

# *Anticarsia gemmatalis* nucleopolyhedrovirus from soybean crops in Tamaulipas, Mexico: diversity and insecticidal characteristics of individual variants and their co-occluded mixtures

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## Abstract

In 1999, *Anticarsia gemmatalis* nucleopolyhedrovirus (AgMNPV) was introduced into a major soybean-growing region in Tamaulipas, Mexico, for control of its lepidopteran host, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae). The virus introduction proved to be highly successful in controlling this agronomically important pest. In order to determine the genotypic diversity and insecticidal traits of Mexican AgMNPVs, we obtained 30 field-collected isolates from Tamaulipas State. Five distinct variants (genotypes 1–5) were identified from plaques replicated in *A. gemmatalis* larvae by examination of restriction profiles using *Hind*III. Initial screening indicated that none of the variants, or co-occluded mixtures of variants in different proportions, was more pathogenic than the 30 field isolates mixture or a reference variant from Brazil (AgMNPV-2D). Mean occlusion body production also was similar among genotype variants, the mixture of 30 field isolates and AgMNPV-2D treatments, but was significantly reduced in 1 co-occluded mixture. Speed of kill also was similar among variants (except genotype 1) and their mixtures. Lethal concentration metrics indicated that these results were unlikely due to selection of variants with reduced pathogenicity during the plaque purification process. We conclude that the mixture of 30 field isolates most likely would prove suitable for use as a biological insecticide in the soybean-growing region of Mexico.

Key Words: baculovirus; genotypic mixtures; pathogenicity; speed-of-kill; virus production

## Resumen

En 1999, el nucleopoliedrovirus de *Anticarsia gemmatalis* (AgMNPV) fue introducido a una importante región de producción de soya en Tamaulipas, México para controlar el lepidóptero huésped, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae). La introducción del virus demostró ser muy exitosa en el control de esta importante plaga agrícola. Para determinar la diversidad genotípica y las características insecticidas de los AgMNPVs de México, obtuvimos 30 aislados colectados en campos de soya del estado de Tamaulipas. Mediante el uso de la enzima de restricción *Hind*III, se identificaron cinco variantes distintas (genotipos 1–5) que replicaron en *A. gemmatalis*. Los ensayos indicaron que ninguna de las variantes, ni mezclas coocuidas de las mismas en diferentes proporciones, tuvieron una patogenicidad mayor que la de la mezcla de 30 aislados silvestres, o de una variante de referencia de Brasil (AgMNPV-2D). La producción de cuerpos de oclusión fue similar entre las variantes, la mezcla de 30 aislados silvestres y el AgMNPV-2D, aunque la producción fue significativamente reducida en una mezcla de variantes coocuidas. El tiempo medio de matar también fue similar entre las variantes (excepto el genotipo 1) y las mezclas de variantes. Ensayos de concentración-mortalidad indicaron que durante el proceso de purificación de placa no hubo selección de variantes con menores características de patogenicidad. Concluimos que la mezcla de 30 aislados silvestres podría ser el ingrediente activo de un insecticida biológico en esta región de producción de soya en México.

Palabras Clave: baculovirus; mezclas genotípicas; patogenicidad; tiempo de matar; producción viral

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The velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae), is a major defoliating pest of soybean crops in the New World, and is distributed from the USA (about 40°N) through Argentina (about 39°S) (Sosa-Gómez 2004). Control of this pest usually is achieved by application of synthetic insecticides such as pyrethroids or insect growth regulators, and by growing soybean

expressing insecticidal proteins of *Bacillus thuringiensis* (Bacillaceae) in regions where the use of transgenic plants is permitted (Panizzi 2013).

Larvae of *A. gemmatalis* are infected naturally by a nucleopolyhedrovirus (AgMNPV; Baculoviridae: *Alphabaculovirus*) (de Castro Oliveira et al. 2006). Infection begins when larvae consume foliage

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contaminated with viral occlusion bodies that break down in the insect midgut and release occlusion derived virions that infect midgut epithelial cells. The infection then spreads through the body of the larva and death occurs several days later. Viral occlusion bodies are released from infected cadavers for horizontal transmission. However, AgMNPV transmission is not hastened by viral enzymes such as cathepsin and chitinase, as observed in several other nucleopolyhedroviruses (Lima et al. 2013).

