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Authors: Rios-Velasco, Claudio, Gallegos-Morales, Gabriel, Berlanga-Reyes, David, Cambero-Campos, Jhonathan, and Romo-Chacón, Alejandro

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MORTALITY AND PRODUCTION OF OCCLUSION BODIES IN *SPODOPTERA FRUGIPERDA* LARVAE (LEPIDOPTERA: NOCTUIDAE) TREATED WITH NUCLEOPOLYHEDROVIRUS

Claudio Rios-Velasco[1,*], Gabriel Gallegos-Morales[2], David Berlanga-Reyes[1], Jhonathan Cambero-Campos[3] and Alejandro Romo-Chacón[1]

[1]Centro de Investigación en Alimentación y Desarrollo, A.C. Unidad Cuauhtémoc, Chihuahua, Av. Río Conchos S/N Parque Industrial. CP. 31570, Cuauhtémoc, Chihuahua, México
Phone (+52 625) 5812920, Ext. 112

[2]Departmento de Parasitología, Universidad Autónoma Agraria Antonio Narro, Buenavista, CP. 25315, Saltillo, Coahuila, México

[3]Unidad Académica de Agricultura, Universidad Autónoma de Nayarit, Xalisco, Nayarit, México, Tepic-Compostela Km 9

*Corresponding author; E-mail: claudio.rios@ciad.mx

Abstract

The fall armyworm (FAW), *Spodoptera frugiperda* is the main corn pest in Latin America. As an alternative to chemical insecticide applications, one isolate of *Spodoptera frugiperda* nucleopolyhedrovirus from soil of an experimental field in Saltillo, Coahuila, Mexico was evaluated. Egg masses of the pest were superficially inoculated by the immersion method, with different nucleopolyhedrovirus concentrations expressed as occlusion bodies/mL (OBs/mL). Artificial diet was also inoculated with different viral concentrations and used to conduct bioassays on first to sixth instar larvae to determine the number of OBs produced per larva. Another assay was performed under greenhouse conditions to evaluate four baculovirus formulations. The use of the immersion method on egg masses caused the highest mortality rates recorded in the larval stage (74.37%), mainly in the first instar, with 64.05% mortality. The number of OBs produced per larva ranged from 5.15×10^6 to 2.3×10^9, where fifth and sixth instar larvae produced the highest amount. OBs produced by weight unit of larva were registered and higher values were found in the last larval instars. In the baculovirus formulations tested under greenhouse conditions, the mixture with maize flour and starch resulted in significantly increased mortality rates.

Key Words: Virus**,** Baculoviridae, Fall armyworm, eggs, occlusion bodies, mortality

Resumen

El gusano cogollero (*Spodoptera frugiperda*) es la principal plaga del maíz en Latinoamérica. Como una alternativa a las aplicaciones de insecticidas químicos, se evaluó un aislado de nucleopoliedrovirus de *Spodoptera frugiperda* de suelo de un campo experimental en Saltillo, Coahuila, México. Se inocularon masas de huevecillos de la plaga, por el método de inmersión, con diferentes concentraciones del nucleopoliedrovirus, expresadas como cuerpos de oclusión/mL (COs/mL). Además se inoculó dieta artificial con diferentes concentraciones virales usadas para realizar bioensayos sobre larvas del primero al sexto instar para determinar el número de COs producidos por larva. Otro ensayo fue realizado bajo condiciones de invernadero, para evaluar cuatro formulaciones de virus. El uso del método de inmersión de masas de huevecillos causó la mayor mortalidad en el estado larval (74.37%), principalmente en el primer instar, con un 64.05% de mortalidad. El número de COs producidos por larva fue de 5.15×10^6 a 2.3×10^9, donde las larvas del quinto y sexto instar produjeron la mayor cantidad de COs. Se registraron los COs producidos por unidad de peso de la larva, y los valores mayores fueron encontrados en los últimos instares larvales. En las formulaciones del virus evaluadas bajo condiciones de invernadero, la mezcla con harina y almidón de maíz resultó en un incremento significativo en la mortalidad.

