2011) or improved *in vivo* production are being investigated (Bhutia et al., 2012; Elvira et al., 2013).

Several approaches have been used to produce fast acting baculoviruses, such as development of recombinant viruses. These viruses can express specific toxin (Inceoglu et al., 2006), hormone (Elvira et al., 2010) or enzyme (Gramkow et al., 2010). However, public aversion to liberate genetically modified organisms (GMOs) (Szewczyk et al., 2006) has focused on the research o new isolates or genotypes with improved phenotypic characteristics. Additionally, the use of natural fast killing genotypes have been evaluated (Behle and Popham, 2012).

The inactivation caused by solar radiation under field conditions is the main environmental factor limiting the widespread use of these biocontrol agents (Ignoffo and García, 1992; Ignoffo et al., 1997). Actually, there have been developed some protective agents against UV that have been included into the viral formulations (Bernal et al., 2013; Mondragón et al., 2007; Zhu et al., 2013). Additionally, microencapsulation of baculoviruses has been investigated as a promising alternative for the formulation baculoviruses control agents. This technique allows coating small solid particles in a thin uniform layer of coating material, protecting the virus from environmental conditions and improving the storage stability (Behle and Popham, 2012; Villamizar et al., 2010).

The use of synergism between two different pathogens is a strategy that could be useful to improve the baculoviruses pest control. For instance, a synergistic effect was observed in a *Lymantria dispar* nucleopolyhedrovirus and *S. frugiperda* granulovirus mixture (Hoover et al., 2010). This effect is attributed to the presence of enhancin protein, that alter the peritrophic membrane of the larvae, allowing rapid entry of viral particles

to the cells, thereby decreasing time of kill and increasing the pathogenicity (Hoover et al., 2010; Mukawa and Goto, 2007).

Other strategies to enhance the activity of baculoviruses have included the use of chemical or biological substances added to virus formulations such as inorganic acids (Cisneros et al., 2002) and sublethal concentrations of chemicals (McCutchen et al., 1997) or botanical insecticides (Zamora-Avilés et al., 2013) among others.

Knowledge of the biology of the baculovirus progress toward improving formulations of baculovirus based biopesticides because there is no doubt that they have a much lower risk to the environment than traditional chemical pesticides (Szewczyk et al., 2006).

Although baculoviruses are an efficient tool for pest control, their evaluation in field conditions presented inconsistent results (Villamizar et al., 2009). In this sense, deleterious effect of environmental conditions as temperature and solar radiation on the viral particles (Ignoffo and García, 1992), as well as the low storage stability are the main problems that can limit the results. Others factors that affects the performance of biopesticides includes density of the pest, crop phenology and insect eating habits, among other factors. To set the appropriate parameters for the use of a biopesticide, it is necessary to evaluate the product under greenhouse and field conditions (Grzywacz et al., 2008; Gupta et al., 2007; Lasa et al., 2007). Crops validation of biopesticides is the only way they can be subjected to real conditions of field, climate, natural density of pest and management practices

SCOPE OF RESEARCH

Despite the high potential of SfMNPV as a biopesticide, in Colombia no product based on this virus has so far been registered. The aim of this study was to develop a strategy based on a local SfMNPV to control fall armyworm in Colombia.

The first step was the study of the natural diversity of SfMNPV isolates in Colombia. The **chapter II** describes the analysis of thirty-eight SfMNPV isolates collected from infected larvae in three different geographical regions of Colombia. The molecular characterization was made using restriction endonuclease analysis (REN) and physical map of selected isolate (SfCOL) was constructed by comparison with a previously characterized Nicaraguan isolate (SfNIC). The genome size of SfCOL was estimated and genomic differences were described by sequencing. The biological activity of SfCOL was compared against both native and foreign insect colonies, demonstrating the importance of the use of native isolates for control local pests. The SfCOL insecticidal properties and its potential as active ingredient for biopesticide development were demonstrated.

The following step was to study the intraspecific diversity of SfCOL isolate. In **chapter III** ten distinct genotypes were purified by plaque assay from SfCOL isolate. The genetic differences among genotypes were studied by sequencing and physical maps were constructed. Molecular characterization of different genotypes showed the presence of distinctive sizes deletions localized in a variable region. The variable regions among genotypes were sequenced and two ORFs previously not reported in SfMNPV genomes were described. The insecticidal activity from each genotypic variant and their mixtures was studied. Antagonistic interaction between genotypes was observed, being SfCOL-A variant the most pathogenic. This chapter suggests the use of SfCOL-A as the active ingredient for biopesticide development.

The **chapter IV** describes the effect of OBs formulation over the insecticidal activity of SfCOL-A variant compared to SfCOL wild type (SfCOL-wt) in laboratory, greenhouse and field conditions. SfCOL wild type and SfCOL-A genotypic variant were formulated as a wettable powder by microencapsulating the occlusion bodies with the methacrylic acid polymer Eudragit S100®. In order to determine the effect of the formulation over viral

efficacy, different bioassays were performed with both formulated (SfCOL-A-F, SfCOL-wt-F) and unformulated (SfCOL-A and SfCOL-wt) viruses. High protection against inactivation by UV irradiation through the formulation was observed and formulated viruses maintained efficacies higher than 80% under greenhouse conditions. The formulated virus efficiently reduced insect damage under field conditions. The plant damage on maize plantation was inferior to thresholds of economical impact when formulated virus was used.

The final **chapter V** describes the most relevant results and the conclusions obtained in this thesis that is focused into development of a strategy for control fall armyworm in maize crops in Colombia. This is the first research work that use molecular tools to analyze the natural diversity of SfMNPV in Colombia and apply the knowledge generated in the development of a strategy for pest management.

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CHAPTER II

Spodoptera frugiperda MNPV as a potential biological insecticide: genetic and phenotypic comparison of field isolates from Colombia

ABSTRACT

Thirty eight isolates of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV), collected from infected larvae on pastures, maize and sorghum plants in three different geographical regions of Colombia, were subjected to molecular characterization and were compared with a previously characterized Nicaraguan isolate (SfNIC). Restriction endonuclease analysis (REN) using six different enzymes showed two different patterns among Colombian isolates, one profile was particularly frequent (92%) and was named SfCOL. The physical map of SfCOL was constructed and the genome was estimated to be 133.9 Kb, with few differences in terms of number and position of restriction sites between the genomes of SfNIC and SfCOL. The *Pst*I-K and *Pst*I-M fragments were characteristic of SfCOL. These fragments were sequenced to reveal the presence of seven complete and two partial ORFs. This region was collinear with SfMNPV *sf20* to *sf27.* However, two ORFs (4 and 5) had no homologies with SfMNPV ORFs, but were homologous with *Spodoptera exigua* MNPV (*se21* and *se22/se23*) and *Spodoptera litura* NPV (*splt20 and splt21*). Biological characterization was performed against two different colonies of *S. frugiperda*, one originating from Colombia and one from Mexico. Occlusion bodies (OBs) of the SfCOL isolate were as potent (in terms of concentration-mortality metrics) as SfNIC OBs towards the Mexican insect colony. However, SfCOL OBs were twelve times more potent for the Colombian colony than SfNIC OBs and three times more potent for the Colombian colony than for the Mexican colony. SfCOL and SfNIC showed a slower speed of kill (by ~50 h) in insects from the Colombian colony compared to the Mexican colony, which was correlated with a higher production of OBs/larvae. SfCOL is a new strain of SfMNPV that presents pathogenic characteristics that favor its development as the basis for a biopesticide product in Colombia.

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INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an important and widely distributed pest in tropical and sub-tropical areas of the Americas. This species can cause severe yield losses in maize, rice and sorghum (Murua and Virla, 2004; Sparks, 1979). Chemical control is widely employed against this pest but can have an adverse impact on populations of non-target organisms and excessive use can favour the development of pest resistance (Chandler and Summer, 1991). The multinucleocapsid nucleopolyhedrovirus (NPV) of *S. frugiperda* (SfMNPV) has been isolated from fall armyworm populations in North, Central and South America (Berretta et al., 1998; Escribano et al., 1999; Loh et al., 1982; Shapiro et al., 1991). Some isolates have been evaluated under field conditions as potential biopesticides to control *S. frugiperda* on maize (Armenta et al., 2003; Cisneros et al., 2002; Moscardi, 1999; Williams et al., 1999) resulting in important levels of larval mortality combined with significant mortality from natural parasitism (Armenta et al., 2003; Castillejos et al., 2002).

Three SfMNPV isolates from Nicaragua, United States and Brazil have been biologically and genetically characterized and their genomes have been completely sequenced (Harrison et al., 2008; Simón et al., 2004; 2011; Wolff et al., 2008). With the aim of selecting a candidate SfMNPV isolate for use in biocontrol experiments in Mexico and Honduras, four SfMNPV isolates were collected from geographically distant populations (United States, Nicaragua and Argentina) and were characterized by restriction endonuclease (REN) analysis (Escribano et al., 1999). The Nicaraguan isolate (SfNIC) was selected for formulation and field trials in Honduras and Mexico as it showed the highest insecticidal activity against a Mexican population of *S. frugiperda*, as measured by lethal concentration metrics (Williams et al., 1999).

The population genetic structure and genomic organization of the SfNIC isolate have been the subject of intensive study. Following plaque purification, the SfNIC population was found to comprise nine genotypes (named SfNIC-A to I). The principal variation between the genotypes was located in a single region of the genome, between map units 14.8 and 26.8. This region includes *Pst*I-F, -L, -K fragments (Simón et al., 2004, 2005b). These genetic differences were reflected in important differences in the insecticidal activity, speed of kill, occlusion body (OB) production or number of virions within OBs of each of the genotypes (Simón et al., 2004; 2008).

In Colombia, *S. frugiperda* causes losses of up 60% in maize and the need for sustainable control measures against this pest is well recognized in this region (ICA, 2008; Quintero et al., 2004). The aim of this study was to select a SfMNPV isolate for use against *S. frugiperda* in a control program currently under development in Colombia. To achieve this, the natural diversity of SfMNPV isolates collected in Colombia was evaluated by molecular and biological characterization and compared with the Nicaraguan isolate that has been extensively characterized.

MATERIALS AND METHODS

Insects-source and rearing

Larvae of *S. frugiperda* were obtained from two laboratory colonies. One colony was established at the Universidad Pública de Navarra (UPNA) with pupae received from ECOSUR, Tapachula, southern Mexico. This colony was refreshed periodically with pupae from a colony maintained in ECOSUR over a period of approximately 5 years. The second colony was established in the Biological Control Laboratory of the Colombian Corporation of Agricultural Research (CORPOICA) using larvae collected from maize fields close to Bogota, Colombia. Both colonies were maintained at 25 ºC, 75% RH (relative humidity) and 16h light:8h dark photoperiod on a wheat germ-based semi-synthetic diet (18% corn flour, 3.4% brewer's yeast, 3.2%

wheatgerm, 1.5% casein, 0.45% ascorbic acid, 0.11% nipagin, 0.05% formaldehyde, 0.13% benzoic acid and 1.5% agar) described by Greene et al. (1976).

Virus isolates and amplification

Thirty-eight *S. frugiperda* larvae with typical signs and symptoms of baculovirus infection were collected in pastures, maize and sorghum crops located in three departments of Colombia: Córdoba, Tolima and Meta. OB suspension obtained from the field collected samples were amplified in a single passage in *S. frugiperda* fourth instars from the Colombian colony by the droplet feeding method (Hughes and Wood, 1981). For this, 25 starved larvae were inoculated orally with an OB suspension and reared individually until death. The OBs from cadavers were purified as described by Caballero et al. (1992). The OB pellet was resuspended in two volumes of milli-Q water and the concentration was determined using an improved Neubauer haemocytometer (Hawksley Ltd., Lancing, UK) under phase contrast microscopy at x400. Purified OBs were stored at 4 ºC.

Viral DNA extraction and REN analysis

Virions were released from OBs by mixing 100 µl of purified OB suspension (~10^9 OBs/ml), with 100 µl of 0.5 M Na_2CO_3, 50 µl of 10% sodium dodecyl sulphate (SDS) 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 × *g*, 5 min). The supernatant containing the virions was treated with 25 µl proteinase K (20 mg/ml) for 45 min 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 0.1x TE (Tris-EDTA) buffer for 10 min at 60°C. DNA concentration was estimated by spectrophotometric absorbance at 260 nm wavelengths. For REN analysis the 38 Colombian isolates were compared with reference to SfNIC (Simón et al., 2004). A sample of 2 µg of

viral DNA was mixed with 10 U of one of the following restriction enzymes *Pst*I, *Bam*HI, *Hin*dIII, *Eco*RI, *Bgl*II and *Sma*I (Takara, Shiga, Japan) and incubated for 4 to 12 h at 37°C. Reactions were stopped by mixing with 4 µl of loading buffer solution (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). An isolate showing the predominant REN profile was named SfCOL and was selected for further molecular and biological characterization.

Physical mapping

The physical map of SfCOL was achieved by comparison with the complete genome sequence and the physical map of the Nicaraguan isolate (Simón et al., 2004; Simón et al., 2005a; Simón et al., 2011). For this, restriction fragments obtained after digestion of SfCOL DNA with different enzymes were compared with those of Nicaraguan isolate and co-migrating fragments and isolate specific fragments were identified. Fragments absent in the SfCOL profiles were eliminated from the physical maps and the isolate-specific fragments were included. On this basis, preliminary physical maps were constructed. Physical maps were confirmed by terminal sequencing of SfCOL isolate-specific *Pst*I fragments.

Two differential bands that were found to be characteristic in the SfCOL *Pst*I profile, namely *Pst*I-K (4.9 Kb) and *Pst*I-M (3.6 Kb) fragments, were used for cloning and sequencing. The selected fragments were cloned into pUC19 plasmid using low melt agarose ligation. The ligation reaction was prepared by mixing 50 ng of purified vector pUC19, 10x ligase buffer, 2U of ligase enzyme and 15 µl of *Pst*I fragments obtained after melting excised pieces of agarose, incubated at 16ºC overnight and used to transform One Shot *Escherichia coli* cells (Invitrogen). The cells were plated on LB-ampicillin (100 mg/ml) plates containing IPTG (0.1 mM) and X-gal (40

µg/ml). White colonies that included the DNA fragments were amplified in liquid LB containing ampicillin (100 mg/ml) at 37ºC overnight. DNA was then extracted by alkaline lysis, digested with the relevant endonuclease and separated on 1% agarose and compared with SfCOL total genomic DNA digests.

The *Pst*I-K (4.9 Kb) and *Pst*I-M (3.6 Kb) fragments were completely sequenced by the primer walking method and assembled. Sequence information was analyzed for the presence of open reading frames (ORFs) using Open Reading Frame Finder (NCBI). Homology searches were performed at the nucleotide and amino acid levels using all putative ORFs. DNA and protein comparisons with entries in the updated GenBank/EMBL, SWISS PROT and PIR databases were performed using BLAST (NCBI) (Altschul et al., 1990; Pearson, 1990).

Biological characterization

The insecticidal activity of the SfCOL isolate was compared with that of SfNIC strain. The median lethal concentration (LC_{50}) and mean time to death (MTD) were determined in *S. frugiperda* larvae originating from both the Mexican and the Colombian laboratory colonies. For this, second-instars 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 the following five concentrations for each isolate: $1.92x10^3$, $9.60x10^3$, $4.80x10^4$, $2.40x10^5$ and $1.20x10^6$ OBs/ml. This range of concentrations was previously determined to kill between 5 and 95% of experimental insects from the Mexican colony (Simón et al., 2004), based on an average volume of 0.077 ml consumed by each larva (Simón et al., unpublished data). Control larvae were treated identically but drank from a sucrose and dye solution that did not contain OBs. Larvae that ingested the suspension within 10 min were transferred to individual wells of a 24-well tissue culture plate with a piece of semisynthetic diet. Bioassays were performed using 24 larvae per virus concentration and 24 control larvae.

Insects were reared at 25°C, and mortality was recorded every 8 h until death or pupation. The bioassay was performed on three occasions. Virus induced mortality was subjected to probit analysis using the PoloPlus program (LeOra-Software, 1987).

Time mortality data were subjected to Weibull survival analysis using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993). Equivalent effective OB concentrations for time mortality assays were based on ~90% mortality estimates from the concentration-mortality bioassay. Groups of 24 overnight starved second instars from the Mexican colony, were inoculated with $2.5x10^6$ OBs/ml of SfNIC OBs and $1.5x10^6$ OBs/ml for SfCOL, using the droplet feeding method, reared on semisynthetic diet at 25 °C, and checked at intervals of 8 h until death. Similarly, insects from the Colombian colony were inoculated with $1.2x10^9$ OBs/ml for SfNIC, and $2.2x10^6$OBs/ml for SfCOL. The assay was performed on three occasions.

OB production of SfNIC and SfCOL isolates was determined in cohorts of 24 overnight-starved second instars inoculated with an OB concentration that resulted in ~90% mortality and reared on semisynthetic diet at 25 °C until death. The assay was performed three times. All the larvae that died from NPV disease (at least 20 for each virus treatment per replicate, a total of ~60 larvae per virus treatment) were individually collected and stored at -20ºC. For OB counting, each larva was homogenized in 100 µl of distilled water and counted in triplicate in a Neubauer hemocytometer. The results were analyzed by Kruskal-Wallis and Mann-Whitney nonparametric statistics using the SPSS program (SPSS version 10.0). Values of *P* were subjected to false discovery rate adjustment for multiple pairwise comparisons (Benjamini and Hochberg, 1995).

RESULTS

REN patterns and genome size of Colombian NPV isolates

Two different REN profiles were identified in the 38 isolates from Colombia following treatment with six restriction endonucleases. Of these, 35 isolates (92.1%) were identical in all six REN profiles. This predominant profile was named SfCOL, and was selected for further molecular and biological characterization. The selected isolate originated from pasture in Monteria, Córdoba Department. The three other isolates presented identical REN profiles that differed only slightly from that of SfCOL (data not shown).

The REN profiles of the SfCOL DNA obtained after digestion with *Pst*I, *Bam*HI and *Hind*III were very similar to the corresponding SfNIC profiles and digestion fragments were designated alphabetically (Figure 1). Co-migrating fragments were detected by stain intensity in *Hind*III (H-I and M-N), the *Pst*I (fragments A-B, I-J and K-L) and in the *Bam*HI REN patterns (fragments A-B).

The REN profile of SfCOL isolate showed two fragments, *Pst*I-K (4.9 Kb) and *Pst*I-M (3.6 Kb), that were absent in SfNIC whereas the SfCOL isolate lacked a 7.5 Kb fragment present in SfNIC (*Pst*I-F) (Figure 1, Table 1). The *Bam*HI profile of the Colombian isolate was also similar to that of SfNIC except that *Bam*HI-F fragment (5.1 Kb) present in SfNIC was absent in SfCOL. *Hind*III profiles were also similar, except that *Hind*III-C and -D fragments (14.8 and 13.1 Kb) present in the SfNIC profile were absent in SfCOL, whereas an additional fragment *Hind*III-A (28.8 Kb) was present in SfCOL. The remaining fragments co-migrated in SfNIC and SfCOL profiles, suggesting that those fragments were similar.