In Brazil, the use of AgMNPV as the basis for a biological insecticide to control velvetbean caterpillar was successful during the 1990s, and in the following decade when virus-based control of this pest was used on approximately 2 million ha of soybean. This made the *A. gemmatalis* control program the most successful example of baculovirus-based pest control in the world (Moscardi et al. 2011). The use of the virus was adopted in neighboring countries, such as Paraguay and Uruguay (Haase et al. 2015). Testing also was performed in the United States (Fuxa & Richter 1999), as well as in Argentina and Colombia (Haase et al. 2015). However, AgMNPV use has declined as transgenic soybean has become increasingly common in several areas (Moscardi et al. 2011).

In Mexico, an AgMNPV isolate from Brazil was introduced in 1999 and used in a series of field trials in the Huastecas soya-growing region that includes the state of Tamaulipas (Ávila-Valdez & Rodríguez-del-Bosque 2008). Beginning in 2005, growers in this region were encouraged to adopt a system of integrated pest management involving the use of an AgMNPV-based insecticide produced by infecting larvae on soybean crops from field infected cadavers. Dead infected larvae were collected by hand, dried, mixed with kaolin, and stored until required for pest control. Currently, this procedure is organized by Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), a federal government research organization where approximately 15,000 ha of soybean have been treated with AgMNPV annually (Williams et al. 2013).

Natural nucleopolyhedrovirus populations usually comprise a mixture of several genotypes, each of which differs in key traits, such as pathogenicity (measured as dose-mortality metrics), speed of kill, and occlusion body production (Erlandson 2009). Presently, there is a growing understanding of the role that genotypic interactions play in the phenotype of nucleopolyhedroviruses. These interactions can have a marked influence on the ecology of the virus and its transmission (López-Ferber et al. 2003; Hodgson et al. 2004; Simón et al. 2006; Hitchman et al. 2007; Clavijo et al. 2009).

It is now possible to produce combinations of viral variants that are occluded together within occlusion bodies that possess insecticidal properties greater than those of the initial genotypes or wild-type isolates from which they originated. A key aspect involves the replication of viral variants within the same host cell (as well as enveloping and variant occlusions) to form genetically heterogeneous occlusion-derived virions and occlusion bodies (Clavijo et al. 2010). This technology has been applied to the production of nucleopolyhedroviruses with precise genotype mixtures produced to control several major lepidopteran pests (Caballero et al. 2009; Bernal et al. 2013; Arrizubieta et al. 2013, 2015).

Because natural isolates of AgMNPV are genotypically diverse (Crozier & Ribeiro 1992; Ribeiro et al. 1997; Maruniak et al. 1999; de Brito et al. 2016) we posed the question whether insecticidal activity of Mexican isolates could be enhanced by using variant mixture technology. Natural isolates from *A. gemmatalis* larvae collected from soybean fields in Tamaulipas were subjected to plaque purification, and characterized using restriction endonucleases. Pathogenicity and occlusion body production then were characterized from individual AgMNPV genotypes as well as their mixtures.

## Materials and Methods

### INSECT COLONY

A laboratory colony of *A. gemmatalis* was started using apparently healthy larvae collected in Sep 2013 and Sep 2014 from soybean crops grown at the INIFAP-Las Huastecas field station (22.5663°N, 98.1661°W) in Tamaulipas State, Mexico. The colony was maintained in the Instituto de Ecología AC insectary in Xalapa, Veracruz State, at  $26 \pm 2$  °C, 60 to 80% humidity, and 14:10 (L:D) photoperiod. Larvae were reared individually on a semisynthetic diet modified from Greene et al. (1976), and adults were provided with continuous access to 10% honey solution. Eggs were collected from nylon mesh walls of cages used to hold adult moths, and were disinfected by immersion in 0.05% sodium hypochlorite solution for 5 min, then rinsed in distilled water.