Palabras Clave: Virus, Baculoviridae, cogollero del maíz, huevos, Cuerpos de oclusión, mortalidad

The fall armyworm (FAW), *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is the main corn pest in Latin America (Castillejos et al. 2002), causing reductions in biomass and grain yield in infested crops (Hernandez-Mendoza et al. 2008). This pest causes yield losses of 15-73% when it infests 55-100% of the plants. Control of the FAW greatly depends on chemical insecticides, requiring 2 to 4 applications during plant development, which represents an extra production cost (Hruska & Gould 1997). In addition, injudicious use of chemical insecticides often induces insecticide-resistance in both target and secondary pests, reduces the abundance of natural enemies and other insects in maize fields, and pollutes the environment. Also insecticide misuse may result in a chronic pesticide poisoning in farm workers (McConnell & Hruska 1993; Hunt et al. 1999; Tillman & Mulrooney 2000; Méndez et al. 2002).

An alternative method to control the FAW is the use of natural enemies, mainly entomopathogens (Rios et al. 2011a). The control of this insect pest with viruses has been successful in recent years, specifically nucleopolyhedroviruses (NPV) (Hughes et al. 1984; Shapiro et al. 1991). The *S. frugiperda* NPV (SfNPV, Baculoviridae) biopesticide is a widely studied and effective microbial control agent against FAW larvae, due to its specificity, biosecurity, persistence, and high level of virulence (Herniou et al. 2003; Fuxa 2004). However, *in vivo* production of entomopathogenic viruses is expensive (Lasa et al. 2009). The immediate deposition of NPV in the correct site (feeding zone) is a factor that largely determines its effectiveness in the field, because FAW larvae, after the second instar, are protected deep in the corn whorl, where they remain until completing their larval stadium (Jones et al. 1997). Biopesticide formulations have an important role in pathogen stabilization, ease of use, and inactivation rate of OBs. The use of feeding stimulants added to clays contaminated with OBs can result in an improved pest control (Castillejos et al. 2002; Lasa et al. 2009). However, most of the research on SfMNPV has been carried out on larval stages and under laboratory conditions (Lasa et al. 2009; Rios-Velasco et al. 2011b). There is no research data published evaluating mortality of FAW larvae treated in the egg stage and subsequent production of OBs. Therefore, in this study we determined (i) the mortality of FAW larvae treated in egg stage with a native isolate of NPV, (ii) production of OBs in larvae fed with a NPV-contaminated diet in laboratory conditions, and (iii) mortality of FAW larvae fed on maize plantlets contaminated with mixtures of viral OBs and natural substances (clays and feeding stimulants) under greenhouse conditions.

Materials and Methods

Egg masses and FAW larvae were obtained from a laboratory colony established under controlled conditions (25 ± 2 °C, 12:12 L:D h and 50-60% RH). They were fed with artificial diet (Southland Products Incorporated, Lake Village, Arkansas). The OBs of a Mexican isolate (AN_2) of SfMNPV (Rios-Velasco et al. 2011b) were amplified *in vivo* on third-instar FAW and purified by filtration and centrifugation as described by Muñoz et al. (2001). The concentrations of viral OBs were quantified using a Neubauer hemacytometer (Blau Brand, Germany) and expressed in OBs per mL (OBs/mL of water) and stored in aliquots of 500µL of distilled water at 0 °C until required.

Egg masses were disinfected with 0.2% sodium hypochlorite and placed on dry paper. Six concentrations of OBs were evaluated, ranging from 8×10^2 to 8×10^6 OBs/mL, 0.1% Tween was added to the suspensions, and a control treatment without OBs was used (Table 1). Six egg masses were taken from a laboratory colony per replicate for each concentration. Therefore a total of 18 egg masses were used with over 100 eggs, and from these 50 eggs per tested concentration were taken to determine the incidences of mortality in each of the subsequent developmental stages, i.e., larval instars, pre-pupae, pupae and adults. This study was repeated 3 times on different dates. Emergence of first-instar-larvae, larval, pre-pupal and pupal death and/or development to adult stages were recorded to register the specimens killed by the action of virus. For the bioassay, the egg immersion technique was used, consisting of egg masses being immersed in 10 mL of a suspension containing different NPV concentrations (8×10^2, 2×10^3, 8×10^3, 8×10^4, 2×10^5 and 8×10^6 OBs) for 5 s and placed on blotting paper to allow drying at room temperature. Afterwards, they were transferred to transparent one-ounce plastic cups (Envases Cuevas S.A. de C.V., Estado de México, México) containing untreated artificial diet.