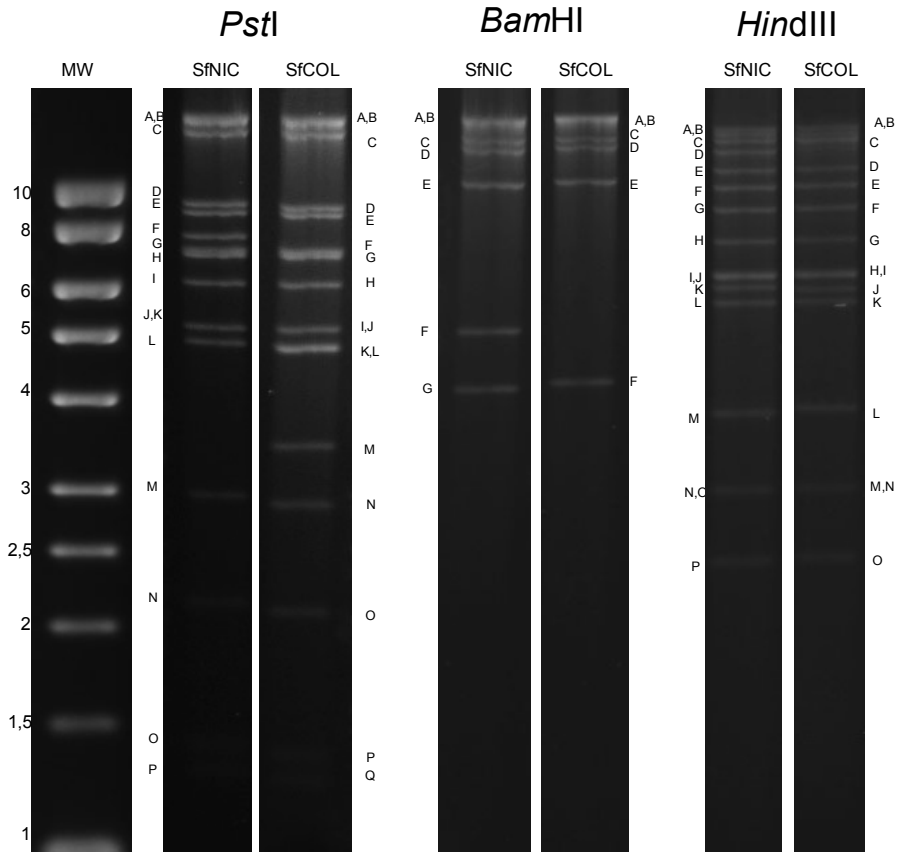


Figure 1. REN patterns of Colombian SfMNPV isolate DNA (SfCOL) digested with *Pst*I, *Bam*HI and *Hin*dIII as indicated, generated by electrophoresis in 1% agarose gel. The REN patterns were compared with those of Nicaraguan isolate (SfNIC) generated with the same enzymes. All DNA fragments are marked with a letter corresponding to their sizes. The first lane indicated the molecular weight (MW) that corresponds to a 1 Kb DNA marker (Stratagene).

Table 1. Molecular sizes of *Pst*I, *Bam*HI and *Hind*III restriction endonuclease fragments of SfMNPV genomic DNA from Nicaraguan (SfNIC) and Colombian (SfCOL) isolates.

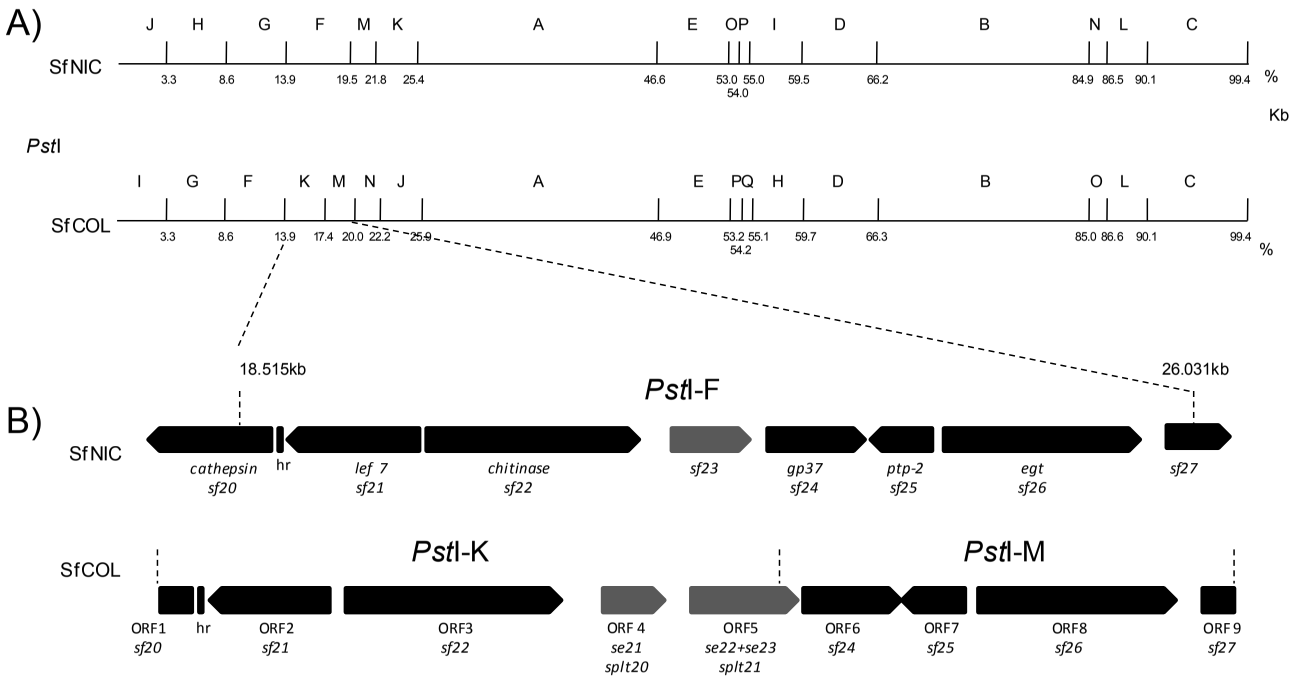
	Restriction size fragments (bp)					
Fragment	***Pst*I**		***Bam*HI**		***Hind*III**	
	SfNIC	SfCOL	SfNIC	SfCOL	SfNIC	SfCOL
A	28,084	28,084	53,872	54,655	17,385	28,843
B	24,926	24,926	28,992	28,992	16,151	17,385
C	12,465	12,465	16,158	19,460	14,826	16,151
D	8,864	8,864	14,179	16,158	13,072	11,008
E	8,481	8,481	10,26	10,260	11,008	8,452
F	7,517	7,079	5,119	4,216	8,452	7,498
G	7,079	6,932	4,216	158	7,498	7,182
H	6,932	6,124	158		7,182	6,231
I	6,124	5,182			6,231	6,175
J	5,182	4,899			6,175	5,886
K	4,899	4,887			5,886	5,552
L	4,769	4,769			5,552	3,775
M	2,953	3,575			3,775	2,988
N	2,112	2,953			2,988	2,428
O	1,339	2,112			2,428	2,131
P	1,228	1,339			2,131	930
Q		1,228			930	676
R					676	608
S					608	
Total	132,954	133,899	132,954	133,899	132,954	133,899

The sizes of the SfCOL REN fragments were estimated by comparison with the known sizes of SfNIC fragments and the complete genome sequence (Simón et al., 2005a) (Figure 1, Table 1). The SfCOL genome was estimated to be 133.9 Kb in length, calculated from the average sum of restriction fragment sizes across the three endonuclease digests, which was similar to that of SfNIC (132.9 Kb).

Physical maps of the SfCOL genome

The physical map was constructed based on the map of SfNIC (Simón et al., 2005a). The *Pst*I-F (7.5 Kb) fragment present in SfNIC was absent in SfCOL, whereas two additional fragments *Pst*I-K (4.9 Kb) and *Pst*I-M (3.6 Kb) were apparent in SfCOL (Figure 1). The sum of the sizes of these two additional fragments (8.5 Kb) was similar to that of the SfNIC *Pst*I-F fragment, suggesting that the SfCOL genome likely presents an insertion of ~1.0 Kb in this region. No other differences were observed. These observations led us to believe that the SfCOL *Pst*I-K and *Pst*I-M fragments were the result of an additional restriction site, not present in the *Pst*I-F region of the SfNIC genome. To confirm this, the *Pst*I-K and *Pst*I-M fragments were sequenced. This region was 8,462 bp in length encompassing SfCOL *Pst*I-K (4,887 bp) and *Pst*I-M (3,575 bp) fragments (Figure 2) that correspond to the genomic region between m.u. 13.9 and 20.0 of the SfCOL genome. These sequences were homologous and collinear to SfNIC *Pst*I-F that correspond to the genomic region between nucleotides 18,515 and 26,031 (Simón et al., 2005a; Simón et al., 2011). Seven complete ORFs were identified and two partial ORFs located at the ends of the *Pst*I-K and *Pst*I-M regions; these were named ORF1 – ORF9. The partial ORF1 in SfCOL *Pst*I-K fragment corresponds to the 5' end of SfNIC *Pst*I-F fragment, whereas the other partial ORF (ORF9) corresponds to the 3' end of SfNIC *Pst*I-F fragment. A homologous repeat region (44 bp) with imperfect palindrome was identified between ORF1 and ORF2. ORF1 corresponds with *cathepsin*, ORF2 with *late expression factor 7* (*lef7*), ORF3 with *chitinase,* ORF4 and ORF5 with proteins of unknown function, ORF6 with *gp37*, ORF7 with *protein tyrosine phosphatase-2* (*ptp-2)*, ORF8 with *ecdysteroid UDP-glucosyltransferase* (*egt)* and ORF9 with an unknown function protein homologous to *sf27* (Figure 2B).

Figure 2. (A) Comparison of *Pst*I physical maps of SfNIC and SfCOL genomes. The position and orientation of the SfMNPV *polyhedrin* (*polh*) and *ribonuclease reductase* (*rr*) genes are indicated by arrows. (B) ORFs present in the *Pst*I-F fragment of the SfNIC genome, and partial ORFs found in the 5' end of the *Pst*I-K and 3' end of the *Pst*I-M fragments of SfCOL profile. The homologous genes are indicated below the arrows



The SfCOL deduced amino acid sequences were compared with SfNIC (Simón et al., 2011), SfMNPV from United States (Sf3AP2) (Harrison et al., 2008), *Spodoptera exigua* MNPV (SeMNPV) (Ijkel et al., 1999) and *Spodoptera litura* NPV (SpltNPV) (Pang et al., 2001) genomes (Table 2). The percentage of amino acid sequence identity (Id) and similarity (Sim) revealed that five complete ORFs (2, 3, 6, 7 and 8) and two partial ORFs (1 and 9) presented homology with SfMNPV genomes (Id 94 - 100%; Sim 96 - 100%), however, the ORF4 and ORF5 did not present homologies with SfNIC or Sf3AP2. These ORFs shared homology with *splt20* and *splt21* ORFs of the SpltNPV genome respectively (Id 96 - 98%; Sim 98%) and with *se21* and *se22* + *se23* ORFs of the SeMNPV genome (Id 70 - 79%; Sim 79 - 86%). In contrast, the sequenced region of SfCOL lacked ORF *sf23* present in the other SfMNPV genomes. The genomic organization of SfCOL is more closely related to that of Sf3AP2, SfNIC and SeMNPV than to SpltNPV, although orthologs of *splt20* and *splt21* were detected in SfCOL with a high degree of similarity (Table 2).

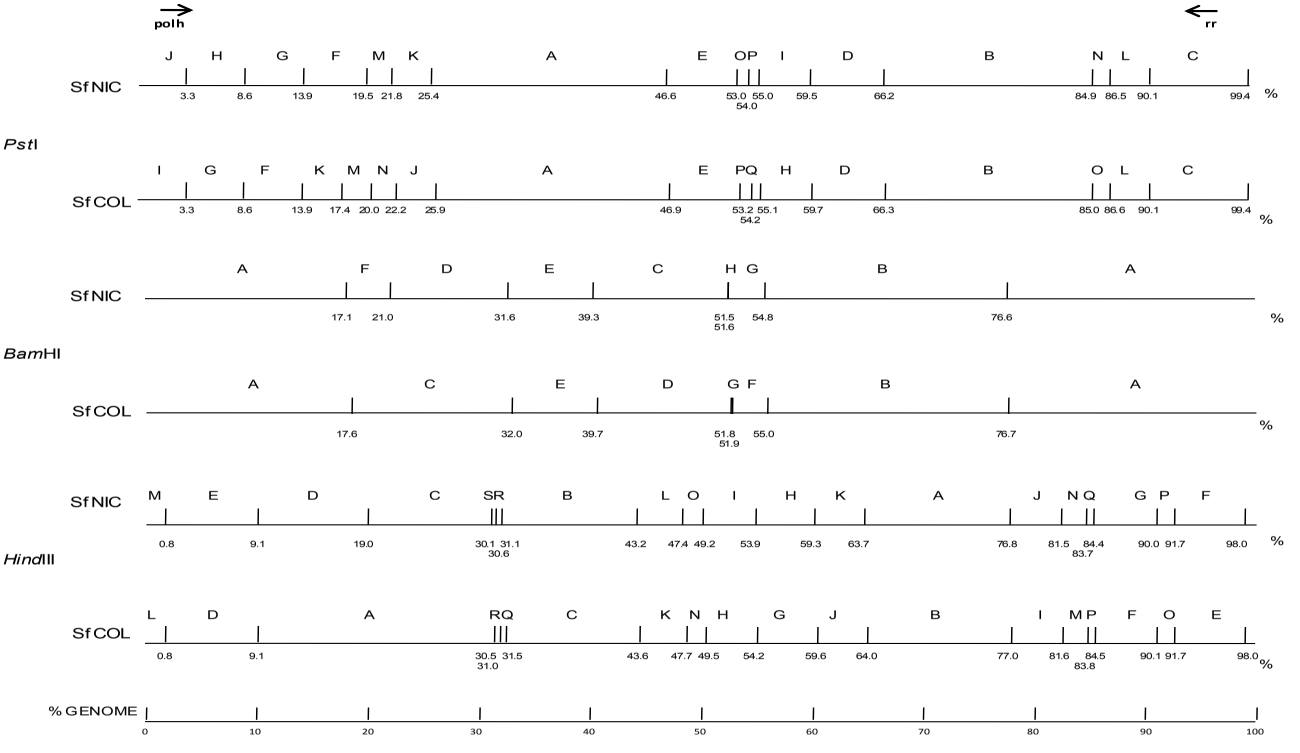
The sequence analysis of SfCOL *Pst*I-K and *Pst*I-M fragments also revealed the absence of the recognition sites *Hind*III and *Bam*HI enzymes that were present in the SfNIC genome. Using this information physical maps were constructed for *Bam*HI and *Hind*III (Figure 3). The SfCOL genome was cleaved into 6 and 18 visible fragments with *Hin*dIII and *Bam*HI enzymes, respectively, wheras the SfNIC genome was digested to produce 7 and 19 fragments by each of these enzymes. The SfCOL REN profile lacked the SfNIC *Hin*dIII-D fragment (13.0 Kb) due to the lack of a *Hin*dIII site in the SfCOL genome. Consequently, the SfCOL *Hin*dIII-A fragment (28.8 Kb) is the result of the sum of the SfNIC *Hind*III-C (14.8 Kb) and -D (13.0 Kb) fragments (Figure 3). Treatment of SfCOL DNA with *Bam*HI revealed that the 5.1 Kb fragment present in the SfNIC profile (*Bam*HI-F) was absent and had been incorporated in the *Bam*HI-C fragment (19.5 Kb),

that resulted from the sum of the SfNIC *Bam*HI-D (14.1 Kb) and -F (5.1 Kb) fragments (Figure 3).

Table 2. Percentage of deduced amino acid sequence identity and similarity of the proteins encoded by SfCOL ORFs to their orthologues in the SfNIC (Simón et al., 2010), Sf3AP2 (Harrison et al., 2008), SeMNPV (Ijkel et al., 1999) and SpltNPV (Pang et al., 2001) genomes.

SfCOL ORFs	Gene family	size aa	ORF number/% Identity (Similarity)			
			SfNIC	Sf3AP2	SeMNPV	SpltMNPV
1	*cathepsin*	85	*sf20*/97(97)	*sf20*/97(97)	*se16*/90(91)	*splt17*/90(98)
2	*lef-7*	320	*sf21*/98(98)	*sf21*/98(98)	*se17*+*se18*/56(67)	*
3	*chitinase*	573	*sf22*/98(99)	*sf22*/99(99)	*se19*/85(90)	*splt18*/83(90)
4	*unknown*	162	*	*	*se21*/70(79)	*splt20*/96(98)
5	*unknown*	286	*	*	*se22*+*se23*/78(86)	*splt21*/98(98)
6	*gp37*	262	*sf24* /94(96)	*sf24*/94(96)	*se25*/83(88)	*splt22*/88(94)
7	*ptp-2*	167	*sf25*/99(100)	*sf25*/99(100)	*se26*/68(83)	*splt23*/63(83)
8	*egt*	525	*sf26*/99(100)	*sf26*/96(96)	*se27*/76(91)	*splt24*/78(91)
9	*unknown*	77	*sf27*/100(100)	*sf27*/100(100)	*se28*/64(71)	*splt25*/82(89)
TOTAL	% Ident (Sim)		98(99)	98(98)	74(83)	85(93)

Figure. 3. Physical maps of the SfCOL genome compared with those of SfNIC. Restriction maps for *Pst*I, *Bam*HI and *Hind*III are shown. The circular DNA of each genome is represented in linear form. The first nucleotide of the genome corresponds to the *polyhedrin* gene. Numbers of map units (m.u.) representing restriction sites are indicating below the maps. Scales of m.u. are indicated at the bottom. The SfCOL genome size was estimated to be 133.7 Kb.



Biological characterization

The biological activity of SfCOL OBs was compared with that of SfNIC OBs using laboratory colonies of *S. frugiperda* from Mexico and Colombia (Table 3). The LC_{50} values of the SfNIC and SfCOL OBs were very similar in larvae from the Mexican colony. In contrast, SfNIC OBs (LC_{50} $5.37x10^5$ OBs/ml) were approximately twelve-fold less pathogenic than SfCOL OBs (LC_{50} $4.57x10^4$ OBs/ml) to the Colombian colony. Overall, SfCOL OBs were approximately three-fold more potent to the Colombian colony larvae than to the Mexican colony larvae. However, the SfNIC isolate was five-fold less potent to the Colombian colony than to the Mexican colony. No mortality was observed in the controls.

MTD values were estimated for virus concentrations that resulted in ~90% larval mortality (Table 3). SfNIC and SfCOL presented similar MTD values for each of the colonies analyzed. With a MTD of 160 h and 168 h for SfNIC and SfCOL, respectively, these viruses were significantly slower to kill Colombian colony insects than Mexican insects (with a MTD of 125 h and 119 h for SfNIC and SfCOL, respectively. The slower speed of kill was correlated with a higher OB production in the Colombian colony insects compared to those from the Mexican colony.

Table 3. Estimated LC_{50} values, relative potencies and mean time to death (MTD) values of SfNIC and SfCOL isolates in *S. frugiperda* second instars from two different populations originating from Mexico and Colombia.

Origin insect culture	Virus	LC_{50} (OBs/ml)	Relative Potency	Fiducial limits (95%)		MTD (h)	Fiducial limits (95%)	
				Low	High		Low	High
Mexico	SfNIC	$1.52x10^5$	1.0	-	-	125[b]	121	129
	SfCOL	$1.14x10^5$	1.3	0.84	2.11	119[b]	115	123
Colombia	SfNIC	$5.37x10^5$	0.2	0.16	0.48	160[a]	154	166
	SfCOL	$4.57x10^4$	3.3	2.14	5.52	168[a]	162	174

OB production of the different isolates was determined in second instars that had consumed a ~90% lethal concentration of OBs. The results were not normally distributed and were subjected to non-parametric analysis and are shown as medians with the corresponding interquartile ranges (Figure 4). No differences were observed in OB production among the SfCOL and SfNIC isolates in each of the two insect colonies tested. Both isolates produced significantly greater numbers of OBs in insects from the Colombian colony, with approximately three-fold more OBs/larva (range $6.4x10^7$ to $7.5x10^7$ OBs/larva) than observed in the Mexican colony insects (range $1.8x10^7$ to $2.1x10^7$ OBs/larva).