### PREVALENCE OF GENOTYPIC VARIANTS IN AGMNPV ISOLATES

Collections of *A. gemmatalis* larvae from 3 soybean fields resulted in 17 out of 87 larvae (20%) that died from virus infection in 2013, and 13 out of 150 larvae (9%) in 2014. Each of these 30 virus-killed larvae was homogenized in 1 mL sterile water, and the homogenate filtered through a fine metal gauze (80 µm pore size) to remove insect debris. The resulting occlusion body suspension was used to inoculate groups of 12 fourth instars from the laboratory colony using the diet surface contamination technique of Lacey & Kaya (2007). Inoculated larvae were incubated in darkness at  $25 \pm 1$  °C for 7 d, after which virus-killed larvae were placed in 1.5 mL microcentrifuge tubes and stored at -20 °C until required. Virus death was confirmed by microscopic examination of Geimsa-stained smears of larval tissues to identify the presence of occlusion bodies.

To produce a pooled sample representing all the virus variants present in the population, isolates amplified in the previous step were thawed, occlusion bodies extracted in 1 mL sterile water and counted using a Neubauer cell counting chamber (Hawksley, Lancing, United Kingdom). Equal numbers of occlusion bodies of each isolate then were mixed to produce a suspension of  $7.1 \times 10^8$  occlusion bodies per mL which comprised 30 isolates from the wild-type population.

### PLAQUE PURIFICATION OF GENOTYPIC VARIANTS

To obtain plaque purified variants, occlusion bodies of the mixture of 30 field isolates were mixed in a solution of 10% (w/v) sucrose and 0.05% Fluorella Blue food dye. This mixture then was used to inoculate a group of 12 fourth instar *A. gemmatalis* with a concentration of  $1 \times 10^8$  occlusion bodies per mL using the droplet feeding method of Hughes & Wood (1981). Inoculated larvae were reared individually on semisynthetic diet for 48 h, after which hemolymph containing budded virus was extracted, pooled, and used to prepare 9 serial dilutions ( $10^{-1}$ – $10^{-9}$ ) in TC-100 medium (Gibco, Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) that contained 10% fetal bovine serum and antibiotics (Biowhittaker, Lonza Group Ltd., Basel, Switzerland). Samples of 200 µL from each dilution were applied to wells containing  $5 \times 10^5$  *Spodoptera frugiperda* JE Smith (Lepidoptera: Noctuidae) cells (Sf9). This process was performed in triplicate. After 1 h incubation, the medium was removed, cells were overlaid with 0.25% Seaplaque agarose (Lonza, Allendale, New Jersey, USA) with 1 mL TC-100 + FBS and antibiotics, then incubated for an additional 7 d. Cells then were microscopically inspected and clearly isolated plaques removed using a sterile Pasteur pipette. Each plaque was suspended in 100 µL TC-100 medi-

um where 10  $\mu$ L was injected into groups of 10 fourth instar *A. gemmatalis* using a 1 mL insulin syringe and a microinjector (Burkard, Rickmansworth, United Kingdom). Injected larvae were reared on semisynthetic diet and virus-killed individuals were placed in 1.5 mL tubes and stored at  $-20^{\circ}\text{C}$ .

Infected cadavers were thawed and homogenized in 1 mL distilled water while occlusion bodies were filtered through metal gauze then counted using a Neubauer Improved cell counting chamber (Hawksley, Lancing, United Kingdom), and adjusted to a concentration of  $1 \times 10^9$  occlusion bodies per mL. To extract genomic DNA, 80  $\mu$ L of each occlusion body suspension were mixed with 100  $\mu$ L 0.5 M sodium carbonate, 50  $\mu$ L of 10% (w/v) SDS, and adjusted to a final volume of 500  $\mu$ L. The mixture was incubated for 10 min at  $60^{\circ}\text{C}$  and debris removed by centrifugation at 8,000 rpm for 5 min. The supernatant containing occlusion derived virions was treated with 3  $\mu$ L proteinase K (20 mg per mL) for 1 h at  $50^{\circ}\text{C}$  and DNA purified by treatment with phenol-chloroform followed by chloroform + isoamyl alcohol (24:1). DNA was precipitated with ethanol and re-suspended in  $0.1 \times \text{TE}$  (Tris-EDTA) buffer (pH 7.2).