To determine the number of OBs produced by FAW, larvae in all 6 instars were inoculated with NPV. Fifty larvae of each of the 6 instars were placed individually in plastic cups containing artificial diet inoculated with treatments ranging from 2.0×10^3 to 2.0×10^9 OBs/mm^2 (Table 2). Larvae were fed with the contaminated diet for 24 h and then individually reared to untreated diet. Subsequently, larvae were weighed and examined daily to observe any symptoms of disease caused by NPV until their death. Ten larvae of each instar were considered as controls. To facilitate the collection of OBs, dead larvae were frozen at -20 °C and then placed individually in a plastic Eppendorf tube containing 2 mL of sterile distilled water (Lasa et al. 2007), thawed, homogenized, filtered and centrifuged at 1000 g for 5 min to remove the sclerotized parts of the larvae (cuticle

TABLE 1. LARVAL, PRE-PUPAE AND PUPAE MORTALITIES OF *SPODOPTERA FRUGIPERDA* TREATED IN THE EGG STAGE WITH DIFFERENT CONCENTRATIONS OF NUCLEOPOLYHEDROVIRUS.

Concentration (OBs/mL)	No. of treated eggs (average)	n[z]	Mortality rate (%)					Survival to adult stage (%)
			Instar larval			Pre-pupae	Pupae	
			First	Second	Third			
8×10^6	426.00	50	100.00	0.00	0.00	0.00	0.00	0.00
2×10^5	157.00	50	98.00	0.00	0.00	0.00	0.00	2.00
8×10^4	302.33	50	90.00	0.00	2.00	2.00	0.00	6.00
8×10^3	208.66	50	46.00	24.00	0.00	8.00	0.00	22.00
2×10^3	412.00	50	32.33	18.00	3.00	12.66	2.00*	32.00
8×10^2	305.00	50	18.00	10.00	5.00	14.00	4.00	49.00
Control	224.00	50	0.00	0.00	0.00	2.00*	0.00	98.00

[z].- Sample of eggs taken from the total eggs treated with the indicated concentration of OBs/mL.
*Death by unknown causes. Note: From the fourth to sixth instars larvae no mortality occurred (data nota shown).

TABLE 2. PRODUCTION OF OCCLUSION BODYS IN *SPODOPTERA FRUGIPERDA* LARVAE THAT HAD BEEN TREATED WITH NUCLEOPOLYHEDROVIRUS BY FEEDING 50 LARVAE OF EACH OF THE 6 INSTARS FOR 24 H ON NPV-INOCULATED DIET AND THEN REARING THEM ON UNTREATED DIET. THESE ASSAYS WERE PERFORMED 3 TIMES.

Instar	Number of larvae inoculated	Treatment (OBs/mm^2)	Number positive for virus infection	Weights of insects collected (mg±SD)	OBs/larvae (Mean ±SD)	OBs/mg of larval weight
First	50	2×10^3	47	1.4 ± 1.0 e*	$5.15 \times 10^6 \pm 0.49$ d	$3.68 \times 10^6 \pm 26.3$ d
Second	50	2×10^4	44	6.8 ± 1.9 e	$8.8 \times 10^7 \pm 0.33$ d	$1.3 \times 10^7 \pm 754.6$ a
Third	50	2×10^5	42	33.9 ± 5.8 d	$1.06 \times 10^8 \pm 0.71$ d	$3.13 \times 10^6 \pm 122.8$ e
Fourth	50	2×10^7	43	154.8 ± 4.6 c	$1.09 \times 10^9 \pm 0.37$ c	$7.04 \times 10^6 \pm 309.2$ b
Fifth	50	2×10^8	32	276.3 ± 7.4 b	$1.7 \times 10^9 \pm 0.43$ b	$6.3 \times 10^6 \pm 123.3$ c
Sixth	50	2×10^9	29	340.9 ± 8.5 a	$2.31 \times 10^9 \pm 0.79$ a	$6.8 \times 10^6 \pm 125.2$ b