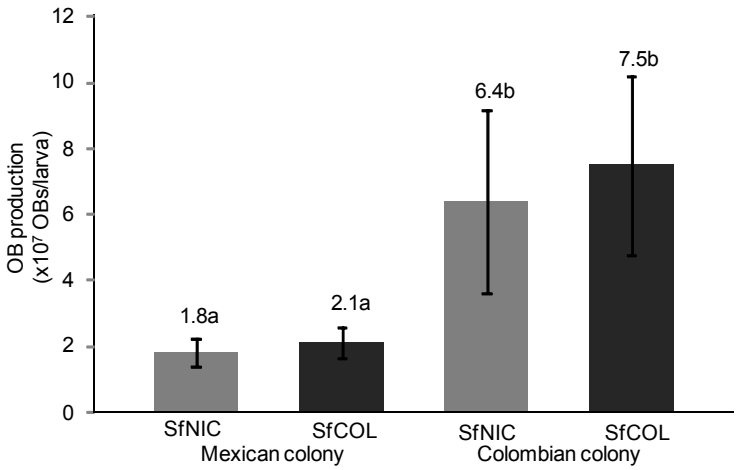


Figure 4. Median OB production per larva of SfNIC and SfCOL isolates using larvae from the Mexican and Colombian colonies. Values followed by identical letters did not differ significantly for pairwise comparisons (Mann–Whitney U-test followed by Benjamini and Hochberg (1995) false discovery rate adjustment of *P* values for multiple comparisons). Vertical lines indicate the interquartile range.

DISCUSSION

The aim of this study was to select a native SfMNPV isolate that could be used as the basis for a biological insecticide product to control *S. frugiperda* in Colombia. Field NPV isolates were collected from different host plants in various regions in Colombia. Thirty-eight isolates were examined by REN analysis. Baculovirus REN profiles from the same lepidopteran host species in different geographic regions frequently show variation in the number and position of restriction sites. This simple technique has been used to reveal the occurrence of distinct strains in different geographical isolates of SfMNPV (Berretta et al., 1998; Escribano et al., 1999; Loh et al., 1982; Shapiro et al., 1991). In this study, 35 out of the 38 isolates displayed identical REN profiles with six different enzymes, suggesting that all 35 isolates are the same virus strain. An isolate from Monteria (Córdoba, Colombia) was selected as being representative of the predominant isolates and named SfCOL. Few differences in the presence and distribution of REN cleavage sites were observed between the SfCOL and the SfNIC genomes (Simón et al., 2004; Simón et al., 2011). This is a common feature between distinct geographical NPV isolates that differ minimally in their REN profiles and physical maps, normally due to point mutations and small deletions or insertions (Chen et al., 2002; Zhang et al., 2005).

Although the REN profiles of Colombian isolates appear to be unique, the variations relative to SfNIC occurred in a region of the SfMNPV genome that commonly differs between strains (Harrison et al., 2008; Simón et al., 2004; 2005a; Wolff et al., 2008). Variability in this region was also observed among genotypes of SeMNPV that is highly collinear with the SfMNPV genome (Muñoz et al., 1998; Simón et al., 2005a). Localized regions of variation are also a common feature in other baculoviruses; strains of *Autographa californica* MNPV (AcMNPV) or SeMNPV differ in specific genomic regions that tend to comprise a high prevalence of auxillary genes (Dai et al., 2000; Erlandson et al., 2007; Stiles and Himmerich, 1998).

Auxillary genes such as *cathepsin* and *egt* have been identified within this SfMNPV genomic region (Flipsen et al., 1995; Slack et al., 1995). In addition, a homologous region (*hr*) was also detected. *Hrs* are implicated in important functions such as origins of DNA replication and transcriptional enhancement (Pearson and Rohrmann, 1995) and have been found also near to regions of variability in others baculovirus genomes (de Jong et al., 2005; Hayakawa et al., 2000; Li et al., 2002). Sequencing of this SfCOL variable region including *Pst*I-K and *Pst*I-M fragments, revealed an insertion of ~1.0 Kb compared to SfNIC. This region was collinear with that of SfNIC or Sf3AP2, however within SfCOL two ORFs that do not have their orthologues in SfNIC or Sf3AP2 genomes were identified. These two ORFs were closely related to *splt20* (96%) and *splt21* (98%), but less closely related to *se21* (70%) *and se22+se23* (78%) (Ijkel et al., 1999; Pang et al., 2001). In contrast, SfCOL lacked the *sf23* ORF of unknown function, which is found in SfMNPV genomes (Harrison et al., 2008; Simón et al., 2011). The heterogeneity found at the genome level between SfNIC and SfCOL isolates could be attributed to exchanges of genetic material. Heterologous recombination has been shown to occur in cell culture (Hajós et al., 2000) and has been suggested to occur between *Rachiplusia ou* MNPV (RoMNPV) and AcMNPV in natural populations (Croizier et al., 1988). In addition, recombination has been postulated as possible major cause of genetic heterogeneity in NPV wild populations (Croizier and Ribeiro, 1992). Genomes of different baculoviruses can co-infect the same cell and closely related viruses that normally infect different host species can be forced to co-infect the same cell (Herniou et al., 2003). In this case, the geographic distribution of the natural host of SfMNPV, *S. frugiperda* and that of SpltNPV, *S. litura,* do not overlap. While *S. frugiperda* is distributed across tropical and subtropical regions of the Americas (Sparks, 1979), *S. litura* is found in Asia and Oceania (Takatsuka et al., 2003) and no infestations of *S. litura* have been reported in the Americas. However, SfCOL virus may have

acquired these two ORFs by recombination with a SpltNPV virus when both viruses coinfected a susceptible host with a geographical distribution that overlapped the distribution of each virus. The range of hosts that both SfMNPV and SpltNPV infect in nature has not been characterized in detail. It may be possible. Therefore, that a moth species infected by SpltNPV migrated into the natural distribution area of SfMNPV and its host(s). If common, this behavior could increase the likelihood of recombination between these viruses and the generation of novel variants.

Differences in gene content between SfCOL and SfNIC are likely to have effects on the phenotypic characteristics of the isolates. Baculoviruses isolated from the same species at different sites frequently vary in their pathogenicity and virulence (Cory and Myers, 2003; Erlandson et al., 2007). Differences in biological activity are common among virus isolates from distinct geographical regions (Erlandson, 2009; Fuxa, 2004) or among cloned variants derived from a single wild-type virus obtained by *in vitro* (Lynn et al., 1993; Ribeiro et al., 1997; Simón et al., 2004, 2005b) and *in vivo* cloning techniques (Muñoz et al., 2000). The selection of an isolate as a biocontrol agent requires geographical isolates to be tested against insect populations from the locality of the program. Compared to exotic isolates, native baculovirus isolates might also be easier to register as the basis for a biopesticide product in pesticide regulatory systems. The analysis of baculovirus phenotype using long-term laboratory cultures of insects may be hindered if the colony susceptibility does not accurately reflect the range of susceptibilities of naturally occurring populations of the pest in question. To address this, two different *S. frugiperda* populations were tested; a long-standing laboratory culture from Mexico and a recently established colony from field collected insects in Colombia. The Colombian and Nicaraguan isolates presented similar lethal concentration values when tested against the Mexican colony insects. However, the SfCOL isolate was twelve-fold more pathogenic than SfNIC isolate against larvae from Colombian colony.

In contrast, SfCOL showed a slower speed of kill in insects from the Colombian colony compared to the Mexican colony, which was correlated with a higher production of OBs/larvae. The extended infection period might increase production of viral progeny (Wilson et al., 2000). This is a common phenomenon within baculoviruses as reported previously for other baculoviruses (Muñoz and Caballero, 2000; Simón et al., 2004; 2008). 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).

Given the high activity of SfCOL OBs towards the Colombian colony insects, SfCOL is likely better suited for the development as a biological insecticide for the control of *S. frugiperda* populations in Colombia than the SfNIC isolate. Similarly, insects from a Honduran *S. frugiperda* population were more susceptible to the neighboring SfNIC isolate than to geographically distant isolates (Escribano et al., 1999) and local isolates of *Trichoplusia ni* SNPV (TnSNPV) or AcMNPV were more infective towards local pest populations than those from geographically distant origins (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. In conclusion, the SfCOL isolate showed improved insecticidal characteristics to the insects from a local colony compared to an isolate from Nicaragua. The SfCOL isolate may prove suitable as the basis for a biopesticide for control of *S. frugiperda* infestations in Colombia.

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CHAPTER III

Deletion genotypes reduce occlusion body potency but increase occlusion body production in a Colombian SfMNPV

ABSTRACT

A Colombian field isolate (SfCOL-wt) of *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) is a mixture of different genotypes. To evaluate the insecticidal properties of the different genotypic variants, 83 plaque purified virus were characterized. Ten distinct genotypes were identified (named A through J). SfCOL-A was the most prevalent (71±2%; mean ± SE) showing a *Pst*I restriction profile indistinguishable to that of SfCOL-wt. The remaining nine genotypes presented genomic deletions of 3.8 - 21.8 Kb located mainly between nucleotides 11,436 and 33,883 in the reference genome SfMNPV-B, affecting the region between open reading frames (ORFs) *sf20* and *sf33*. The insecticidal activity of each genotype from SfCOL-wt and several mixtures of genotypes was compared to that of SfCOL-wt. The potency of SfCOL-A occlusion bodies (OBs) was 4.4-fold higher than SfCOL-wt OBs, whereas the speed of kill of SfCOL-A was similar to that of SfCOL-wt. Deletion genotype OBs were similarly or less potent than SfCOL-wt but six deletion genotypes were faster killing than SfCOL-wt. The potency of genotype mixtures co-occluded within OBs were consistently reduced in two-genotype mixtures involving equal proportions of SfCOL-A and one of three deletion genotypes (SfCOL-C, -D or –F). Speed of kill and OB production were improved only when the certain genotype mixtures were co-occluded, although OB production was higher in the SfCOL-wt isolate than in any of the component genotypes, or mixtures thereof. Deleted genotypes reduced OB potency but increased OB production of the SfCOL-wt population, which is structured to maximize the production of OBs in each infected host.

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INTRODUCTION

Viruses in the family Baculoviridae are characterized by a high intraspecific heterogeneity in isolates from distant geographic regions (interpopulation diversity) (Escribano et al., 1999; Rowley et al., 2010) and also within single isolates that can exist as mixtures of genotypes (intrapopulation diversity) (Cory et al., 2005; Hodgson et al., 2001; Muñoz et al., 1999; Redman et al., 2010; Simón et al., 2004). Genotypic variants can be the result of recombination during simultaneous infections by multiples genotypes or can be due to genetic drift by mutation during replication (Erlandson, 2009; Possee and Rorhmann, 1997). Moreover, genomic variability has important effects on baculovirus fitness parameters related to the pathogenicity, speed of kill and production of viral occlusion bodies (OBs) (Erlandson, 2009; Simón et al., 2004).

Genotypic variability of the *Spodoptera frugiperda* multiple nucleopolyhedrovirus (SfMNPV) has been determined by characterization of different geographical isolates (Barrera et al., 2011; Escribano et al., 1999; Rowley et al., 2010) and by isolating the genotypic variants present in particular field isolates using *in vitro* techniques (Harrison et al., 2008; Simón et al., 2004). In a Nicaraguan field isolate (SfNIC) the OBs of all genotypic variants were less pathogenic, as determined by concentration-mortality metrics, than the wild type OBs or those of experimental mixtures of genotypes (López-Ferber et al., 2003; Simón et al., 2004; 2006). In contrast, in a commercial isolate of *S. exigua* multiple NPV (SeMNPV), deletion genotypes reduced the activity of the wild-type population (López-Ferber et al., 2003; Muñoz et al., 1998; Muñoz and Caballero, 2000; Simón et al., 2004; 2006). These findings have emphasized the need to evaluate interactions between genotypes within wild-type nucleopolyhedrovirus populations that can be highly advantageous during the process of selecting the active material for the development of baculovirus-based insecticides.

Previous studies on SfMNPV as a potential biological control agent in Colombia identified the SfCOL isolate as the most pathogenic of a total of 38 Colombian field isolates and the Nicaraguan isolate SfNIC previously characterized (Barrera et al., 2011). The objectives of the present study were to determine the genotypic diversity present in the SfCOL isolate and evaluate the contribution of the genotypic variants to the insecticidal properties of the natural isolate by examining key phenotypic traits, such as pathogenicity, virulence and OB productivity, of single genotypes alone and in mixtures with the dominant genotype.

MATERIALS AND METHODS

Insects source and rearing

Larvae of *S. frugiperda* were obtained from a laboratory colony established in the Biological Control Laboratory of the Colombian Corporation of Agricultural Research (Corpoica) using larvae collected from experimental maize crops located in Corpoica Research Center "La Libertad" (Villavicencio, Colombia). Specific permission requirements were not necessary to collect larvae as this location is not in a protected area and *S. frugiperda* is not a protected species. This insect colony was periodically refreshed with field-collected insects and maintained at 25±1 °C, 75±5% RH (relative humidity) and 16 h light: 8 h dark photoperiod on a wheatgerm-based semisynthetic diet (Greene et al., 1976). However, during rearing this colony collapsed and a second colony was established shortly after that with larvae collected from maize crops at the same site.

Virus isolate and amplification

The Colombian isolate SfCOL (Barrera et al., 2011) was amplified in *S. frugiperda* larvae. OBs from cadavers were purified (Caballero et al., 1992), and resuspended in milli-Q water. OB concentrations were determined using an improved Neubauer hemocytometer (Hawksley Ltd., Lancing, UK) under phase contrast microscopy at x400. Purified OBs were stored at 4 °C.

***In vitro* virus cloning**

For the isolation of individual genotypes, SfCOL infected larvae were surface decontaminated with 70% ethanol. Hemolymph was taken by bleeding at 48 h post infection (h.p.i.) and diluted in sterile phosphate buffered saline (PBS). Sf9 cells were prepared at 2 x 10^6 cells/well in 6-well tissue culture plates and incubated at 27 °C for 3 h, with TC100 medium supplemented with 1% penicillin/streptomycin (Gibco). The medium was then removed and 0.1 ml of serial 10-fold dilutions (from 10^{-2} to 10^{-6}) of hemolymph were inoculated onto cells. After 1 h, the inoculum was removed and 3 ml of TC100 medium supplemented with 5% fetal calf serum (FSC, Gibco), 3% (w/v) SeaPlaque agarose and antibiotics were added to each well. Agarose was overlaid with 3 ml TC100 medium supplemented with antibiotics. The overlaid liquid was replaced every day. After 10 days, 248 well isolated plaques were picked individually using a sterile Pasteur pipette and transferred to a vial containing 0.1 ml of PBS. Volumes of 5 µl of this suspension were injected into *S. frugiperda* fourth instars for viral amplification. Virions were injected into larvae in order to avoid the potential loss of genotypes that were defective in genes necessary for oral infectivity, such as the *per os* infection factors (*pifs*).

Purification of OBs, DNA extraction and restriction endonuclease analysis

OBs were purified from dead diseased larvae by titration and centrifugation (Caballero et al., 1992) and quantified by counting three times in a Neubauer hemocytometer. For DNA extraction, virions were released from OBs by mixing 100 µl of purified OB suspension at 1 x 10^9 OBs/ml with 100 µl of 0.5 M Na_2CO_3, 50 µl of 10% sodium dodecyl sulphate (SDS) in a final volume of 500 µl and incubating for 10 min at 60 °C. Undissolved OBs were removed by low-speed centrifugation (3,800 × *g*, 5 min). Supernatant containing the virions was treated with 25 µl of proteinase K (20 mg/ml) for 15 min at 50 °C. Viral DNA was extracted using saturated phenol-chloroform followed by

alcohol precipitation. The resulting pellet was resuspended in 50 to 100 µl of 0.1×TE (Tris-EDTA) for 10 min at 60 °C. DNA concentration was estimated using a UV-spectrophotometer at 260 nm (ND1000 - Thermo Scientific).

For restriction endonuclease analysis, samples were compared with the SfCOL-wt profile (Barrera et al., 2011). A sample of 2 µg of viral DNA was mixed with 10 U of one of the following restriction enzymes: *Pst*I, *Bam*HI or *Hin*dIII (Takara), and incubated for 4 to 12 h at 37°C. Reactions were stopped by mixing with 4 µl of loading buffer solution (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). Genotypes that differed in one or more restriction profiles were each assigned a letter from the alphabet (SfCOL-A, -B, etc.).

Construction of physical maps

Physical maps of the purified genotypes were constructed by comparison with the physical maps of SfCOL (Barrera et al., 2011). For this, restriction fragments obtained after treatment with different enzymes were compared with those of SfCOL, and co-migrating as well as genotype-specific fragments were identified. Based on this, preliminary physical maps were constructed. Two differential SfCOL genotype-specific *Pst*I fragments that were found to be characteristic of the *Pst*I profiles of SfCOL-C and -D genotypes, namely *Pst*I-E' fragment (7.5 Kb) and *Pst*I-C' fragment (9.1 Kb), respectively, were each cloned into pUC19 plasmid. The ligation reaction was prepared by mixing 1 µl (50 ng/µl) of purified vector pUC19, 5 µl of 10x ligase buffer, 1 µl (2U/µl) of ligase enzyme and 15 µl of the corresponding *Pst*I fragment obtained from excised pieces of agarose, incubated at 16 °C overnight and used to transform *Escherichia coli* cells (Invitrogen ElectroMAX DH10B-T1). The cells were plated on LB-ampicillin (100 mg/ml) plates containing IPTG (0.1 mM) and X-gal (40 µg/ml). White colonies that

included the DNA fragment were amplified in liquid LB containing ampicillin (100 mg/ml) at 37 °C overnight. DNA was then extracted by alkaline lysis, digested with the relevant endonuclease and separated on 1% agarose and compared with total DNA digested from the SfCOL isolate.

The cloned fragments were sequenced by primer walking using universal M13 and M13 reverse primers (Sistemas Genómicos, Paterna, Valencia, Spain). Sequence information was analyzed for the presence of open reading frames (ORFs) using Open Reading Frame Finder (NCBI). Homology searches were performed at the nucleotide and amino acid levels using all putative ORFs. DNA and protein comparisons with entries in the updated GenBank/EMBL, SWISS PROT and PIR databases were performed using BLAST (NCBI). Additionally, to confirm the deleted ORFs in SfCOL genotypes, some ORF regions were amplified by PCR using specific primers for different ORFs based on sequence from SfCOL (Barrera et al., 2011) and SfMNPV-B genomes (Simón et al., 2011) (Table 1). These amplifications were compared with those obtained from SfCOL-wt, to confirm the deletions within these regions.

Relative proportion of complete SfCOL-A genotype in the wild-type population

Once the physical maps were constructed and the gene content of each genotype had been determined, specific primers were designed in a region common to all genotypes and in a specific region of the complete SfCOL-A genotype in order to estimate the relative proportion of the complete genotype in the wild-type population by qPCR. A set of specific primers were designed that amplified in the *DNA polymerase* gene, common to all genotypes, and in the *egt* gene, present only in SfCOL-A (Table 1) using sequence information from the SfMNPV-B genome (Simón et al., 2011). Non-template controls were analyzed for each set of primers designed in order to verify the absence of non-specific background signal.

Table 1. Summary of the primers used to confirm the deletions within the SfCOL genotypes and to perform qPCR.