Restriction endonuclease analysis was conducted with 2  $\mu$ g of viral DNA incubated with 20 U of *Hind*III (New England Biolabs, Ipswich, Massachusetts, USA) for 4 h at  $37^{\circ}\text{C}$  following the manufacturer's recommendations. The reaction was stopped by adding loading buffer, and subjected to electrophoresis in 1% agarose gel in Tris-borate-EDTA (pH 8.3) buffer. Restriction fragments were stained with SYBR<sup>®</sup> Green (Sigma-Aldrich, St. Louis, Missouri, USA), visualized and photographed using a transilluminator (UV ChemiDoc XRS+ System with Image Lab Software; Bio-Rad, Hercules, California, USA). As a reference, occlusion bodies of a previously characterized Brazilian genotype AgMNPV-2D (de Castro Oliveira et al. 2006) were supplied kindly by V. Romanowski (Instituto de Biología y Biología Molecular, La Plata, Argentina) and amplified in *A. gemmatalis* larvae from the insect colony as described earlier.

## PRODUCTION OF CO-OCCLUDED MIXTURES OF GENOTYPIC VARIANTS

Co-occluded mixtures of genotypic variants were produced by mixing different quantities of occlusion bodies of each genotype in the following proportions: (i) Mixture 1: genotypes 1, 2, and 5 in a ratio of 1:1:1; (ii) Mixture 2: genotypes 3, 4, and 5 in a ratio of 1:1:1; (iii) Mixture 3: genotypes 1, 2, 3, 4, and 5 (in equal proportions 1:1:1:1:1); (iv) Mixture 4: genotypes 1, 2, 3, 4, and 5 in the proportions in which they were identified in cell culture plaques (0.20, 0.07, 0.63, 0.03, and 0.07, respectively). The occlusion body mixtures were adjusted to a concentration of  $1 \times 10^8$  occlusion bodies per mL in 10% (w/v) sucrose solution with blue food dye, and inoculated into groups of 12 fourth instar *A. gemmatalis* using the droplet feeding method. Inoculated larvae were reared individually until death, and occlusion bodies containing co-occluded genotypes were extracted and counted prior to use in bioassays.

## BIOLOGICAL ACTIVITY OF GENOTYPIC VARIANTS AND MIXTURES

To screen for evidence of improved biological activity of AgMNPV variants in co-occluded mixtures, occlusion bodies of individual variants (and their mixtures) were subjected to bioassays using a single concentration expected to result in about 70% mortality in preliminary tests (data not shown). First instar *A. gemmatalis* were allowed to molt overnight, then inoculated with  $2 \times 10^5$  occlusion bodies per mL using the droplet feeding method. Twenty-four larvae that consumed the occlusion body suspension within 10 min were transferred

individually to wells of a 24-well cell culture plate, provided with diet, and incubated at  $25 \pm 1^{\circ}\text{C}$  in darkness. Larval mortality was checked at 24 h intervals until all larvae had died or pupated. Control larvae were treated identically, but did not consume occlusion bodies. The bioassay was performed on 3 occasions using different batches of insects.