*Values followed by identical letter did not differ significantly according to Tukey's test ($P > 0.05$).
The results were subjected to ANOVA without prior transformation.

and head). The resulting OB suspension was adjusted to a final volume of 2 mL. Sterile distilled water was used as control. The assays were performed 3 times using the same number of insects of each larval instar (Table 2).

The number of OBs recovered from each larva was determined using a Neubauer hemacytometer under a phase contrast microscope at 400X, taking 2 samples from each larva. The relationship between OBs production and host weight (OBs/mg) was estimated based on larval weight recorded on the day before death. OBs production and larval weight data were subjected to an analysis of variance (ANOVA). Values of OBs per mg of larval weight were normally distributed and were subjected to ANOVA using Statistical Analysis System Version 9.0 (SAS 2002). Means were separated by Tukey's test ($P < 0.05$). The bioassay was performed 3 times.

Clay-based formulations (bentonite, diatomaceous earth and zeolite) and a phagostimulant based on maize flour and maize starch (Grupo Industrial Maseca S.A. de C.V., Monterrey Nuevo León, México) (Fig. 1) were evaluated under greenhouse conditions in "El Bajío" Experimental Station of the Universidad Autónoma Agraria Antonio Narro (N 25° 23' W 101° 00') Buenavista, Saltillo, Coahuila, México. Initially, OBs were mixed with the formulations forming a paste. Next, the paste was dried at room temperature with a thickening agent (Xanthan gum 0.5%) and then covered with foil to prevent inactivation of the OBs. Subsequently, the paste was macerated in a porcelain mortar and screened through a wire sieve, obtaining a wettable powder. These preparations were stored at 4 °C until used.

To evaluate the phagostimulant and clay-based formulations, they were mixed with 500 mL of water to obtain a suspension containing 2.8 × 10^8 OBs of SfMNPV-AN_2 and the adjuvant Pegodel™ 1% (Syngenta) was added. Pastes consisting of maize flour + maize starch (4.83 g), bentonite (4.0 g), zeolite (6.2 g) and diatomaceous earth (2.0 g) were used. Each suspension was sprayed on maize plantlets (30 cm height approximately) kept under greenhouse conditions, with 4 to 5 plantlets per pot with 5 replicates. Plantlets were sprayed at dew point with 20-25 mL of wettable powder per plantlet. After the plants had dried, 30 ± 1 neonate larvae (obtained from a laboratory