Primer	Sequence	Nucleotide position in SfMNPV genome	SfCOL size (nt)	SfCOL genotypes									
				A	B	C	D	E	F	G	H	I	J
sf24-sf25	5'-acttttgtcgtccgctgtgaa-3'	*	925	925	∅	∅	∅	∅	∅	925	∅	∅	∅
	5'-gcgggtatatctcgcagcgca-3'	23.603-23.622											
egt	5'-acagtttggtcaagacacgccga-3'	24.781-24.803	457	457	∅	∅	∅	∅	∅	∅	∅	∅	∅
	5'-gagcgcttcgacggcttcgt-3'	25.218-25.237											
sf27-sf28	5'-tgttggaactccaggcggatc-3'	26.007-26.027	799	799	∅	∅	∅	∅	∅	∅	∅	∅	∅
	5'-gccgtggtcgtggttggca-3'	26.791-26.809											
sf28	5'-agccgatattatttgacggcttcgt-3'	26.602-26.628	206	206	206	206	206	∅	206	∅	∅	∅	∅
	5'-gccgtggtcgtggttggca-3'	26.791-26.809											
sf29	5'-cggatcccatgcgcatcgtcg-3'	27.917-27.937	550	550	550	550	550	∅	550	∅	550	550	∅
	5'-agcgtgtttaaagaagaacgcggc-3'	27.390-27.411											
sf82	5'-aacagcggcaatcggacgca-3'	79.177-79.196	329	nt	nt	nt	329	nt	nt	nt	nt	nt	nt
	5'-gtcagttgctgttggcacacg-3'	79.485-79.505											
sf84	5'-acgctttcgccccaccacat-3'	80.145-80.164	612	nt	nt	nt	612	nt	nt	nt	nt	nt	nt
	5'-cgcgccaacagcaaaggagc-3'	80.737-80.756											
sf86	5'-tgagctcctccggcgtctcc-3'	82.065-82.084	338	nt	nt	nt	338	nt	nt	nt	nt	nt	nt
	5'-tatcctggcgtggcgagcga-3'	82.383-82.402											
sf87	5'-tgatctcgctccgcgggat-3'	83.415-83.434	471	nt	nt	nt	471	nt	nt	nt	nt	nt	nt
	5'-taatgggggccgcgaaggtgc'-3	82.964-82.983											
sf88	5'-tcccggcggagcgagatcat-3'	83.416-83.435	419	nt	nt	nt	419	nt	nt	nt	nt	nt	nt
	5'-ggtcgtcgccgatggtgctt-3'	83.815-83.834											
sf89-sf90	5'-cgcggtgttccctgctctccg-3'	84.534-84553	662	nt	nt	nt	662	nt	nt	nt	nt	nt	nt
	5'-ccttgatgctcgccgcccaa-3'	85.176-85.195											
sf91	5'-gagcgctgcctacgtgcgat-3'	86.785-86.804	612	nt	nt	nt	612	nt	nt	nt	nt	nt	nt
	5'-acgcgcagagaacacccagc-3'	87.377-87.396											
sf92	5'-tgcacagtgaacgcatcaacatca-3'	88.273-88.296	1.342	nt	nt	nt	1.342	nt	nt	nt	nt	nt	nt
	5'-gagagcacttgccgccggt-3'	89.595-89.614											
qPCR *DNA polymerase*	5'-caacgtcgacaatcaaatgg-3'	89.480-89.499	109	109	109	109	109	109	109	109	109	109	109
	5'-cgcatctcgtttctggtga-3'	89.570-89.589											
qPCR *egt*	5'-ggtcgtcggttgtgaaatcg-3'	24.102-24.121	100	100	∅	∅	∅	∅	∅	∅	∅	∅	∅
	5'-tcgaccagtgcttgaatgta-3'	24.182-24.201											

The sizes of the PCR fragments are indicated according to SfCOL (*sf24* to *sf29*) and SfMNPV-B (*sf82* to *sf92* and qPCRs) sequence sizes [10,18]. nt indicates not tested. ∅ symbol represents absence of amplification and * indicates no homologs present in the SfMNPV-B genome.

SfCOL wild-type DNA was extracted from OBs as previously described. Five different DNA extractions were performed in each of three replicates. All DNA measures were performed twice. The frequency of SfCOL-A was obtained by calculating the average of the fifteen samples (mean ± standard error). DNA concentration was quantified by spectrophotometry and by agarose gel electrophoresis, and diluted to 0.01 ng/µl. All reactions were performed using SYBR Green fluorescence in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The reaction mixture (10 µl) contained 5 µL SYBR Premix Ex *Taq* (2x), 0.2 µl of ROX Reference Dye (50x), 0.1 µl of each SfMNPV primer (10 pmol/µl) (Table 1) and 1 µl of DNA template. qPCR was performed under the following conditions: 95 °C for 30 s, followed by 45 elongation cycles of 95 °C for 5 s and 60 °C for 30 s and finally a dissociation stage of 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s. Data acquisition and analysis were handled by Sequence Detector Version 2.2.2. software (Applied Biosystems). Known dilutions of SfCOL-A CsCl-purified DNA (10^{-7} – 10^{-1} ng/µl) were used as internal standards for each qPCR reaction. Melting-curve analysis was performed to confirm specific replicon formation in qPCR.

Production of OB mixtures and co-occluded mixtures of genotypes

Experimental mixtures were produced for the combinations of genotypes SfCOL-(A)+(C), -(A)+(D) and -(A)+(F) as previously described (López-Ferber et al., 2003; Simón et al., 2005). Briefly, the purified OB suspensions of each genotype were quantified by counting and adjusted to the same concentration. OBs of each genotype were then mixed in equal proportions and used as peroral inocula in insect bioassays described below.

To produce co-occluded genotype mixtures, SfCOL-(A+C), -(A+D) and -(A+F), the methodology described previously was followed; after mixing the OBs of the different genotypes, ODVs were released by alkali disruption with a dissociation buffer (1 vol. OB: 1 vol. Na_2CO_3 0.5M: 5 vol. H_2O).

Undissolved OBs and other particulate materials were pelleted by low speed centrifugation at 2,700 x g, 5 min. ODV suspension was then injected (5 µl/larva) into *S. frugiperda* fourth instars that were individually maintained on semisynthetic diet until death. OBs containing co-occluded genotypes were recovered from virus killed larvae and used to perform bioassays. Co-occlusion was not checked, as previous studies on SfMNPV have demonstrated that this methodology can generate co-occlusion of multiple genotypes within the same OB and co-envelopment within the same ODV (Clavijo et al., 2010).

Insect bioassays

The mean lethal concentration (LC_{50}), mean time to death (MTD) and total OB production (OBs/larva) of SfCOL-wt and each of the cloned genotypes were estimated by peroral bioassay following the droplet feeding technique (Hughes and Wood, 1981) using the first insect colony. Bioassays were also performed using experimental mixtures of OBs, SfCOL-(A)+(C), SfCOL-(A)+(D) and SfCOL-(A)+(F), and co-occluded mixtures, SfCOL-(A+C), -(A+D) and -(A+F), comprising equal proportions of each genotype using the second colony established one year later. Second-instars of both S. *frugiperda* colonies were starved for 8 to 12 h at 26°C and then allowed to drink from an aqueous suspension containing 10% (wt/vol) sucrose, 0.001% (wt/vol) Fluorella blue, and OBs at one of the following five concentrations: $1.2x10^6$, $2.4x10^5$, $4.8x10^4$, $9.6x10^3$ and $1.92x10^3$ OBs/ml. This range of concentrations was previously estimated to result in 95 to 5% mortality (Barrera et al., 2011). Larvae that ingested the suspension within 10 min. were transferred to individual plastic cups with semisynthetic diet. Bioassays with 24 larvae per virus concentration and 24 larvae as control were performed three times. Larvae were reared at 25 °C and mortality was recorded every 12 h until the insects had either died or pupated. Virus induced mortality was subjected to logit regression using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993).

Time mortality data were subjected to Weibull survival analysis using the GLIM program (Crawley, 1993) and OB concentrations used for time mortality assays were those that resulted in ~90% larval mortality in the previous bioassays. For SfCOL-wt and the individual genotypes (SfCOL-A to -J) the 90% lethal concentrations were $1.4x10^7$, $1.1x10^6$, $9.3x10^6$, $7.0x10^6$, $2.7x10^7$, $3.7x10^6$, $1.5x10^7$, $1.9x10^6$, $5.8x10^7$, $3.9x10^7$ and $7.2x10^7$ OBs/ml, respectively. For experimental mixtures of occlusion bodies the 90% lethal concentrations were $1.4x10^6$, $2.7x10^6$ and $2.4x10^6$ OBs/ml for SfCOL-(A)+(C), SfCOL-(A)+(D) and SfCOL-(A)+(F), respectively. Finally, for co-occluded mixtures SfCOL-(A+C), SfCOL-(A+D) and SfCOL-(A+F) the 90% lethal concentrations were $3.7x10^6$, $1.3x10^6$ and $6.9x10^6$ OBs/ml, respectively. Groups of 24 second instars were infected using the droplet feeding method (Hughes and Wood, 1981) and mortality was checked at intervals of 8 h until death. The assay was performed on three occasions. Groups of 24 control larvae were treated identically but did not feed on OB suspensions.

The production of OBs in insects infected by SfCOL-wt, single genotypes and experimental OB mixtures and co-occluded mixtures were determined in groups of 24 overnight-starved second instars inoculated with the LC_{90} used in the speed of kill assay and reared on semisynthetic diet at 25 °C until death. The whole assay was performed on three occasions. All the larvae that died from polyhedrosis disease (at least 20 for each virus treatment per replicate, a total of ~60 larvae per virus treatment) were individually collected and stored at -20 °C. For OB counting, each larva was individually homogenized in 100 µl of distilled water and counted in triplicate in a Neubauer hemocytometer. The results were analyzed by Kruskal-Wallis and Mann-Whitney nonparametric statistics using the SPSS program (SPSS version 10.0). Critical probability values were subjected to false discovery rate adjustment for multiple pairwise comparisons (Benjamini and Hochberg, 1995).

RESULTS

Identification of genotypic variants

Out of 248 plaque picks, just 83 plaque picks were successfully amplified and caused fatal infection following injection in insect larvae. Ten different genotypes (named SfCOL-A to -J) were identified by analysis of plaques using *Pst*I, *Bam*HI and *Hin*dIII endonucleases (Figure 1A). The SfCOL-F genotype was the most frequently isolated genotype, which was present in 45 of amplified plaques (representing 54% of the clones), followed by SfCOL-H (N=21, 25%), SfCOL-E (N=5, 6%), SfCOL-I (N=4, 4.8%) and -G (N=3, 3.6%). The remaining genotypes appeared in only a single amplified plaque in each case (Figure 1B). All of these variants could be differentiated using the *Pst*I enzyme (Figure1A).

SfCOL-A genotype with the complete genome showed a *Pst*I restriction profile indistinguishable to that of SfCOL-wt, suggesting its high frequency in the population. While, all the others genotypes lacked specific fragments present in SfCOL-A. The *Pst*I-F fragment was absent in the SfCOL-D and -F genotypes, which differed only in the *Pst*I-D fragment that was absent in the SfCOL-D profile. The *Pst*I-K fragment was absent in five of nine deletion variants (SfCOL-C, -D, -F, -H and -J), and all genotypes (except SfCOL-A) lacked the *Pst*I-M and *Pst*I-N fragments. The *Pst*I-J fragment was absent in SfCOL-E, -G, -I and -J variants. Finally, *Pst*I-O and –L fragments were absent in SfCOL-J. In all cases missing restriction fragments were associated with the presence of additional fragments. One fragment of 9.1 Kb was shared by the SfCOL-D and -F variants. Characteristic bands were observed for unique genotypes, being of 6.3, 5.8, 4.2, 2.7, 1.9 and 1.3 Kb in SfCOL-I, -G, -E, -B, -H and -J, respectively (Figure 1A and 2, Table 2). No submolar bands were observed in these genotypes and the restriction profiles remained invariant for at least two passages in insects.

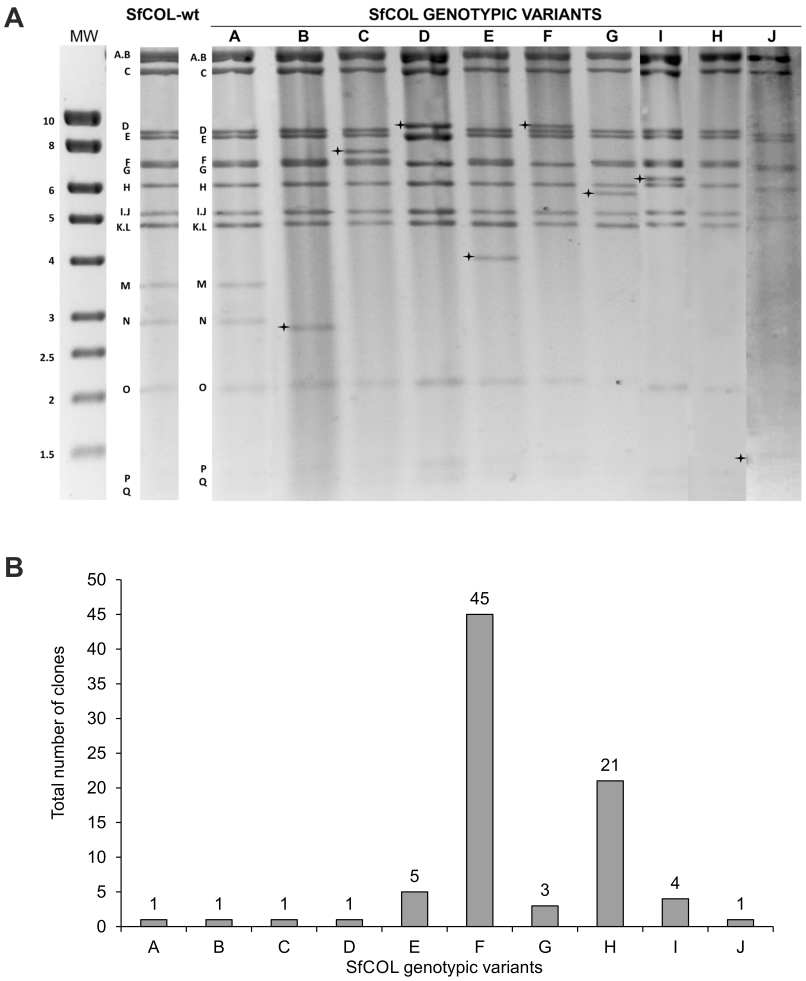

Figure 1. Restriction profiles and frequency of SfCOL genotypic variants. A. REN analysis of SfCOL-wt and genotypic variants DNAs digested with *Pst*I following electrophoresis in 1% agarose gel. All DNA fragments of SfCOL-wt and SfCOL-A are marked with a letter corresponding to their sizes. The cross marks the polymorphic fragments of each variant. The first lane indicates the molecular weight (MW) (1Kb DNA marker, Stratagene). B. Number of clones obtained for each of the genotypes (total number of clones 83).

Genotype restriction fragment sizes were estimated by comparison with SfCOL-wt fragments (Barrera et al., 2011) and the SfMNPV-B completely sequenced genome (Simón et al., 2011). The SfCOL-A genome was estimated to be 133.9 kb in length, while the remaining variants were estimated between 112.0 Kb and 130.1 Kb (Table 2).

Table 2. Restriction fragments (Kb) generated by *Pst*I treatment of SfCOL-wt DNA and component genotypic variants.

Fragment	SfCOL-wt	Genotypic variants									
		A	B	C	D	E	F	G	H	I	J
A	28.084	28.084	28.084	28.084	28.084	28.084	28.084	28.084	28.084	28.084	28.084
B	24.926	24.926	24.926	24.926	32.926	24.926	24.926	24.926	24.926	24.926	24.926
C	12.465	12.465	12.465	12.465	12.465	12.465	12.465	12.465	12.465	12.465	12.465
D	8.864	8.864	8.864	8.864	9.133	8.864	9.133	8.864	8.864	8.864	8.864
E	8.481	8.481	8.481	8.481	8.481	8.481	8.864	8.481	8.481	8.481	8.481
F	7.079	7.079	7.079	7.548	6.932	7.079	8.481	7.079	7.079	7.079	7.079
G	6.932	6.932	6.932	7.079	6.124	6.932	6.932	6.932	6.932	6.932	6.932
H	6.124	6.124	6.124	6.932	5.182	6.124	6.124	6.124	6.124	6.300	6.124
I	5.182	5.182	5.182	6.124	4.899	5.182	5.182	5.800	5.182	6.124	5.182
J	4.899	4.899	4.899	5.182	4.769	4.887	4.899	5.182	4.899	5.182	1.300
K	4.887	4.887	4.887	4.899	2.112	4.769	4.769	4.887	4.769	4887	1.339
L	4.769	4.769	4.769	4.769	1.339	4.200	2.112	4.769	2.112	4.769	1.228
M	3.575	3.575	2.700	2.112	1.228	2.112	1.339	2.112	1.921	2.112	
N	2.953	2.953	2.112	1.339		1.339	1.228	1.339	1.339	1.339	
O	2.112	2.112	1.339	1.228		1.228		1.228	1.228	1.228	
P	1.339	1.339	1.228								
Q	1.228	1.228									
Total	133.899	133.899	130.071	130.032	123.674	126.672	124.538	128.272	124.405	128.772	112.004

Additional fragments that did not appear in the SfCOL-wt restriction profiles are indicated using an apostrophe ('). The fragments sizes were confirmed by sequencing and/or by comparison with restriction fragments of the SfNIC genome.

Mapping of genotypic variants

The physical maps of the genotypes were constructed based on the physical maps of SfCOL-wt (Barrera et al., 2011) (Figure 2). Genotypic variability was mainly located in a single region of the SfMNPV-B genome, between nt 11,436 and 33,883 (Simón et al., 2011), involving *Pst*I-F, -K, -M, -N and -J fragments which included 23 complete ORFs and two partial ORFs (*sf12* to *sf35*). In this region, SfCOL-J had the largest deletion of 21.8 Kb when compared with SfCOL-A, followed by SfCOL-D, -H, -F, -E, -I and -G with deletions of 10.2, 9.4, 9.3, 7.2, 5.6 and 5.1 Kb, respectively.

The smallest deletion in this region of 3.8 Kb was observed in SfCOL-B and –C genotypes. Additionally a *Pst*I recognition site was absent in the SfCOL-D variant genome at nt 87,999 in the SfMNPV-B genome, which indicates an indel or mutation point at this site. Finally, SfCOL-J had a deletion in the region between nt 112,930 and 119,810 of the SfMNPV-B genome.

The variable ORFs within SfCOL-C, -D and -F genotypes were confirmed by sequencing of *Pst*I-C' and *Pst*I-E' fragments, whereas those found in SfCOL-B, -E, -G, -H, -I and -J genomes were determined by PCR amplification using specific primers that amplified between ORFs *sf24* and *sf29* (Table 1). The variable region (12.8 Kb) among genotypes was assembled and the variable ORFs were observed between *sf20* (*cathepsin*) and *sf29*. An extended region of variability was present in SfCOL-J, which possibly included a deletion within *sf33,* corresponding to the *arif-1* gene (Figure 2). The first ORF affected (left end) was *cathepsin* (*sf20*), which presented a partial deletion of ~60% of the gene in the SfCOL-D and –F genotypes. A region including *lef-7, chitinase,* ORF4 (similar to *splt20*) and ORF5 (similar to *splt21*) was absent in the SfCOL-D, -F, -H and -J genotypes. All deletion variants lacked *gp37*, *ptp-2*, *egt* and *sf27*, except the SfCOL-G genotype that presented *gp37* and *ptp-2*.