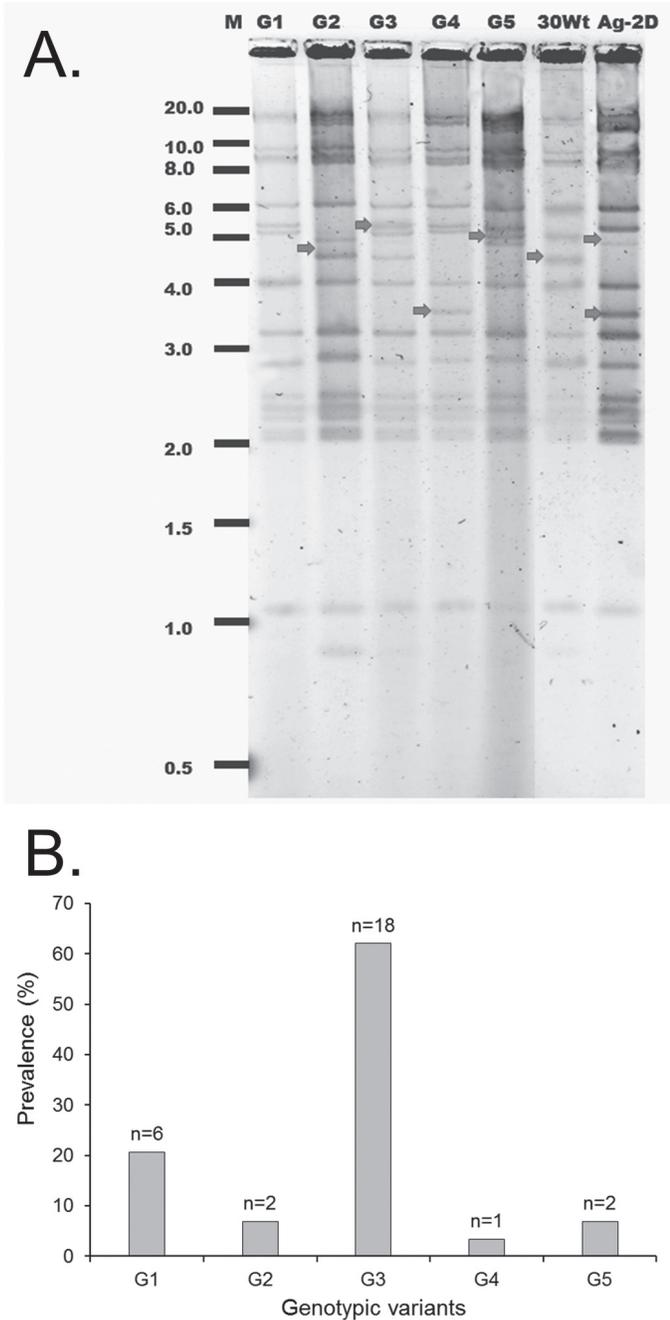
To determine occlusion body production, and speed of kill for individual variants as well as mixtures, groups of 24 fourth instar *A. gemmatalis* that had molted in the previous 12 h, were inoculated with  $1 \times 10^8$  occlusion bodies per mL in 10% (w/v) sucrose solution with blue food dye. This inoculation rate was expected to result in about 90% mortality. In the case of the genotype 3 variant and co-occluded mixture 2,  $1 \times 10^9$  occlusion bodies per mL was used as inoculum to obtain equivalent mortality (about 90%) responses given the lower pathogenicity of these occlusion bodies. Larvae that ingested the occlusion body suspension in 10 min were reared individually on semisynthetic diet at  $25 \pm 1^{\circ}\text{C}$  and examined at 12 h intervals until death. Twelve virus-killed larvae were selected at random, homogenized in 1 mL of 0.1% SDS, and triplicate samples of occlusion bodies counted using a Neubauer cell counting chamber. The procedure was performed on 3 occasions with different batches of insects. Control larvae were treated identically, but did not consume occlusion bodies. Mean time to death was estimated by Weibull survival analysis in GLIM (Crawley 1993). The validity of the Weibull model was confirmed by comparing the results with those of the Kaplan-Meier model (Crawley 1993). The average number of occlusion bodies per larva was calculated for each replicate and subjected to 1-way ANOVA following logarithmic transformation ( $\log_{10}[x]$ ). Mean separation was performed by Tukey's test ( $P < 0.05$ ).

To determine whether plaque purification resulted in the selection of variants with reduced pathogenicity in *A. gemmatalis* larvae, occlusion bodies from 52 plaque purified variants were mixed in equal proportions and subjected to concentration-mortality bioassays. For comparison, occlusion bodies from the mixture of 30 field isolates also were subjected to similar bioassays. Groups of 24 recently molted second instars were allowed to drink 1 of the following concentrations of occlusion bodies following the droplet feeding technique:  $1 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $5 \times 10^5$  or  $1 \times 10^6$  occlusion bodies per mL. Inoculated larvae were reared individually at  $25 \pm 1^{\circ}\text{C}$  until death or pupation. Both bioassays were performed on 3 occasions and results were subjected to logit regression in GLIM 4. Changes in model deviance are given in terms of  $\chi^2$  statistics. Minor over-dispersion of larval mortality data was taken into account by scaling the binomial error distribution; analyses are reported as F statistics (Crawley 1993).