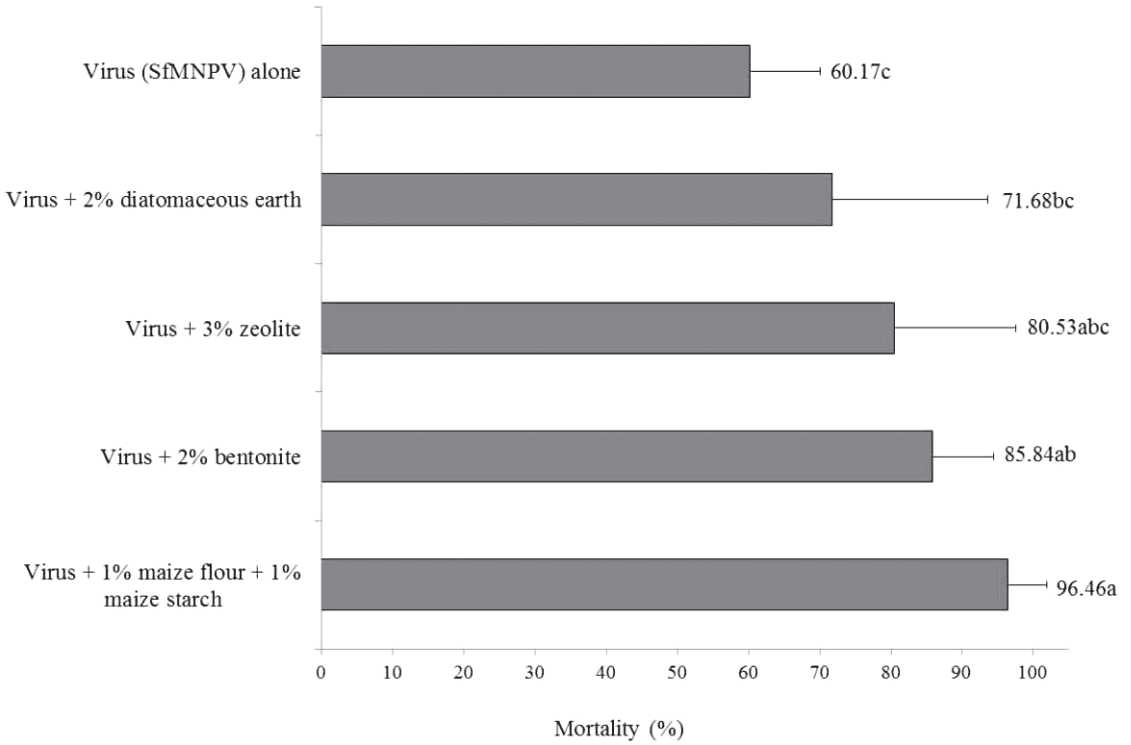

Fig. 1. Mortality rates of *Spodoptera frugiperda* when first instars were fed on maize plantlets treated with SfMNPV occlusion bodies (OBs) alone or as formulations (wettable powder) amended with a thickening agent (xanthan gum at 0.5%) and an adjuvant (Pegodel™ at 1%, Syngenta). Error bars indicate standard deviation from the mean of mortality responses observed among replicates. The value beside each error bar indicates the percent mortality, and letters indicate significant differences compared with the control and treatments involving virus OBs alone according to Tukey's test ($P > 0.05$). Concentrations of test substances are given as percentage wt/wt.

colony) were placed on the plantlets in each pot. The same number of larvae were used per treatment in all replicates. These larvae were starved for 6 h before being used. Control plantlets were sprayed only with the 1% Pegodel™ adjuvant. In the virus control treatment, plantlets were sprayed with OBs suspension plus adjuvant and thickening agent but without clay and phagostimulant substances. Larvae were fed for 24 h on the treated plants. Next 20 of these larvae were randomly selected from each pot and individually transferred into one-ounce plastic cups containing artificial virus-free diet, reared at 25 °C, and examined for virus mortality each day for 1 week. Each assay was performed 3 times (replicates) using the same batches of larvae. The experiment was conducted with a randomized complete block design with 6 treatments including the control and a treatment with virus alone. Mortality rate was corrected using Abbott's formula (Abbott 1925) before statistical analysis. Mortality data were normalized using arcsine transformation and then subjected to ANOVA using the Statistical Analysis System Version 9.0 (SAS 2002). Means were separated by Tukey's test ($P < 0.05$).

Results and Discussion

The egg masses treated with NPV showed mortality in the larval stage, mainly in the first 3 instars. The highest mortalities were observed in the first (neonate larvae) and second instars (Table 1). From the fourth to sixth larval instars there was no mortality, but this occurred in pre-pupae, and pupae in some of the concentrations used (Table 1), which showed the presence of OBs when macerated and observed with phase-contrast microscope at 400X. From an average of 300 eggs treated with different OB concentrations, 74.37% died in the larval stage (64.05% in neonate, 8.66% in the second, and 1.66 % in the third instar), 6.11% died in the pre-pupa, 0.66 % in the pupae, and no mortality was observed in the adult stage (Table 1). The highest mortality was registered in the larvae from eggs treated with 8×10^6, 2×10^5 and 8×10^4 OBs per mL, which caused 100, 98 and 90% mortality, respectively (Table 1). No death occurred in subsequent larval instars (Table 1). Ibarra & Del Rincon-Castro (2001) mentioned that the egg immersion method of treating insects with a virus is so highly efficient because hatching larvae have the habit of feeding on the chorion, which is heavily contaminated with the virus in this treatment method. Therefore, one strategem in the integrated management of this pest can be the treatment of the egg stage with this virus.