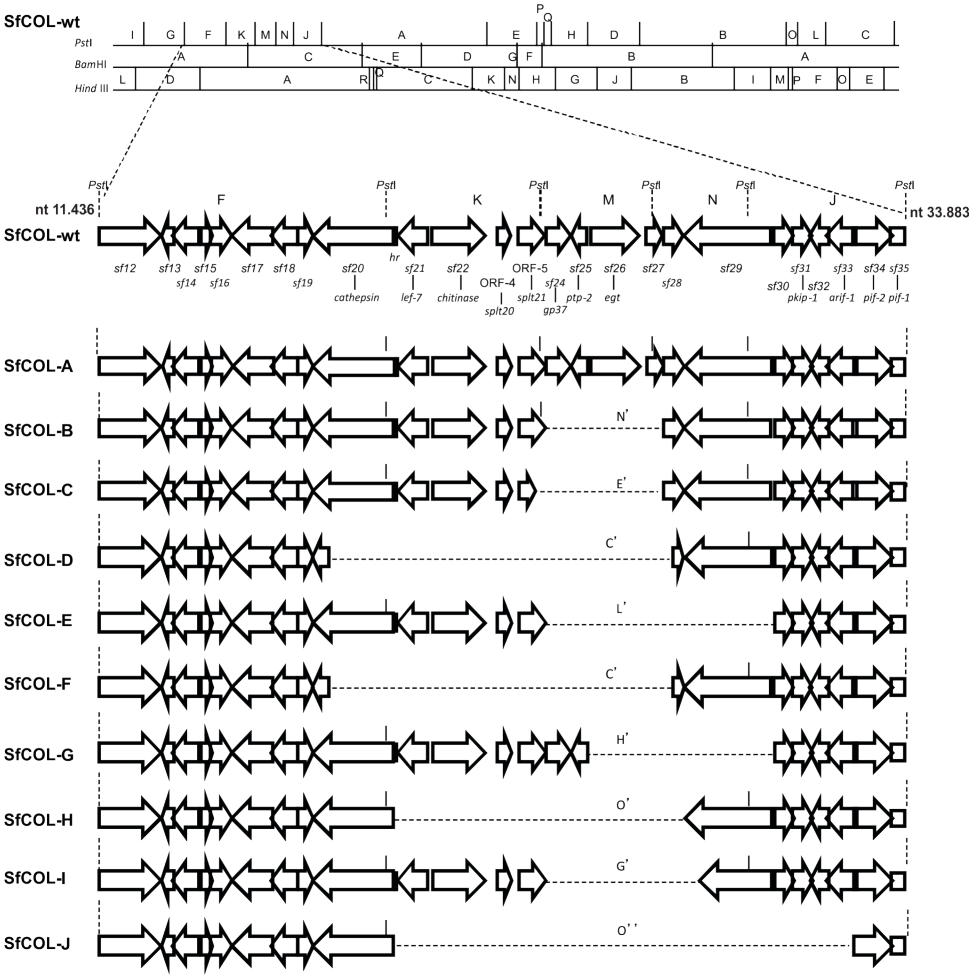

Figure 2. Physical maps of the variable regions of SfCOL genotypic variants. Schematic representation of the gene order of SfCOL genotypic variable region, including *Pst*I-F, -K, -M, -N, and -J fragments. The arrows represent the ORFs and point to their directions of transcription. Gene name and homologous ORFs in SfMNPV genome are indicated below the SfCOL-wt genome.

The *sf28* ORF (nt 26,351-26,998) appeared to be present in SfCOL-A because *sf27-sf28* (nt 26,007-26,809) and *sf28* (nt 26,602-26,809) primers amplified in this genotype. In addition, sequencing of the specific fragments of SfCOL-C, –D and -F genotypes revealed that the *sf28* ORF was present

in the SfCOL-C variants, but was partially deleted in SfCOL-D and -F genotypes. In the last two genotypes, an amplification of the expected size was obtained with *sf28* primers, however these primers amplified 251 nt downstream from the start codon (Simón et al., 2011). Finally, in SfCOL-B the amplification obtained with *sf28* primers and the size of the specific fragment (2.7 Kb), suggested the presence of this ORF. In contrast, the lack of amplification of the *sf29* ORF in SfCOL-E, -G and -J variants suggested the absence of this ORF. SfCOL-D genotype DNA was used to amplify the ends of the ORFs included in the *Pst*I-D fragment (*sf82* to *sf92*) (Simón et al., 2011). All ORFs were amplified with identical sized products to those of SfCOL-wt, indicating the presence of these ORFs in SfCOL-D (Table 1). However, no *Pst*I recognition site was detected at nt 87,999 within the *sf92* gene, as the fragment amplified with *sf91* forward and *sf92* reverse primers of 2,829 bp did not digest with this enzyme, probably due to a point mutation, as the amplified PCR products were identical to SfCOL-wt.

Relative proportion of SfCOL-A genotype in the wild-type population

Sequence analysis revealed that the *egt* gene and *sf27* ORF were the only two genes absent in all deleted genotypes and were only present in the complete SfCOL-A genotype (Figure 2), which permitted the use of both genes as qPCR markers for this genotype, although the *egt* gene was selected for this purpose. qPCR analysis revealed that the SfCOL-A genotype accounted for 70.75±2.32% (mean ± SE) of the genotypes in the wild-type population.

Biological activity of genotypic variants

The biological activities of the SfCOL genotypic variants OBs were compared with that of SfCOL-wt OBs. The OBs of all genotypes were orally infective, however SfCOL-A OBs were approximately 4.4-fold more potent (in terms of concentration mortality-metrics) than SfCOL-wt OBs. In contrast, the potency of OBs of SfCOL-B, -C, -E, -G or -I did not differ significantly from that of SfCOL-wt, and four genotypic variants (SfCOL-D, -F, -H and –J)

were significantly less pathogenic than SfCOL-wt OBs (Table 3). Variants SfCOL-D, -F, -H and –J did not liquefy the infected larvae, likely due to absence of *chitinase* in all of them and/or *cathepsin* in SfCOL-D and –F.

Table 3. Estimated 50% lethal concentration (LC_{50}), relative potency and mean time to death (MTD) values of SfCOL genotypic variant OBs in *Spodoptera frugiperda* second instars.

Virus	LC_{50} (OBs/ml)	Fiducial limits (95%)		Relative potency	*P* value	MTD (h)	Fiducial limits (95%)	
		Low	High				Low	High
SfCOL-wt	$1.03x10^5$	$4.40x10^4$	$1.40x10^5$	1.0	-	167de	160	175
A	$2.34x10^4$	$1.24x10^4$	$4.30x10^4$	4.4	0.001	178e	171	186
B	$1.99x10^5$	$1.11x10^5$	$3.52x10^5$	0.5	0.102	154bcd	146	162
C	$9.02x10^4$	$5.05x10^4$	$1.61x10^5$	1.1	0.740	151bc	144	158
D	$3.31x10^5$	$1.87x10^5$	$5.85x10^5$	0.3	0.005	158cd	151	166
E	$1.32x10^5$	$7.39x10^4$	$2.33x10^5$	0.8	0.538	140b	133	148
F	$2.53x10^5$	$1.43x10^5$	$4.51x10^5$	0.4	0.025	124a	119	130
G	$7.17x10^4$	$3.93x10^4$	$1.28x10^5$	1.4	0.357	160cd	153	168
H	$2.85x10^5$	$1.61x10^5$	$5.04x10^5$	0.4	0.012	125a	120	131
I	$1.95x10^5$	$1.10x10^5$	$3.45x10^5$	0.5	0.110	134ab	127	141
J	$4.60x10^5$	$2.58x10^5$	$8.22x10^5$	0.2	<0.001	126ab	119	133

Logit regressions were fitted using GLIM program. A test for non-parallelism for alltreatments was significant (X^2 = 58.2, d.f. = 10, *P.*= 0.001). The SfCOL-wt, SfCOL -B, -C, -D, -F, -H, and -I genotypes presented a common slope (±SE) of 0.728 ± 0.079 whereas SfCOL-A, -E, -G and -J genotypes presented a common slope (±SE) of 0.940 ± 0.091. Relative potencies of the genotypes were calculated as the ratio of LC_{50} values relative to that of the wild-type isolate. MTD values were estimated by Weibull analysis; values labeled with different letters differed significantly (t-test, $P<0.05$).

Mean time to death values were estimated for virus concentrations that resulted in ~80% larval mortality. SfCOL-F and –H were the fastest-killing genotypic variants, followed by SfCOL-J, -I, and -E, that were significantly more virulent than SfCOL-A, -D and -G genotypic variants, that were similar to SfCOL-wt (Table 3).

The results of OB production of the different SfCOL genotypic variants were not normally distributed and were subjected to non-parametric analysis (Figure 3A).

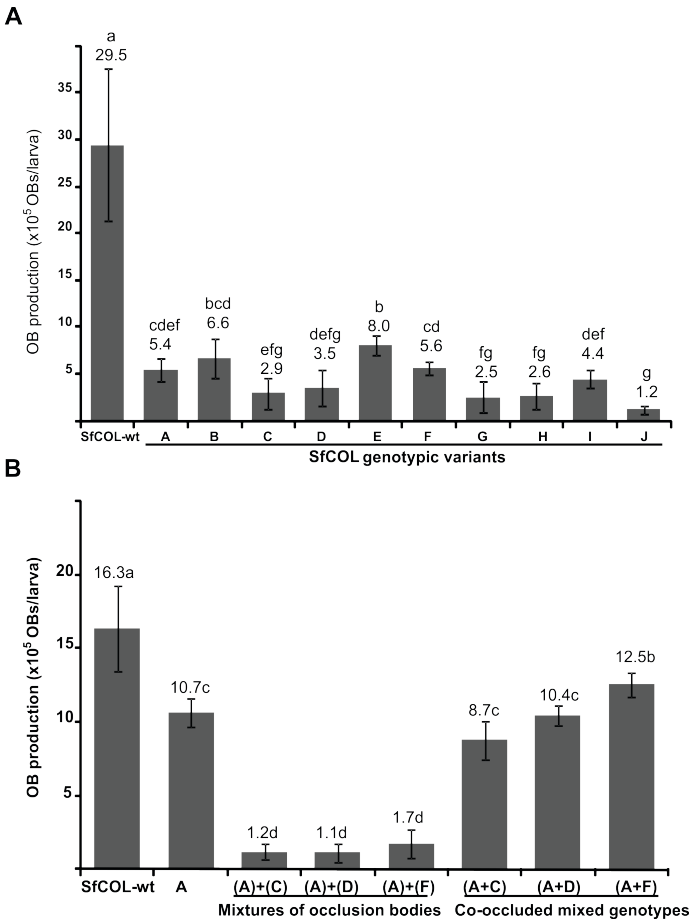

Figure 3. Production of occlusion bodies (OBs) in second instar *S. frugiperda*. A. Median OB production per larva of SfCOL-wt and genotypic variants in *S. frugiperda* inoculated in the second instar. B. Median OB production per larva of SfCOL-wt, SfCOL-A and OB mixtures and co-occluded genotype mixtures comprising equal proportions of SfCOL-A and –C, -D or –F genotypes. Vertical lines indicate the interquartile range. Values followed by different letters indicate significant differences (P≤0.05).

Median OB production in insects infected by SfCOL-wt was significantly higher than that of any of the individual genotypic variants. Significant differences in OB production were also detected between the component genotypes; SfCOL-E had the highest median OB production and SfCOL-J the lowest. There was no significant relationship between speed of

kill and median OB production in insects infected by these genotypes (correlation coefficient R^2 = 0.028).

Biological activity of genotypic mixtures

In order to study the interaction between genotypes, bioassays were performed using insects from a colony that had been recently refreshed with field-collected larvae. Experimental mixtures of OBs were prepared using the most pathogenic genotype (SfCOL-A) in equal proportions with one of the least pathogenic genotypes (SfCOL-D and -F) or a variant of intermediate potency (SfCOL-C) (Table 4).

Mixtures of OBs of SfCOL-A with SfCOL-C, –D or -F OBs had potencies similar to that of SfCOL-wt OBs (Table 4). In effect, the pathogenicity of SfCOL-A genotype was reduced due to dilution by less pathogenic genotype OBs. MTD values in insects that consumed mixtures of OBs were similar to that of insects that consumed SfCOL-wt OBs or SfCOL-A OBs (Table 4). In all cases, median OB production in insects that consumed OB mixtures was approximately 10-fold lower than in insects infected by SfCOL-wt (Figure 3A), and was not significantly affected by the genotypic composition of OB mixtures. Co-occlusion of genotype A in mixtures with genotypes -C, -D or -F resulted in OBs that were of similar pathogenicity to SfCOL-wt OBs (Table 4). In contrast, the MTD of insects that consumed co-occluded mixtures of SfCOL-(A+D) or –(A+F) were significantly reduced compared to insects infected by SfCOL-(A+C), SfCOL-A alone or SfCOL-wt (Table 4).

Table 4. Estimated LC_{50} values, relative potencies and mean time to death (MTD) values of SfCOL-wt, SfCOL-A OBs alone, mixtures of OBs, and co-occluded genotype mixtures, in similar proportions, in *Spodoptera frugiperda* second instars.

Virus	LC_{50} (OBs/ml)	Fiducial limits (95%)		Relative potency	*P* value	MTD (h)	Fiducial limits (95%)	
		Low	High				Low	High
SfCOL-wt	1.09×10^5	6.68×10^4	1.76×10^5	1.0	-	142b	132	151
A	2.69×10^4	1.54×10^4	4.62×10^4	3.7	0.005	144b	134	154
Mixtures of occlusion bodies								
(A)+(C)	4.44×10^4	3.22×10^4	6.17×10^4	2.5	0.010	161b	147	177
(A)+(D)	9.02×10^4	6.55×10^4	1.25×10^5	1.2	0.580	160b	146	175
(A)+(F)	7.84×10^4	5.70×10^4	1.08×10^5	1.4	0.320	142b	131	154
Co-occluded mixed genotypes								
(A+C)	5.36×10^4	3.09×10^4	9.20×10^4	1.9	0.145	142b	132	151
(A+D)	7.69×10^4	4.48×10^4	1.33×10^5	1.3	0.517	115a	108	121
(A+F)	8.00×10^4	4.26×10^4	1.39×10^5	1.2	0.573	120a	112	128

Logit regressions for mixtures of occlusion bodies (OBs) were fitted using GLIM program with a common slope (±SE) of 0.850 ± 0.069. A test for non-parallelism was not significant (X^2 = 7.31, df = 4, *P* = 0.12). Logit regressions for co-occluded mixed genotypes were fitted using GLIM program with a common slope (±SE) of 0.661 ± 0.083. A test for non-parallelism was not significant (X^2 = 2.76, df = 4, *P* = 0.599). Relative potencies were calculated as the ratio of effective concentrations relative to that of the wild type isolate. MTD values were estimated by Weibull analysis; values followed by different letters differed significantly (t-test, P<0.05).

Median OB production in insects that consumed co-occluded genotypes SfCOL-(A+F) was significantly higher than that of SfCOL-(A+C) or –(A+D), both of which were similar to the median OB production value of insects infected by SfCOL-A alone (Figure 3B). No significant relationship was observed between MTD and median OB production values in insects that consumed co-occluded genotype mixtures.

Finally, differences in MTD and OB production between SfCOL-wt and SfCOL-A in assays performed with the individual genotypic variants and with their mixtures, may be related to the insect colony used, as the second colony succumbed faster to SfMNPV infection and produced fewer OBs, than the first colony.

DISCUSSION

The composition of the genotypic variants that comprise the active ingredient can be controlled both in terms of variant selection and relative abundance for the development of baculovirus-based insecticides, and this technology can be protected by patent (Caballero et al., 2007). Given that particularly pathogenic or fast-killing traits will usually result in improved pest control and reduced crop damage following application of the bioinsecticide, these traits have generated the greatest interest during the development of these products (Erlandson, 2009).

In this study, the genotypic diversity of the SfCOL-wt field isolate was studied by plaque purification. The plaques produced by SfCOL genotypic variants were small, as previously described for SfNIC genotypes (Simón et al., 2004). In addition, a low proportion of plaque picks were amplified following injection in *S. frugiperda* larvae. This could be due to the fact that plaque picks did not contain enough virus particles to kill the host, or that cell culture conditions favored the proliferation of other variants, including defective genotypes, which contain large deletions that can affect viral replication (Dai et al., 2000; Pijlman et al., 2001; Simón et al., 2004). Genotypes with shorter genomes can have replication advantages over genotypes with larger genomes leading to potential over estimates of their frequencies using cell culture quantification techniques (Cory et al., 2005; Simón et al., 2004). This was observed in the present study in which SfCOL-F was the most frequent isolated genotype, representing 54% of the clones. However, the indistinguishable restriction profiles of SfCOL-A and SfCOL-wt and the absence of visible submolar bands in SfCOL-wt (Barrera et al., 2011), suggest that SfCOL-A is likely to be the dominant genotype in the natural population. The dominance of a particular genotype in a virus population has been reported in other wild-type baculoviruses, notably SeMNPV and SfMNPV isolates (Muñoz et al., 1999; Simón et al., 2004). This was also confirmed by qPCR analysis of SfCOL-wt; SfCOL-A genotype

accounted for 71% of the genomes amplified from the wild-type population, although this variant represented just 1.2% of the amplified clones. In theory, the restriction profile of SfCOL-A should be different from that of SfCOL-wt, as this variant comprised 71% of the genotypes in the population, however it was indistinguishable probably due to the high frequency of other genotypes with restriction profiles similar to that of SfCOL-A, such as SfCOL-H.

It is known that deletion genotypes can be generated during replication *in vitro* (Dai et al., 2000), and the difference between the frequencies of cell culture isolated genotypes and those present within the wild-type population might suggest that minority genotypes were artifacts produced during propagation of SfCOL in cell culture. However this difference has also been observed previously in the Nicaraguan isolate of SfMNPV (Simón et al., 2004; 2008b). Variants isolated *in vitro* at low frequency from the Nicaraguan isolate were demonstrated to be present in the wild-type population (Simón et al., 2008b). This led us to believe that SfCOL rare genotypes were not artifacts of cell culture. In this sense, SfCOL-A was selected as the reference genotype due to its high frequency in the wild-type population and because it had the largest genome.

Strikingly, the main variable region among SfCOL genotypes was similar in size and collinear with that of SfMNPV isolates from Nicaragua and Missouri (Harrison et al., 2008; Simón et al., 2004). Genotypic variation in baculoviruses is very common in natural populations and can be the result of natural intragenomic recombination in hot spot regions that contain *bro* genes, homologous regions or transposable elements (Erlandson, 2009). Sequence analysis of the variable region in SfCOL variants revealed the presence of a single *hr,* that is identical and collinear with *hr2* reported in other SfMNPV isolates (Harrison et al., 2008; Simón et al., 2011; Wolff et al., 2008). The variability around the *hr* also seems to be a common characteristic in other nucleopolyhedroviruses including SeMNPV (Muñoz et al., 1999), *H. armigera* NPV (Ogembo et al., 2007) and *Bombyx mori* NPV

(López-Ferber et al., 2001), suggesting that *hrs* are hot spots for intragenomic variation (Erlandson, 2009). In SfMNPV, this region comprises ORFs between *sf20* to *sf29* that encode non-essential proteins with auxiliary functions, including the *egt* gene that can affect the speed of kill phenotype of these viruses (Harrison et al., 2008; Simón et al., 2005). The *egt* gene encodes an ecdysteroid UDP-glucosyltransferase that can extend the infection period allowing increased production of progeny OBs in each infected insect (Wilson et al., 2000). Using a fast-killing phenotype for pest control has been considered advantageous, since pest feeding damage on plants is reduced as larvae die earlier (Behle and Popham, 2012). Nine defective SfCOL genotypic variants lacked the *egt* and *sf27* genes, however only six of them presented a faster killing phenotype compared to SfCOL-wt, suggesting that other factors are likely involved in these phenotypes.

All the SfCOL variants were orally infective as their OBs produced mortality in larvae, in contrast to that observed in SfNIC variants (Simón et al., 2004), indicating that none of them lacked the *pif* genes that are essential for peroral transmission (Peng et al., 2012). However, differences were observed in OB pathogenicity and speed of kill; the genotypic variants with the largest deletions were significantly less pathogenic than SfCOL-wt. Similarly, variants with the largest deletions were significantly less pathogenic than the other variants present in the SfNIC population (Simón et al., 2004, 2005), and are therefore likely to be subject to selection for variation in transmissibility under varying biotic and abiotic conditions. *Chitinase* and *cathepsin* were deleted in the least pathogenic SfCOL variants and have been reported to be absent in several genotypes cloned from other SfMNPV isolates (Harrison et al., 2008; Simón et al., 2005; Vieira et al., 2012). These genes appear to act together to facilitate the release of OBs from dead larvae with corresponding improvements in horizontal transmission (Slack and Arif, 2007; Vieira et al., 2012). Additionally, the genomic deletion common to the low potency variants included *lef-7* and

ORFs similar to *splt20* and *splt21*, suggesting that the last two ORFs could be involved in the pathogenic characteristics of these viruses as *lef-7* is known to be involved in the replication of late genes (Gomi et al., 1997). Also, some SfCOL genotypes lacked ORF *sf29* that has been reported as a viral factor that may determine the number of ODVs occluded in each OB and thereby modulate the infectivity of OBs (Simón et al., 2008a). However, no differences were observed in OB pathogenicity between SfCOL genotypes that lacked *sf29* and SfCOL-wt in the present study.