## Results

### PREVALENCE OF GENOTYPIC VARIANTS

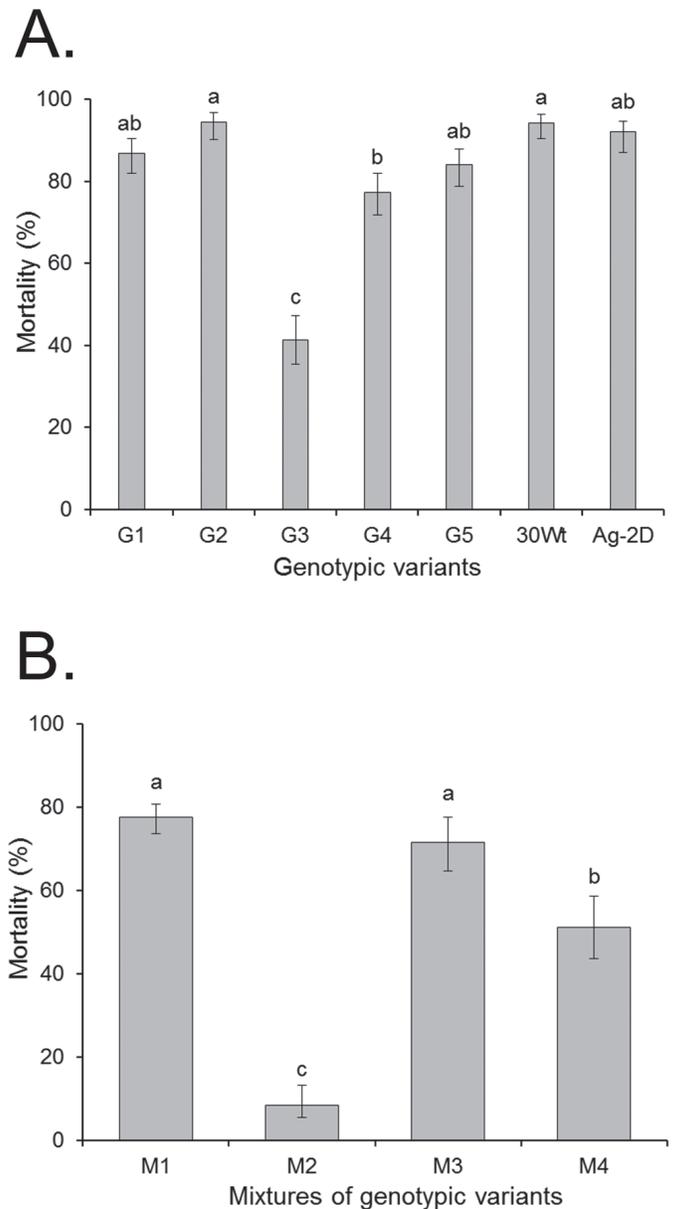
A total of 89 plaques were selected for injection of *A. gemmatalis* larvae. Of these, 52 (58%) isolates caused lethal polyhedrosis disease in larvae. Of the isolates from virus-killed larvae, 29 (56%) were identified as likely to be pure genotypes due to the lack of minor (sub-stoichiometric or sub-molar) fragments, whereas 23 isolates were identified as likely to comprise mixtures of genotypes due to the presence of sub-molar fragments. The number of fragments in genotypic variants was 16 to 20 with 1 clear marker fragment in each variant (Fig. 1A). The most prevalent variant was named genotype 3, occurring in 62% of isolates, and the least prevalent was named genotype 4 at 7% (Fig. 1B) with the remaining isolates at intermediate prevalence.



**Fig. 1.** (A) *Hind*III restriction endonuclease profiles of 5 individual genotypic variants (G1–G5) compared with a mixture of 30 field isolates (30wt) obtained from pooled field-collected larvae and the reference Brazilian variant AgMNPV-2D (Ag-2D). Arrows indicate the position of marker fragments for each of the variants. (B) Prevalence of plaque purified variants in pooled sample of 30 *Anticarsia gemmatalis* larvae that died from polyhedrosis during laboratory rearing (n indicates total number of plaques of each genotypic variant out of a total of 52 plaques).