Assays with OB suspensions of SfMNPV-AN_2 resulted in larval mortality rates of >50% in the first to sixth instars (data not shown). The average number of OBs produced by larvae in the six instars (Table 2) varied broadly according to larval size, as expected. In the first 3 instars, low levels of OB production occurred, i.e., 5.15×10^6, 8.8×10^7 and 1.06×10^8 OBs per larva, respectively. In contrast the highest OB production levels occurred in the fifth and sixth larval instars with 1.74 and 2.31×10^9 OBs/larva, respectively ($F = 148.46$, df = 5, $P < 0.0001$) (Table 2). It should be noted that all control larvae reached the adult stage. Larvae of the first 3 instars showed average weights of 1.4, 6.8 and 33.9 mg respectively, while the fifth and sixth larval instars obtained final average weights of 276.3 and 340.9 mg, respectively ($F = 6877.84$, df = 5, $P < 0.0001$).

For the OB production in larvae, the key parameters are: dose and duration used for larval infection, growth rate, weight gain during the infection period and final weight at death (Sherman 1985). OB production per mg of larval weight ranged between 3.13×10^6 to 1.3×10^7 and differed significantly ($F = 1495.83$, df = 5, $P < 0.0001$) (Table 2). Thus larval weights and OB production levels in the fifth and sixth instars in this study were similar to those obtained by Vásquez et al. (2002), who reported OB production levels per larva of 5.4×10^8 and 7.3×10^8 for fifth and sixth instars, respectively. In fifth instar larvae of *S. exigua*, Lasa et al. (2007) reported production of 1.6×10^9 to 2.29×10^9 OBs/larva, and an average larval weight of 197.6 mg.

In the greenhouse assays, the wettable powders efficiently controlled neonate larvae (Fig. 1). The average mortality rate of larvae fed on maize leaves treated with SfMNPV-AN_2 alone was 60.17%. In control larvae, treatments did not cause mortality. Regarding the four baculovirus-formulations tested, maize flour and maize starch significantly increased larval mortality (96.46 %), mainly in the second instar larvae, compared with virus alone ($F = 37.98$, df = 5, $P < 0.0001$) (Fig. 1), which could mean this formulation has a phagostimulant effect on neonate larvae (Williams & Cisneros 2001). The diatomaceous earth formulation of the NPV caused a mortality rate similar to the virus alone. Lasa et al. (2009) found a mortality rate of 32% when *S. exigua* second instars were treated with virus (SeMNPV) together with maize flour. In this regard, Hostetter et al. (1982) mentioned that the phagostimulant effectiveness depends on the concentration used. The clay-based formulations showed a significant increase in larval mortality, compared with virus alone (Tukey, $P > 0.05$), with mortality rates of 85.84%, 80.53 % and 71.68% for virus-bentonite, virus-zeolite and virus-diatomaceous earth, respectively ($F = 37.98$, df = 5, $P < 0.0001$) (Fig. 1). In this regard, Batista-Filho et al. (2001) mentioned that clays are effective protectors in baculovirus-based formulations, preventing the inactivation of the virus by the UV in sunlight. This protection occurs because the OBs are strongly attached to the clay, giving the formulation greater persistence and stability in field (Christian et al. 2006).

We can conclude that the native isolate SfMNPV-AN$_2$ has a high potential as a biological control in the strategic management of FAW, which can be initiated by applying this virus to the egg stage; moreover, larvae of the last 2 instars can be used for mass production of NPV under laboratory conditions.

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