SfCOL-A OBs were 4.4-fold more potent than SfCOL-wt OBs, indicating that the presence of other genotypes diminished the pathogenicity of the population, as observed in an isolate of SeMNPV (Muñoz et al., 1998). Interactions between genotypes within baculovirus populations can 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 transmissibility, specifically affecting factors that influence the ability of the pathogen to infect, replicate and transmit to a new host. In contrast, all individual genotypes in the SfNIC population had lower pathogenicity than the wild-type isolate; indeed, interactions between SfNIC variants increased the transmissibility of wild-type population (López-Ferber et al., 2003; Simón et al., 2004, 2005). Characterizing interactions between genotypes is therefore crucial during the selection of genotypes or mixtures of genotypes with suitable characteristics for use in biological insecticides. Experimental mixtures of OBs and mixtures of genotypes that were co-occluded in OBs were prepared using the most pathogenic variant (SfCOL-A) and one of three genotypes with lower potency (SfCOL-C, -D or –F). Both types of mixtures decreased the pathogenicity of SfCOL-A, to values similar to that of SfCOL-wt OBs, suggesting a dilution effect of the genotypes when present in mixtures with the most pathogenic genotype SfCOL-A. Similarly, SeMNPV deletion genotypes reduced the pathogenicity of OBs when mixed with complete genotypes (Muñoz et al., 1998). Following inoculation with a

mixture of fast and slow killing genotypes, larvae may experience a survival time that is intermediate between the two viruses (Georgievska et al., 2010; Pijlman et al., 2001). As such, the speed of kill of of SfCOL-(A+D) and -(A+F) mixtures was faster than SfCOL-A alone or SfCOL-wt. A positive speed of kill interaction between these genotypes was only observed when they were co-occluded for reasons that are unclear but which may be related to the prevalence of fast-killing *egt*-deleted genotypes that were transmitted more efficiently when co-occluded with the complete SfCOL-A genotype.

In conclusion, the SfCOL-wt field isolate comprises a high genotypic diversity of which SfCOL-A, the most prevalent genotype in wild-type population, was the most pathogenic and was as virulent as SfCOL-wt, in terms of speed of kill. Deletion genotypes decreased OB potency but increased OB productivity. Although within SfCOL-wt OBs the SfCOL-A was the dominant genotype, SfCOL-wt was more productive than SfCOL-A, suggesting that the other deletion genotypes presented within the SfCOL-wt may modulate increases in OB production. In this sense, it seems that SfCOL-wt is structured to maximize the likelihood of transmission. Mixtures including the most pathogenic SfCOL-A genotype and genotypes with lower pathogenicity, reduced speed of kill but also reduced OB pathogenicity which is undesirable for the development of a biological insecticide. Baculovirus based bioinsecticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest. As such, OB production is usually considered to be a trait of reduced importance during bioinsecticide development, except for its involvement in virus production costs. In this respect, the most pathogenic variant was SfCOL-A which presented the most suitable characteristics as the basis for a biological insecticide to control *S. frugiperda* in Colombia.

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CHAPTER IV

Efficacy of microencapsulated *Spodoptera frugiperda* multiple nucleopolyhedroviruses from Colombia as biological insecticides

ABSTRACT

Baculoviruses are a real alternative in pest management programs, however some limiting characteristics make advise formulations to improve the application and stability in field crops. Wettable powder formulations by microencapsulation of viral occlusion bodies (OBs) of both *Spodoptera frugiperda* multiple nucleopolyhedrovirus from Colombia (SfCOL) and a genotypic variant (SfCOL-A) were developed for control of the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in maize crops. The microencapsulation preserved the insecticidal activity of the OBs after three months of storage at 35ºC, where the rate of inactivation was not greater than 12%, while the unformulated OBs lost between 36 and 46 % of efficacy. Besides, the formulation protected the OBs against inactivation caused by UV-B radiation, retaining its efficacy after 6 hours, in contrast unformulated viral suspensions lost between 50-80% of efficacy. Under greenhouse conditions, the efficacy of the microencapsulated viruses was higher than 80%, similar to the conventional chemical treatment (lufenuron). In field trial, a decrease of percentage of plants with fresh damage was observed when the formulated and unformulated isolates were applied at 8 x 10^{11} OBs/Ha (800g/Ha) dose, being damage levels below the economic injury level (35 %), while the damage in the control treatment was close to 60%. However no differences were observed between unformulated and formulated virus in field trials at 800g/ha applications. The microencapsulation of SfCOL and SfCOL-A OBs provides useful advantages related to storage stability and resistance to UV radiation.

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INTRODUCTION

The fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae) is a severe pest of maize and other crops (Murua and Virla, 2004). Infestations can result in 34 to 60% losses of maize yields (Carnevalli and Florcovski, 1995; ICA, 2008). Commonly the control of *S. frugiperda* in maize crops includes extensive use of broad spectrum and highly toxic chemical insecticides (Andrews, 1988; Ashley et al., 1983; Molina-Ochoa et al., 2003), resulting in an increased probability of poisoning by farmers and of residues accumulation in human and animal food (Lapied et al., 2009). Moreover, chemical insecticides interference with the action of natural enemies and generate resistance development (Chandler and Summer, 1991).

The multiple nucleopolyhedrovirus of *S. frugiperda* (SfMNPV: *Baculoviridae*) has been intensely studied as a potential biological control agent (Barrera et al., 2011; Behle and Popham, 2012; Martinez et al., 2003; Williams et al., 1999). This virus has been isolated from fall armyworm populations in North, Central and South America (Barrera et al., 2011; Barreto et al., 2005; Berretta et al., 1998; Escribano et al., 1999; Loh et al., 1981; 1982). Some isolates have been evaluated under field conditions as potential biopesticides to control *S. frugiperda* on maize crops (Armenta et al., 2003; Barreto et al., 2005; Behle and Popham, 2012; Cisneros et al., 2002; Moscardi, 1999; Valicente and Costa, 1995; Williams et al., 1999). Recently, a SfMNPV field isolate (SfCOL) with potential to develop as biopesticide in Colombia was identified (Barrera et al., 2011). This isolate comprises a high genotypic diversity of which the most prevalent genotype in the population (SfCOL-A) showed the highest pathogenicity among all genotypic variants, even greater than SfCOL wild type (Barrera et al., 2013). This feature makes it a strong candidate for the development of a SfMNPV-based formulation to control *S. frugiperda* in Colombia (Barrera et al., 2013).

Although SfMNPVs are efficient to control insect pests, there are some limitations to their use (Moscardi et al., 2011). The survival of the

viruses in the environment can be affected by different factors including temperature, pH, moisture and exposure to UV light (Szewczyk et al., 2012; Villamizar et al., 2009). Therefore, adequate formulation may substantially improve efficiency and tolerance against adverse environmental factors (Behle et al., 2003; Rodriguez et al., 2012; Szewczyk et al., 2012). Additionally, formulation has been shown important to stabilize the viruses during storage, and distribution and help the handling and application to crops (Behle et al., 2003; Jones and Burges, 1998; Tamez-Guerra et al., 2002).

Several formulations of SfMNPV using different strategies has been studied (Cruz et al., 1997; Valicente and Costa, 1995; Williams et al., 1999) and some of them includes addition of phagostimulants (Castillejos et al., 2002), optical brighteners (Martinez et al., 2003), azadirachtin (Zamora-Avilés et al., 2013) or organic acids (Cisneros et al., 2002). However, addition of chemicals to biopesticide products is not well accepted and microencapsulation represented a promising alternative for SfMNPV formulation (Behle and Popham, 2012; Villamizar et al., 2010). This technique allows to coat small solid particles in a thin uniform layer of coating material, protecting the virus from environmental conditions and improving the storage stability (Villamizar et al., 2010). Recently, a fast killing genotypic variant was formulated by encapsulation and evaluated in field grown cabbage (Behle and Popham, 2012). To set the appropriate parameters for the use of a microencapsulated SfMNPV biopesticide, it is necessary to evaluate the product under greenhouse and field conditions (Grzywacz et al., 2008; Gupta et al., 2007; Lasa et al., 2007a). The aim of the present study was to evaluate the insecticidal activity of both SfCOL and SfCOL-A formulated by microencapsulation with a methacrylic acid polymer Eudragit S100®. For that, formulations were compared to unformulated viruses under laboratory, greenhouse and field conditions.

MATERIAL AND METHODS

Insect colony and virus strain

S. frugiperda larvae were obtained from a laboratory colony established in the Biological Control Laboratory of the Colombian Corporation of Agricultural Research (CORPOICA). The colony was maintained at 25 ºC, 75% RH (relative humidity) and 16 h light -8 h dark photoperiod on a wheat germ based semi-synthetic diet (Greene et al., 1976). The wild type SfMNPV (SfCOL) used in this study was obtained from field diseased larvae from Colombia (Barrera et al., 2011) and the genotypic variant (SfCOL-A) was isolated by plaque purification from SfCOL isolate in Sf9 culture cells (Barrera et al., 2013). Viral occlusion bodies (OBs) were propagated in third instar S*. frugiperda* larvae using the droplet feeding method (Hughes and Wood, 1986). Dead larvae were carefully collected daily and stored at -20ºC. OBs were extracted from dead disease larvae by homogenizing cadavers in SDS solution (0.1%). The obtained suspension was filtrated and centrifuged in a 30% sucrose cushion to purify OBs.

Formulation and quality control analysis

Purified OBs were freeze-dried and microensapsulated using Eudragit S100® according to the methodology described by Villamizar et al. (2010) with some modifications. Eudragit S100® was dissolved in acetone (5%) and freeze-dried virus (1%) was added to the polymer solution. This suspension was included into a solution of Span 80 (3%, v/v) in liquid paraffin and homogenized by an ultraturrax homogenizer at 20ºC ±2. The microcapsules were harvested by centrifugation, washed three times with Tween 80 solution (1%) and freeze-dried.

A quality control analysis was performed with the final product (wettable powder), and viral quantity, moisture content, bacteria content and efficacy were measured. To determine the OBs concentration, 10 mg of microparticles were dissolved in 1 ml of 0.05 M sodium borate buffer (pH 8.0) for 5 min in order to release the OBs, then the concentration was

determined using a Neubauer haemocytometer and a light microscope. To measure moisture content three samples of 0.5 g were tested in a moisture analyzer OHAUS MB 45 at 1008C for 10 min. To evaluate the presence of bacteria content, a sample of 0.1 g of product was suspended in 0.5% (v/v) Tween 80 solution. Ten-fold dilutions were prepared, and 1 ml of each dilution was applied to Petri dishes containing Nutrient-Agar and incubated by 24 h at 37 ºC. Three replicate samples were tested, and results were expressed as average number of colony forming units per gram (CFU/g).

Efficacy of microencapsulated OBs was determined in second-instar *S. frugiperda* larvae by the droplet feeding method (Hughes & Wood, 1981). The larvae were starved for 16 h at 26ºC and then allowed to drink from an aqueous suspension containing 10% (w/v) sucrose, 0.001% (w/v) Fluorella blue, and formulated virus at 1 x 10^7 OBs/ml. This concentration was previously used for quality control analysis of microencapsulated SfMNPV by Eudragit S100® (Gómez et al., 2013). Control larvae were treated identically but drank from a sucrose and dye solution that did not contain OBs. Larvae that ingested the suspension within 10 min were transferred to individual plastic cups with semysinthetic diet. Bioassays were performed using 24 larvae per formulated product and 24 control larvae. Insects were reared at 25ºC, and mortality was recorded until death or pupation. Mortality values were corrected with the value in the negative control by the Schneider-Orelli equation (1947):

$$\text{Efficacy} = ((A - B)) / (100 - B) \times 100$$

where *A* is larval mortality obtained in the treatment and *B* is the mortality in the negative control treatment.

Effect of formulation over SfCOL and SfCOL-A pathogenicity

The mean lethal concentration (LC_{50}) was determined for SfCOL and SfCOL-A both unformulated viruses (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) in second instars *S. frugiperda* larvae using

droplet feeding bioassay technique (Hughes and Wood, 1986). Twenty-four larvae were starved for 16 h and then allowed to drink from aqueous suspension containing 10%(w/v) sucrose, 0.001% (w/v) Fluorella blue and OBs at one of the following concentrations for all the treatments: 1.92 x 10^3, 9.60 x 10^3, 4.80 x 10^4, 2.40 x 10^5 and 1.2 x 10^6 OBs/ml. This range of concentrations was previously determined to kill between 5% and 95% of experimental insects (Barrera et al., 2011). Bioassay was performed three times using 24 larvae per treatment and 24 control larvae treated identically but drank from a sucrose and dye solution that did not contain OBs. Larvae were reared at 25°C and mortality was recorded until the insects had either died or pupated. Virus induced mortality was subjected to logit analysis using the Generalized Linear Interactive Modeling (GLIM) program (Crawley, 1993).

Storage assays

SfCOL and SfCOL-A both unformulated (in aqueous suspensions) and formulated (wettable powders) were evaluated before (time 0) and after accelerated storage conditions (35ºC). The efficacy and bacteria content was evaluated monthly during three months. For efficacy evaluation, bioassay were performed in second instar *S. frugiperda* larvae inoculated with 1 x 10^7 OBs/ml for all treatments by droplet feeding technique (Hughes and Wood, 1986). Bioassays were performed three times with 24 larvae per treatment and 24 larvae as control. The mortality data were expressed as efficacy using the Schneider-Orelli equation (1947). The data were subjected to repeat measure analysis of variance in SPSS program version 10.0 and pairwise treatments were compared by within-subject pairwise comparisons among estimated marginal means with Bonferroni correction.

The bacteria content analysis was performed three times as described above. Data were normalized by log transformation and subjected to repeat measure analysis of variance (ANOVA) in SPSS version 10.0.

Photostability assays

Samples of 200 ul of SfCOL and SfCOL-A both unformulated and formulated, at 2 x 10^7 OBs/ml were placed in a microplate of 96 wells. The first column of the microplate was covered with aluminium film before radiation to protect it against UV light, which was used as control. Then, the microplate was placed inside a chamber at 10 cm from a Lamp Monochromatic UV-B and exposed to the light (between 280 and 320 nm). After 2, 4 and 6 hours of radiation, the samples were collected and mixed with equal volume of a suspension containing 10%(w/v) sucrose, 0.001% (w/v) Fluorella blue to obtain a final concentration of 1 x 10^7 OBs/ml. Bioassay was repeated three times for each treatment using the droplet feeding technique (Hughes and Wood, 1986) as described above. The mortality data was expressed as efficacy using the Schneider-Orelli equation (1947) and the remaining original activity (OAR) was calculated. The data were subjected to repeat measures analysis of variance in SPSS program version 10.0. Test of Mauchly' s sphericity was applied to contrast the variances. The significance of treatment effects at each sample time was determined by within-subject pairwise comparisons among estimated marginal means with Bonferroni correction.

Efficacy in greenhouse conditions

Efficacy assays were conducted on maize plants in greenhouse conditions with unformulated viruses (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) resuspended in water and adjusted to a concentration of 1x10^7 OBs/ml (Gómez et al., 2013). The experimental unit consisted in a row with 12 plants of 35 cm height with six true leaves. The distance between rows was 100 cm. A fully randomized blocks design was performed with five replicates. Chemical treatment with lufenuron (Match 5 EC®, Syngenta) (Chemical Group: Benzoylurea) at the label recommended rate and control treatment with water were included. Treatments were applied by using 2 ml/plant with a hand sprayer. Then, four-second instar larvae were

located per plant (Castillejos et al., 2002). After 48 and 96 hours, larvae were collected and individually reared in the laboratory on artificial diet and checked for virus mortality. The efficacy was measured by percentage of mortality corrected with control treatment and subjected to repeat measures analysis of variance in SPSS program version 10.0. Test of sphericity was performed using Mauchly´s criterion and significance was interpreted in terms of F statistics generated from Pillai's trace.

Efficacy in field conditions

Field trials were carried out from April 2013 to June 2013 in maize crop in the locality of Espinal, in the department of Tolima (Colombia) with a mean annual temperature of 30±2 °C and a mean annual relative humidity of 80±3 %. The crop was planted with the variety 30F32 Pioner at density of approximately 40.000 plants/Ha. Experimental design was in randomized complete blocks. The experimental unit consisted in a plot of 50 m2 including 6 rows of 10 m length with three replicates per treatment and with a gap of 2 m without plants between blocks. Treatments consisted in SfCOL and SfCOL-A both unformulated and formulated and control treatment with water application. Unformulated and formulated virus at 8 x 10^{11} OBs/Ha were applied on maize plants 3 and 17 days after germination of plants as described previously for SfMNPV microencapsulated applied in field conditions (Gómez et al., 2013). Treatments were applied with an air-assisted hydraulic sprayer with a cone nozzle at a pressure of 4 Kg/cm^2. Feeding damage was evaluated at 3, 10, 17 and 24 days after germination (dag). An additional evaluation at 31 dag was made in the first plot. Thirty plants were randomly selected in the three central rows of each plot and were examined for the presence of fresh damage in the youngest leaves. Results were expressed as feeding damage percentage. The data were subjected to repeat measures analysis of variance in SPSS program version 10.0. Test of Mauchly' s sphericity was applied to contrast the variances. The significance of treatment effects at each sample time was determined

by within-subject pairwise comparisons among estimated marginal means with Bonferroni correction.

RESULTS

Quality control analysis of formulated viruses

All the parameters analysed were within the acceptance limits described by Gómez et al. (2013) (Table 1). The results were similar in both formulated SfCOL-F and SfCOL-A-F wettable powders. The concentrations of active ingredient in wettable powders were close to 1.0 x 10^9 OBs/g for both evaluated products. The moisture content and pH values ranged between 1.98% and 2.29% and between 6.22% and 6.14%, respectively. The bacterial contamination values evaluated were close to 4.0 x 10^7 UFC/g and the efficacies ranged between 96.3 and 94.4% for SfCOL-F and SfCOL-A-F respectively.

Table 1. Quality control results of newly formulated SfCOL-F and SfCOL-A-F. Mean values and the error standard for each parameter are expressed.

Characteristic	SfCOL-F	SfCOL-A-F	Acceptance limits*
Concentration (OBs/g)	1.07 x 10^9 ± 0.01	1.13 x 10^9 ± 0.01	≥ 1.00 x 10^9
Moisture content (%)	1.98 ± 0.02	2.29 ± 0.02	< 5
pH	6.22 ± 0.01	6.14 ± 0.01	6 - 8
Bacteria content (UFC/g)	4.3 x 10^5 ± 0.7	4.0 x 10^5 ± 0.7	< 5.0 x 10^8
Efficacy (%)	96.3 ± 5.27	94.4 ± 2.29	≥80%

*Acceptance limits according to Gómez et al. (2013)

Formulation did not affect the viral pathogenicity

The pathogenicity of unformulated viruses did not differ significantly from their respective formulations in both SfCOL and SfCOL-A isolates (Table 2). The LC_{50} values of SfCOL-A both unformulated and formulated OBs were significantly lower than those obtained for SfCOL unformulated OBs, as observed previously (Barrera et al., 2013). The pathogenicity of SfCOL was improved with the formulation reaching values that did not differ significantly

from those of SfCOL-A. Similarly, the relative potency of SfCOL-A was increased 1.88 times when OBs were formulated, although significant differences were not observed.

Table 2. Estimated LC_{50} and relative potencies values of SfCOL and SfCOL-A OBs both unformulated and formulated (F) in *S. frugiperda* second instars.