**BIOLOGICAL ACTIVITY OF GENOTYPIC VARIANTS AND CO-OCCLUDED MIXTURES**

Mortality differed significantly among *A. gemmatalis* larvae inoculated with the different variants, as well as the mixture of 30 field isolates and Ag-2D treatments ( $\chi^2 = 80.69$ ,  $df = 6$ ,  $P < 0.001$ ). Mortality was greatest in larvae treated with genotype 2 and the mixture of 30 field isolates (94%) lowest in the genotype 3 treatment (41%), and interme-

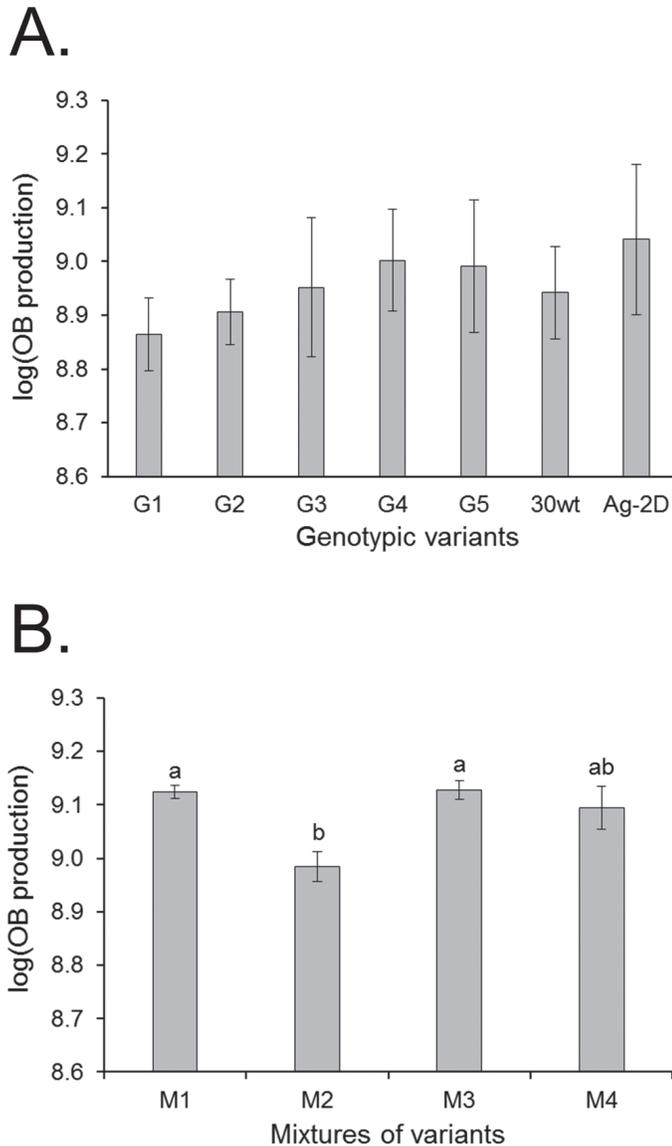


**Fig. 2.** Mortality of second instar *Anticarsia gemmatalis* following inoculation with  $2 \times 10^5$  occlusion bodies per mL of (A) genotypic variants compared with a mixture of 30 field isolates (30wt) and reference Brazilian variant AgMNPV-2D (Ag-2D), and (B) co-occluded mixtures of variants (M1–M4).

diate for all other treatments, including the Ag-2D treatment (Fig. 2A). None of the genotypic variant treatments resulted in mortality greater than the mixture of 30 field isolates; as a result, mortalities across a range of inoculum concentrations were not determined.

Mortality of larvae to co-occluded mixtures of genotypic variants also varied significantly among treatments ( $F = 22.6$ ;  $df = 3,20$ ;  $P < 0.001$ , scale parameter 2.7). The highest prevalence of mortality was observed in co-occluded mixture 1 (78%) and mixture 3 (72%) compared with significantly lower mortality in mixture 4 (51%) and very low mortality (9%) in mixture 2 treatments (Fig. 2B).