TREATMENT	LC_{50} (OBs/ml)	Fiducial limits (95%)		Relative Potency	P value
		Low	Up		
SfCOL	1.58×10^5	1.04×10^5	2.40×10^5	1	
SfCOL-F	1.10×10^5	6.23×10^4	1.97×10^5	1.43	0.258
SfCOL-A	5.75×10^4	3.35×10^4	9.87×10^4	2.75	0.004*
SfCOL-A-F	3.06×10^4	1.77×10^4	5.21×10^4	5.16	<0.00*

Logit regressions for mixtures of occlusion bodies (OBs) were fitted using GLIM program with a common slope (±SE) of 0.489 ± 0.083. A test for non-parallelism was not significant (χ^2 = 7.46. df = 3. *P* = 0.06). Relative potencies were calculated as the ratio of effective concentrations relative to that of the wild type isolate.

Formulated viruses retain their infectivity after accelerated storage

In unformulated viruses, a correlation was observed between the decrease in the activity and the storage time (treatment*time interaction F_3=15.011, *P*=0.003) after three months of accelerated storage conditions (Figure 1). After third month, SfCOL and SfCOL-A efficacies diminished close to 50%. In contrast, the formulated products maintained their efficacies above 80%.

Bacterial contamination before (range 4.2×10^5 and 6.9×10^6 CFU/ml) and after three months of accelerated conditions (range 1.3×10^4 and 9.2×10^7 CFU/ml) in both unformulated and formulated viruses were in acceptance limits (< 5×10^8 CFU/ml) (Gómez et al., 2013) (Figure 2). The initial bacterial contamination values of unformulated isolates were significantly higher than those obtained for formulated viruses (*P*<0.001), and increased significantly after three months of storage (13.2 and 12.0 times in SfCOL and SfCOL-A, respectively), reaching values ranging from 6.23×10^7 to 9.2×10^7 UFC/ml (treatment*time F_3 =23.043, *P*=0.01). In

contrast, the CFU counts of formulated viruses diminished after months (P<0.001).

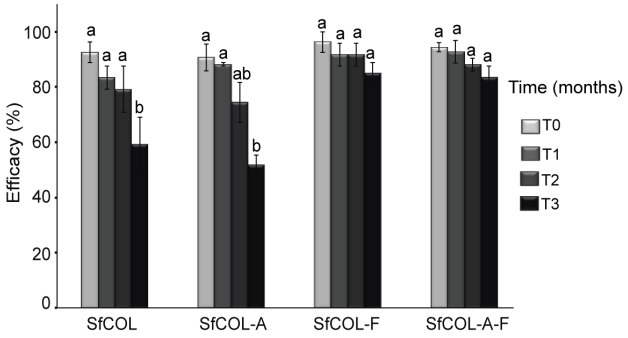

Figure 1. Efficacy of both unformulated (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) viruses at 0 (T0), 1 (T1), 2 (T2), and 3 (T3) months of storage at 35ºC. Bars labelled with identical letters were not significantly different for comparisons between treatments within each time storage (repeated measures ANOVA with intra-subject pairwise comparison of estimated marginal means, P > 0.05 Bonferroni correction)

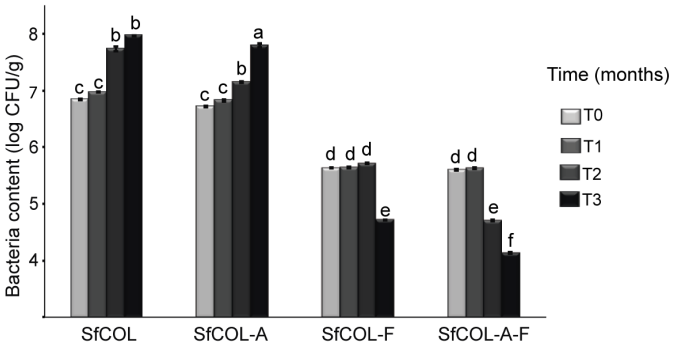

Figure 2. Bacterial contamination after 0 (T0), 1 (T1), 2 (T2), and 3 (T3) months of storage at 35ºC within both unformulated (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) viruses. Bars labelled with identical letters were not significantly different for comparisons between treatments within each time of storage (repeated measures ANOVA with intra-subject pairwise comparison of estimated marginal means, P > 0.05 Bonferroni correction).

Formulated viruses retained their infectivity after UV-B radiation

The original efficacies (before irradiation) of all treatments were higher than 90%, whereas SfCOL efficacy was 84.7%; however, not significant differences among treatments were observed at this time ($F_{3,8}$=2.42, P=0.14) (Table 3). Remaining original activity (ROA) after six hours of irradiation decreased significantly when the isolates were not formulated (treatment*time interaction $F_{9.0}$=2.71, P=0.022). Not significant differences were observed among efficacies of formulated isolates at different times (P=0.054). The efficacy of both unformulated viruses (SfCOL and SfCOL-A) was significantly reduced after two hours of irradiation being inferior to 50% (treatment*time interaction $F_{9.0}$=2.71, P>0.001) (Figure 3). The inactivation percentage of SfCOL-A (87.9 %) was higher than that of SfCOL (50%) after six hours (P<0.001)

Table 3. Efficacy of unformulated (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) viruses after 6 hours of irradiation with UV-B light in laboratory conditions.

TREATMENT	Efficacy before irradiation (%)	Efficacy after 6 hours of irradiation (%)	ROA (%)	Inactivation (%)
SfCOL	84,1	39,1	50,0	50,0 (b)
SfCOL-F	94,2	82,6	98,3	1,7 (c)
SfCOL-A	91,3	10,3	12,1	87,9 (a)
SfCOL-A-F	94,2	88,4	96,8	3,2 (c)

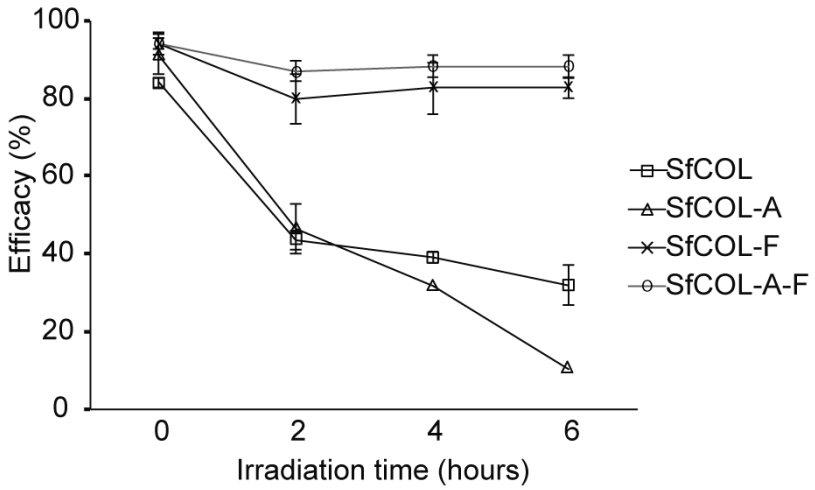

Figure 3. Efficacy of unformulated (SfCOL and SfCOL-A) and formulated (SfCOL-F and SfCOL-A-F) viruses after 2, 4 and 6 hours of irradiation with UV-B light.

Efficacy under greenhouse conditions

The efficacies of unformulated and formulated treatments at two different times were evaluated on maize plants under greenhouse conditions (Figure 4). Significant differences were not found in mortality levels between 48 and 96 hours in all the treatments, either in unformulated and formulated viruses (treatment*time interaction F_1=0.40, P=0.53). Unformulated SfCOL efficacy was significantly lower than the other treatments at 48 and 96 hours ($F_{4,20}$=15.14, P=0.001), being inferior to 61%. No differences were found in the insecticidal activities of SfCOL-A and SfCOL-A-F ($F_{1,20}$=0.40, P=1.00), showing efficacies higher than 90% as found with chemical insecticide.

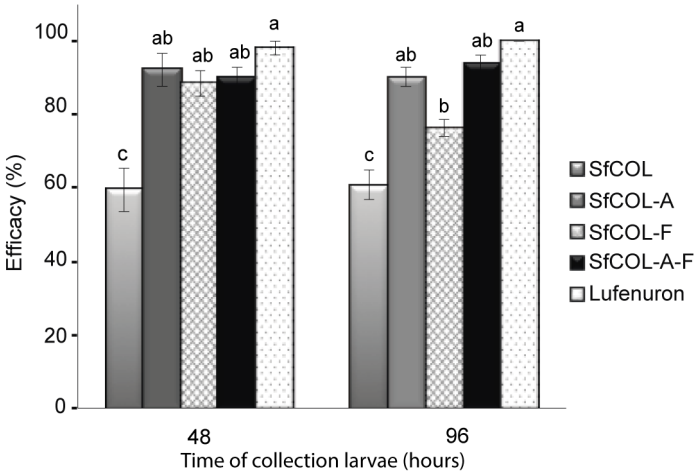

Figure 4. Efficacy of SfCOL and SfCOL-A both unformulated and formulated and chemical insecticide (lufenuron) (measured by mortality percentage) over *S. frugiperda* larvae collected at different times after application (48 and 96 hours) and reared in the laboratory (ANOVA with intra-subject pairwise comparison of estimated marginal means, $P>0.05$ Bonferroni correction).

Efficacy in field conditions

The feeding damage in the plot planted with 30F32 seed ranged between 2 and 10% for all treatments including the control until 10 dag (Figure 5). The feeding damage exceeded economic injury level at 17 dag in plants that had no virus application, reaching to 45.6%, while plants treated with both unformulated and formulated viruses showed feeding damages below 34%, although not significant differences were observed ($F_{4,10}=0.93$, $P=0.482$). In contrast, at 24 dag significant differences were observed between control and virus treatments ($F_{4,10}=19.2$, $P<0.001$). In control plants the fresh damage reached to 58.9%, while this value was fewer than 35% in all the virus treatments, showing no significant differences among the latters.

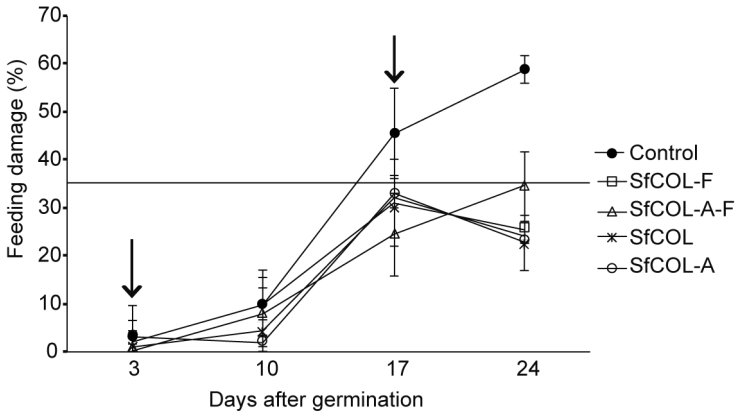

Figure 5. Percentage of maize plants showing *S. frugiperda* fresh feeding damage after applications of SfCOL and SfCOL-A both unformulated and formulated in 30F32 Pioner maize plot. The line indicate the economic injury level for *S. frugiperda* on maize in Colombia (35%). Vertical arrows indicate the moment of application of treatments

DISCUSSION

Several studies have demonstrated the potential use of SfMNPV as biological control agent against *S. frugiperda* (Barrera et al., 2011; Barreto et al., 2005; Behle and Popham, 2012; Berretta et al., 1998; Cisneros et al., 2002). In a previous work a Colombian isolate of SfMNPV (SfCOL) and its genotypic variant (SfCOL-A) demonstrated great potential as biopesticide products to control the fall armyworm *S. frugiperda* in Colombia (Barrera et al., 2011; Barrera et al., 2013; Villamizar et al., 2010). However, the validation under lower controlled conditions such as those found in field is a prerequisite for the product registration. No baculovirus based control programs have been devised for *S. frugiperda* in Colombia. One of the factors that most hamper the development of this virus is its inactivation by several environmental factors (Burges and Jones, 1998; Chang et al., 1998; Jones et al., 1993) and the short shelf-life (Burges and Jones, 1998). Formulation can be used to protect the viruses from some adverse environmental factors, enhance storage stability and maximize application

efficiency (Burges and Jones, 1998; Lasa et al., 2008; Tamez-Guerra et al., 2002). In the present study, SfCOL and SfCOL-A were formulated by microencapsulation and their insecticidal activities were compared with unformulated viruses in laboratory, greenhouse and field conditions.

A quality control analysis was performed after microencapsulation to guarantee the effectiveness in field. The OB concentration obtained in each batch (1.0×10^9 OBs/g) was similar to registered biopesticides (Kolodny-Hirsch et al., 1997). This is important as the concentration of occlusion bodies in formulated products determines the rate of application in the field, being necessary to maintain a constant concentration in each batch of biopesticide. Additionally, moisture content and pH of formulated virus could contribute to stabilize products under storage since neutral or alkaline pH and moisture superior to 5% can favour bacterial growth (Jones and Burges, 1998). Extreme pH values can also be harmful to the OBs. Thus pH between 4 and 6, that not affect OBs, is recommend to prevent the growth of most contaminating organisms (Jones and Burges, 1998; Tamez-Guerra et al., 2002). Moisture and pH can have clear effects on the content of bacteria in a formulation of OBs, however microencapsulated SfCOL and SfCOL-A moisture (between 1.07 and 1.13) and pH (between 6.1 and 6.2) values were in range to prevent bacterial growth. But contaminating bacteria can be also introduced by *in vivo* massive viral production, where the insect's microorganisms could arrive to the final product. This is unacceptable from the viewpoint of product safety and because it can have deleterious effects for the stability of the virus (Jones and Burges, 1998; Lasa et al., 2008). However, formulated viruses presented lower bacteria concentrations compared to unformulated viral suspensions, possibly due to freeze-drying technique used for removing moisture from microcapsules and to the organic solvent used to dissolve the Eudragit S100®, which could help to reduce the content of contaminating bacteria. Additionally, formulation had not effect over insecticidal activity in terms of median lethal concentration.

The main objective of microencapsulation is protecting the virus from environmental conditions and improving the storage stability.

Storage stability has to be considered when developing baculovirus-based biopesticide (Tamez-Guerra et al., 2002). In this sense, studies based on accelerated storage conditions in which product is stored at high temperature provided a useful tool to predict the shelf-life of product over time (Corradini and Peleg, 2007). Microencapsulation of SfCOL and SfCOL-A OBs protected these viruses against degradation during the storage. The efficacies of formulated SfCOL and SfCOL-A were maintained after 3 months storage at 35±2 °C, whereas unformulated OBs efficacies decreased to 60% and 52%, respectively, in the same time. Similarly, the bacteria content increased significantly in unformulated viruses after three months of storage, while in formulated products the bacteria content diminished. The formulation of SfCOL and SfCOL-A could help to maintained viral efficacy by the reduction of the microbial contaminants that may be implicated in accelerating chemical processes that inactivate OBs (Jones and Burges, 1998; Lasa et al., 2008), as previously observed during SeMNPV storage (Lasa et al., 2008).

Radiation from sunlight, especially radiation between 280 and 310 nm is the main constraint affecting the insecticidal activity of baculoviruses when applied in field (Asano, 2005; Ignoffo and García, 1992). Specifically, the SfMNPV is sensitive to UV radiation, where inactivation takes place after 15 minutes of exposure to direct UV light (Mondragón et al., 2007). Addition of optical brighteners such as Tinopal or Calcofluor M2R to SfMNPV formulations provided photo-protection (Martinez et al., 2003; Mondragón et al., 2007). However incorporation of optical brighteners (chemical) to biopesticides products is not well accepted. The microencapsulation technique used in this work protected the OBs from degradation caused by UV radiation, being the remaining original activity superior to 95% after 6 hours of exposure, while unformulated SfCOL and SfCOL-A showed 50%

and 87% of inactivation, respectively. This result suggests that the mixture of genotypes present in the wild type isolate provided more resistance to environmental conditions such as sun radiation, maximizing the likelihood of transmission.

Greenhouse trials were performed to refine the conditions to be used in field trials (Ibarra and Del Rincón, 2001). The efficacies of both SfCOL and SfCOL-A formulations were similar to chemical treatment (lufenuron) commonly used to control *S. frugiperda* (Chandler and Summer, 1991). Additionally, the SfCOL-A unformulated suspension was able to reach the efficacy of the formulated product due to its high pathogenicity. However the unformulated SfCOL showed a efficacy lower than 60%, being necessary the formulation to optimize the insecticidal activity of this isolate. No differences were found in mortality percentage between larvae collected at 48 and 96 hours in all the treatments, indicating that the rate acquisition of deadly dose was before 48 hours. Previous studies concluded that the first 48 hours of feeding are crucial in viral acquisition (Cory and Hoover, 2006; Lasa et al., 2007b). Moreover, it has been indicated that insects acquire infection during the first 6 h following the application (Lasa et al., 2007a).

Rates between 1.5 x 10^{12} OBs/ha and 6 x 10^{12} OBs/ha have been shown to protect efficiently different crops from *S. frugiperda* (Castillejos et al., 2002; Williams et al., 1999). Previous studies on microencapsulated SfMNPV selected 1.5 x 10^{12} OBs/ha as the optimal concentration to protect efficiently maize crops from *S. frugiperda* in Colombia (Gómez et al., 2013). However, the range of doses used was very wide, whereby intermediate doses could protect likewise. Therefore, a lower dose was proved (8 x 10^{11} OBs/ha) and showed that it was enough to reduce crop damage (26%) to values lower than economical injury level (Fernández, 2002), while in control plants damage reached to 59%. Surprisingly, in field no differences were found in the effectiveness between unformulated and formulated viruses. Many biotic and abiotic factors could influence host-pathogen interactions,

for instance density of the pest, crop phenology and insect eating habits among others, thus SfMNPVs evaluation in field conditions can be inconsistent with laboratory results (Villamizar et al., 2009). Additionally, SfCOL degradation due to UV-light occurred slower than SfCOL-A degradation, therefore UV degradation might be compensated by the higher pathogenicity of SfCOL-A, decreasing the effectiveness to that of SfCOL. However, given the high pathogenicity of SfCOL-A a new field trial using lower doses of SfCOL-A should be performed, reducing implementation costs.

In conclusion, both microencapsulated SfCOL and SfCOL-A protect efficiently maize plants against damage caused by *S. frugiperda* in Colombian crops.

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CHAPTER V

General Discussion

The fall armyworm *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) is considered the most serious pest of maize in America (Andrews, 1988; García et al., 2002). Taking into account that this crop is the principal cereal produced in Colombia (Quintero et al., 2004), this species can cause valuable economic damage (up to 60% losses) (Fernández, 2002; ICA, 2008) and the need for sustainable control measures against this pest is well recognized. Several NPVs of *S. frugiperda* (SfMNPV) isolates have been characterized in the distribution area of *S. frugiperda* and showed that SfMNPV isolates have different effectiveness toward different populations of *S. frugiperda*, being insect populations more susceptible to native isolates (Escribano et al., 1999). In this sense, the finding of native's isolates that could be evaluated for the use in a control program targeted at *S. frugiperda* in Colombia is a matter of time. In the search of new strains potentially more efficacious the natural diversity of SfMNPV in Colombia was evaluated (Chapter II). Among 38 isolates originating from Colombia two different REN profiles were identified, being SfCOL the predominant isolate (92%). Examination of the genetic diversity of field populations has revealed also that a particular isolate may be more prevalent than other coexisting virus isolates (Bernal et al., 2013a; Simón et al., 2004), via host-pathogen evolution due to the major adaptation of this isolate to the pest.

Genomic differences among distinct geographical isolates are frequently attributed to point mutations and small deletions or insertions, mostly located in a region containing several auxiliary genes and homologous regions (hr) (Escribano et al., 1999; Harrison et al., 2008; Wolff

et al., 2008). Physical mapping and sequencing of the SfCOL variable region confirmed that SfCOL differed minimally from other SfMNPV isolates in the presence and distribution of REN cleavage sites (Simón et al., 2004; 2011). Auxiliary genes such as *cathepsin, quitinase, egt* or *gp37*, have been identified within this SfMNPV genomic region (Flipsen et al., 1995; Slack et al., 1995) and an hr was also detected. C*athepsin* and *quitinase* act together disrupting the insect exoskeleton and promoting the release and spread of progeny virus (Hawtin et al., 1997). The *egt* gene, which encodes an ecdysteroid-UDP-glycosytransferase (EGT), mediates the inactivation of molting hormones (ecdysone) in insects, preventing molting of the infected larva allowing increased production of progeny OBs in each infected larva (O'Reilly and Miller, 1989). The *gp37* gene is also found within this variable region and has been shown to be associated with the disruption of the peritrophic membrane (PM) (Li et al., 2003). Hrs, which have been found to be implicated in DNA replication and transcriptional enhancement (Pearson and Rohrmann, 1995), have been found also near to regions of variability in other baculovirus genomes (de Jong et al., 2005; Hayakawa et al., 2000; Li et al., 2002). Sequencing also revealed an insertion of 1 kb compared to other SfMNPV genomes, due to the deletion of the ORF homologue to *sf23* of unknown function and the insertion of two ORFs closely related to SpltNPV *splt20* (96% similarity) and *splt21* (98% similarity). The presence of the latters in SfCOL genome suggested heterologous recombination event between SfCOL and SpltNPV when both viruses co-infected a susceptible host with a geographical distribution that overlapped the distribution of each virus. This genomic recombination event could be a viral strategy to gain advantages that could facilitate resistance to host immunity (Streeck et al., 2008), rescue of defective variants (Iwabu et al., 2008), spread resistance (Burke, 1997) or host range expansion (Maeda et al., 1993). Additionally, viral recombination within non-structural genome regions could modify the virulence and regain infectiousness of new sub-lineage (Carrillo, 2012).

There is an increasing evidence that recombination is an important mechanism for maintaining adaptability to changing environments and therefore a mechanism of viral evolution (Erlandson, 2009; van Oers and Vlak, 2007).

The most prevalent and widespread isolate (SfCOL) was also the most pathogenic and productive against a local *S. frugiperda* population. These differences are common among virus isolates from distinct geographical regions (Escribano et al., 1999) or even from the same regions (Arrizubieta et al., 2013; Bernal et al., 2013a), and add up more evidence that native isolates used to be more pathogenic against local insect populations than homologous isolates from elsewhere (Barrera et al., 2011; Erlandson et al., 2007; Escribano et al., 1999). Clearly, there is an adaptive advantage for SfCOL to retain high infectivity and productivity toward the local host population, presumably as a result of continuous host-pathogen coevolution (Cory and Myers, 2003). The increased productivity may also be advantageous for transmission and persistence of SfCOL in field.

The SfCOL may prove useful for a biopesticide product for the control of *S. frugiperda* in Colombia. However the lower virulence of SfMNPV compared to most synthetic insecticides prevented its commercialization. Improvement of the speed of kill has been one of the major research objectives when developing these viruses as the basis for biopesticidal products. Recombinant technology has been used to improve the virulence of baculoviruses (Gramkow et al., 2010; Inceoglu et al., 2006). However, due to the limitations to liberate genetically modified organisms (GMOs), the research has been focused on searching genotype or mixture of genotypes with improved phenotypic characteristics (Caballero et al., 2009). Interactions among conspecific genotypes can 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 insecticidal activity of the wild-type population, with clear implications during

the selection of genotypes or mixtures of genotypes with suitable phenotypic characteristics. Additionally, wild-type isolates that can be collected as natural isolates are not patentable and genotypic characterization is required to select the genotype or mixtures with insecticidal characteristics that favor their inclusion as biopesticide products. Moreover, the genotypic composition of a mixture can be controlled in terms of variant composition and relative abundance for baculovirus-based insecticides development, and this technology can be protected by a patent (Bernal et al., 2013b; Caballero et al., 2007; Caballero et al., 2013).

In this sense, the **Chapter III** aimed to determine the natural diversity within SfCOL. SfCOL isolate comprises a high genotypic diversity, where ten distinct genotypes (named A through J) were identified by *in vitro* cloning technique. SfCOL-A, the most prevalent genotype in wild-type population, showed a REN profile indistinguishable to that of SfCOL. The dominance of a particular genotype in a virus population has been reported in other wild-type baculoviruses (Muñoz et al., 1999; Simón et al., 2004). All the other minor genotypes presented genomic deletions (3.8-21.8 kb) affecting the region between ORFs *sf20* and *sf33*, the same genomic region described in the SfCOL population **(Chapter II**). The genotypic variants with the largest deletions were significantly less pathogenic than SfCOL (Barrera et al., 2013). Similarly, variants with the largest deletions were significantly less pathogenic than the other variants present in the SfNIC population (Simón et al., 2004, 2005). *Chitinase* and *cathepsin* were deleted in the least pathogenic SfCOL variants and have been reported to be absent in several genotypes cloned from other SfMNPV isolates (Harrison et al., 2008; Simón et al., 2005; Wolff et al., 2008). Additionally, the genomic deletion common to genotypes with lower potency included *lef-7* and ORFs similar to *splt20* and *splt21*, suggesting that the last two ORFs could be involved in the pathogenic characteristics of these viruses as *lef-7* is known to be involved in the replication of late genes (Gomi et al., 1997). Also, some SfCOL

genotypes lacked ORF *sf29*, a viral factor that determine the number of ODVs occluded in each OB affecting presumably the viral infectivity (Simón et al., 2008). However, no differences were observed in OB pathogenicity between genotypes that lacked *sf29* and those that included the viral factor. Although, disruption of any of these genes did not block the infection might affect notably the transmissibility of the SfCOL in each insect.

SfCOL-A pure genotype was the most pathogenic genotype, even more than SfCOL wild-type, suggesting interactions between genotypes that diminished the pathogenicity of the population as previously observed in other NPVs (Muñoz et al., 1998; Muñoz et al., 1999). Experimental mixtures of OBs and co-occluded mixtures were constructed using the most pathogenic variant (SfCOL-A) and one of the three genotypes with lower potency (SfCOL-C, -D or –F) **(Chapter III)**. Deletion genotypes decreased OB potency of SfCOL-A to values similar to that of SfCOL but increased OB productivity. The highest productivity of SfCOL suggests that deletion genotypes may modulate increases in OB production with the aim of maximize the likelihood of transmission, a common feature among field isolates (Cory et al., 2005). Since baculovirus-based biopesticides are normally used for inundative applications, in which large quantities of OBs are applied for the rapid suppression of the pest (Williams and Cisneros, 2001), an increased pathogenicity and virulence are desirable characteristics for a baculovirus. In this sense, SfCOL-A genotype presented the most suitable characteristics as the basis for a biological insecticide.

Despite many NPVs species have been isolated and characterized with potential as the basis of biopesticide product, only a few of them have been efficiently registered. To date no product based on SfMNPV has so far been registered. Some limitations have been described to the use of SfMNPV as biological control agent. Viral survival in field can be affected by different factors including temperature, pH, moisture and exposure to UV light (Szewczyk et al., 2012). Formulations aim to maintain stable

insecticidal properties during processing and storage, protect against deleterious environmental conditions and facilitate field application, among others (Behle et al., 2003; Jones and Burges, 1998; Tamez-Guerra et al., 2002). Different strategies have been followed during the formulation process (Cruz et al., 1997; Valicente and Costa, 1995; Williams et al., 1999), and most of them included the addition of phagostimulats (Castillejos et al., 2002), optical brighteners (Martinez et al., 2003), azadirachtin (Zamora-Avilés et al., 2013) or organic acids (Cisneros et al., 2002). However, the inclusion of chemical products into NPV-based biopesticides is not well accepted. In **Chapter IV** microencapsulation of OBs has been studied as this technique allows coating small solid particles in a thin uniform layer of coating material, protecting the virus from environmental conditions and improving the storage stability (Behle and Popham, 2012; Villamizar et al., 2010). The formulated SfCOL and SfCOL-A were compared with unformulated viruses under different conditions. Quality control analysis revealed that parameters such as viral concentration (OBs/g), pH, moisture percentage, bacteria content (CFU/g) and viral efficacy were in acceptance limits according to the limits described previously for microencapsulated SfMNPV (Jones and Burges, 1998; Kolodny-Hirsch et al., 1997; Tamez-Guerra et al., 2002). Microencapsulation did not improve the pathogenicity but at least retained the infectivity in laboratory conditions in second instar *S. frugiperda*. And more interestedly the efficacy and bacterial contamination is maintained under acceptable limits (Corradini and Peleg, 2007) after three months at 35ºC, compared to unformulated viruses in which the efficacy decreased dramatically while bacterial contaminants increased. High levels of bacterial contaminants in the products are detrimental due to accelerate chemical processes that inactivate OBs (Jones and Burges, 1998; Lasa et al., 2008).

Photoprotection provided by the formulation represents another great advantage for NPV-based biopesticides, as the radiation from sunlight,

especially radiation between 280 and 310 nm (UV-B) is the main constraint affecting the insecticidal activity of the baculovirus when applied in the field (Asano, 2005; Ignoffo and García, 1992). Microencapsulation also protected OBs from degradation caused by UV-B radiation, and after 6 hours of exposure the remaining original activity was superior to 95%. In contrast, unformulated SfCOL-A OBs were almost totally inactivated (12% residual activity), while the efficacy of SfCOL decreased to 50%. This result suggests that the mixture of genotypes naturally present in SfCOL wild type provides protection against UV radiation. This UV tolerance and the high productivity of SfCOL, compared to pure genotypes previously observed **(Chapter II),** may increase the likelihood of transmission, suggesting that SfCOL wild type is structured to maximize survival.

Differences between the efficacy under laboratory and field conditions have been attributed to the controlled conditions found in laboratory, while in field several factors can affect the behavior of the virus. Therefore, previous to register a product the insecticidal activity has to be validated under less controlled conditions and more similar to those found in field (Ibarra and Del Rincón, 2001). Greenhouse assays using maize plants showed similar efficacies between formulated SfCOL and unformulated and/or formulated SfCOL-A, being similar to those found with chemical lufenuron commonly used in maize plants in this region. However unformulated SfCOL was clearly less efficient. Additionally, larval mortalities after 48 and 96 hours of exposition were similar, indicating that lethal infection dose was acquired within first 48 hours. Previously, it was shown that SeMNPV infection occurs during the first 6 hours following the application (Lasa et al., 2008). Field assays revealed that microencapsulated SfCOL and SfCOL-A OBs reduced the feeding damage in maize crops under threshold economic impact limit (35%) when 8.0 x 10^{11} OBs/ha were applied. This dose was 20% lower than the dose evaluated previously with SfMNPV microencapsulations in field conditions (1.5 x 10^{12}

OBs/ha and 6.0 x 10^{12} OBs/ha) (Castillejos et al., 2002; Williams et al., 1999). The application concentration directly determines production costs of the biopesticide, therefore is important to establish the lowest dose with the maximum efficiency. Similarly, the frequency of application will depend on the persistence of OBs in the leaves, the transmission of infection from infected to healthy larvae and rate of plant growth (Williams and Cisneros, 2001). Experiments are in process to determine the strategies needed to carry out an efficient control with these microencapsulations.

Surprisingly, the effectiveness of unformulated and formulated viruses was similar under field conditions. This could be due to the fact that in field many biotic and abiotic factors may influence the effectiveness, and additionally SfCOL degradation due to UV-light was lower than that of SfCOL-A. Therefore, in field UV degradation might compensate the higher pathogenicity of SfCOL-A, decreasing the effectiveness to that of SfCOL. Therefore, according to the results presented in this study both SfCOL and SfCOL-A protect efficiently maize plants against damage caused by *S. frugiperda* in Colombian maize crops.

The next investigations will be focused on development of an effective mass production system in order to reduce costs. The possibility of decreasing the biopesticide dose specifically using SfCOL-A to make the product economically viable will be also evaluated. Enhancer (chemical or biological) of the insecticidal activity of these biopesticide products should also be tested attempt to reduce costs. Finally, it will be advisable to determine the efficacy of both biopesticides in maize crops found in different Colombian departments in order to validate their effectiveness in a variety of agro-ecological conditions. All this information would facilitate the incorporation of these biopesticides in integrated pest management strategies against *S. frugiperda* in Colombia.

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CONCLUSIONES

1. Entre los treinta y ocho aislados colectados en cultivos de maíz en diferentes regiones de Colombia, se identificaron dos perfiles de restricción diferentes, siendo el de SfCOL el más prevalente (92%). El tamaño del genoma de SfCOL se estimó en 133.9 Kb, con diferencias mínimas en número y posición de los sitios de restricción respecto al genoma de un aislado de Nicaragua (SfNIC). La región variable del genoma se localiza entre los ORFs *sf20* a *sf27*. El ORF *sf23* está ausente en el genoma de SfCOL, y en su lugar aparecen dos ORFs *splt20* y *splt21* homólogos al NPV de *S. litura,* lo cual sugiere un evento de recombinación entre SfCOL y SpltNPV en una hipotética coinfección del mismo huésped por ambos virus.

2. Los bioensayos realizados con dos poblaciones de *S. frugiperda* de distinto origen geográfico pusieron de manifiesto que SfCOL es igual de activo que SfNIC para la población Mexicana. Sin embargo, SfCOL es 12 veces más activo que SfNIC para la población de insectos local, y tres veces más activo para la población Colombiana que para la Mexicana. Además, SfCOL es menos virulento para insectos de la población Colombiana, lo que se traduce en una mayor producción de OBs/larva.

3. El análisis de la diversidad intra-poblacional demostró que SfCOL contiene al menos diez variantes genotípicas diferentes (SfCOL-A a SfCOL-J), de las cuales SfCOL-A es la más prevalente (71%). SfCOL-A presenta el genoma más largo, mientras que el resto de variantes genotípicas muestran deleciones de diferentes tamaños (3.8–21.8 Kb), afectando la misma región variable localizada entre los ORFs *sf20* a *sf33.*

4. SfCOL-A es 4,4 veces más activo e igual de virulento que el aislado silvestre SfCOL, mientras que los genotipos deletados presentan potencias similares o menores y seis genotipos presentan una velocidad de acción mayor que la de SfCOL. Los bioensayos con mezclas experimentales co-ocluidas que incluyen SfCOL-A y uno de los tres genotipos deletados (SfCOL-C, -D ó –F) en la misma proporción mostraron una patogenicidad menor que la del genotipo puro SfCOL-A. Los genotipos con deleciones

producen más OBs aunque éstos son menos patogénicos, lo que sugiere que el aislado SfCOL está estructurado para maximizar su supervivencia.

5. La microencapsulación de los OBs de SfCOL y SfCOL-A no mejora la patogenicidad; sin embargo, mantiene su eficacia durante al menos tres meses de almacenamiento acelerado y protege los OBs contra la degradación UV-B tras 6 horas de exposición.

6. En ensayos de invernadero, los microencapsulados de SfCOL y SfCOL-A fueron tan efectivas como el tratamiento químico (80%). Mientras que en campo, las aplicaciones de los microencapsulados de SfCOL y SfCOL-A a $8x10^{11}$ OBs/Ha (800g/Ha) protegieron eficazmente los cultivos de maíz contra el daño causado por *S. frugiperda,* manteniendo el nivel de daño fresco por debajo del umbral económico de daños (35%).

7. Los productos microencapsulados de SfCOL y SfCOL-A parecen tener un marcado efecto beneficioso para la formulación del SfMNPV y su utilización como agente de control biológico contra larvas de *S. frugiperda* en el cultivo de maíz. Dadas sus características de eficacia, bioseguridad y compatibilidad, con otros agentes de control, se propone como un componente relevante en el diseño de los programas de control integrado del maíz en Colombia.

CONCLUSIONS

1. Among the thirty-eight isolates collected in maize crops in different regions in Colombia, two different profiles were identified being SfCOL the most prevalent isolate (92%). The SfCOL genome was 133.9 kb long, and showed few differences terms of number and position of restriction sites compared the Nicaraguan isolate (SfNIC). The genomic variable region was between ORFs *sf20* and *sf27*. SfCOL genome lacked ORF *sf23*, instead two ORFs homologue to *S. litura* NPV *splt20* and *splt21* were identified, which suggest a recombination event between SfCOL and SpltNPV in a hypothetic coinfection of the same host by both viruses.

2. Bioassays performed with two *S. frugiperda* populations from distinct geographical origins showed that SfCOL and SfNIC were equally potent for the Mexican insect colony. However, SfCOL was 12 times more potent than SfNIC for the local insect population, and three times more potent for the Colombian colony that for the Mexican colony. Additionally, SfCOL showed lower virulence in insects from the Colombian colony, which was correlated with a higher production of OBs/larva.

3. Intra-population diversity analysis revealed that SfCOL is composed at least of ten distinct genotypes (SfCOL-A to SfCOL-J), of which SfCOL-A was the most prevalent (72%). SfCOL-A presented the largest genome, while the other minor genotypes displayed deletions of different sizes (3.8-21.8 Kb) affecting the same genomic region between ORFs *sf20* to *sf33.*

4. SfCOL-A is 4.4-fold more potent and as virulent as SfCOL, while deletion genotypes are similarly or less potent and six deletion genotypes are faster killing than SfCOL. Experimental mixtures assays revealed that co-occluded mixtures including SfCOL-A and one of the three deletion genotypes (SfCOL-C, -D or –F) in equal proportions presented reduced pathogenicity than SfCOL-A genotype. Deleted genotypes produces more OBs although these are less pathogenic, which suggests that is structured to maximize the survival.

5. Microencapsulation of SfCOL and SfCOL-A OBs do not improve the pathogenicity but preserve the efficacy at least during three months of accelerated conditions storage and protect OBs against UV-B degradation after six hours of exposition.

6. In greenhouse assays, formulated SfCOL and SfCOL-A were as effective as chemical treatment (80%). In field, applications of microencapsulated of SfCOL and SfCOL-A at $8x10^{11}$ OBs/Ha (800g/Ha) protected efficiently maize crops against the damage caused by *S. frugiperda,* maintaining fresh damage percentage below the economic threshold of damage (35%).

7. The microencapsulated SfCOL and SfCOL-A products appear to have a highly beneficial effect for the SfMNPV formulation and its use as biological control agents against *S. frugiperda* larvae on maize crops. Due to the characteristics of efficacy, biosecurity and compatibility with other control agents, we propose it as relevant component in the design of Integrated Pest Management programs for maize in Colombia.

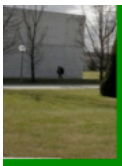
LIST OF PUBLICATIONS

Barrera, G., Simón, O., Villamizar, L., Williams, T., Caballero, P., 2011. *Spodoptera frugiperda* multiple nucleopolyhedrovirus as a potential biological insecticide: Genetic and phenotypic comparison of field isolates from Colombia. Biological Control 58**,** 113-120.

Barrera, G., Williams, T., Villamizar, L., Caballero, P., Simón, O., 2013. Deletion genotypes reduce occlusion body potency but increase occlusion body production in a Colombian *Spodoptera frugiperda* nucleopolyhedrovirus population. Plos One 8**,** e77271.

Barrera, G., Villamizar, L., Gómez, J., Caballero, P., Simón, O., 2013. Efficacy of microencapsulated *Spodoptera frugiperda* multiple nucleopolyhedroviruses from Colombia as biological insecticides. Subtmitted to Biological Control.

This study received financial support from CSIC-COLCIENCIAS Cooperation project 2008CO0029 and COLCIENCIAS Project 1753-636.



Spodoptera frugiperda (Lepidoptera: Noctuidae) cause crop losses in maize that become up to 60% in Colombia, where there is now a well-know demand for the development of sustainable control measures against this pest. The *S. frugiperda* nucleopolyhedrovirus (SfMNPV; Baculoviridae) has been considered as a promising alternative to chemical insecticides. The aim of the present work was to establish the scientific basis for the development of a new bioinsecticide based on a local *Alphabaculovirus* isolated from *S. frugiperda* in Colombia.

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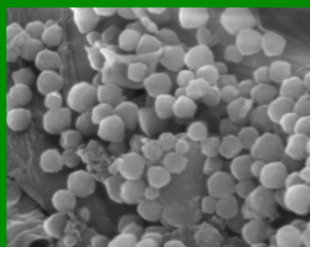

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