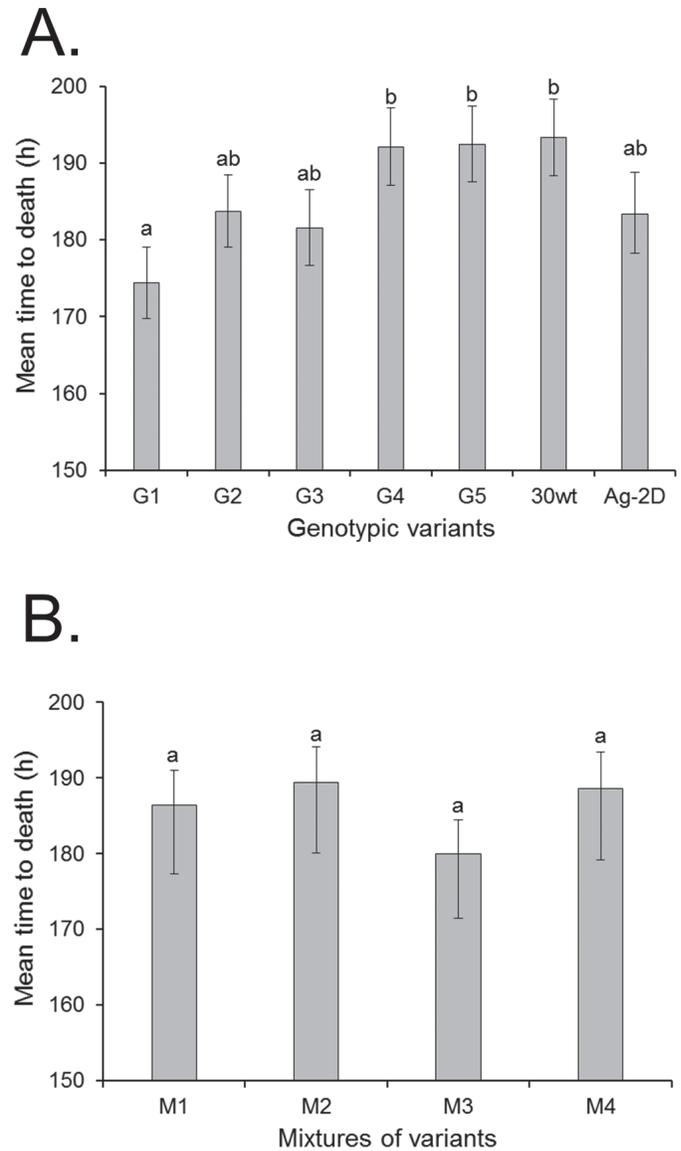
Mortality was similar among larvae inoculated with single variants and the reference treatments comprising the mixture of 30 field isolates and Ag-2D (range 88–94%), as well as among individuals inoculated with variant mixtures (range 82–88%). Mean occlusion body production ranged from  $7.31 \times 10^8$  to  $1.10 \times 10^9$  occlusion bodies per



**Fig. 3.** Logarithm of mean occlusion body (OB) production in fourth instar *Anticarsia gemmatalis* infected by (A) the genotypic variants compared with a mixture of 30 field isolates (30wt) and reference Brazilian variant AgMNPV-2D (Ag-2D), and (B) co-occluded mixtures of variants (M1–M4).

larva (shown as log values in Fig. 3A), but did not differ significantly among insects infected by individual genotypic variants and reference treatments ( $F = 0.333$ ;  $df = 6, 14$ ;  $P = 0.908$ ). In contrast, mean occlusion body production varied significantly in insects treated with co-occluded variant mixtures ( $F = 6.458$ ,  $df = 3, 8$ ;  $P = 0.016$ ). In this case, mean occlusion body production was significantly lower in larvae treated with the mixture 2 compared with the other mixtures (Fig. 3B).

Speed of kill by all genotypic variants were similar to those of the mixture of 30 field isolates and the Ag-2D reference variant, with the exception of genotype 1 which was significantly faster in killing than the treatments involving genotype 4, genotype 5, and the mixture of 30 field isolates (Fig. 4A). None of the mixtures tested differed significantly in mean speed (time) of kill and varied between 180 and 189 h (Fig. 4B). There was no significant correlation between mean time to death and occlusion body production among the individual variants or their mixtures ( $r^2 = 0.031$ ).



**Fig. 4.** Weibull estimates of mean time to death of fourth instar *Anticarsia gemmatalis* infected by (A) the individual genotypic variants compared with a mixture of 30 field isolates (30wt) and reference Brazilian variant AgMNPV-2D (Ag-2D) (shape parameter  $a = 6.776$ ), and (B) co-occluded mixtures of variants (M1–M4) (shape parameter  $a = 7.509$ ).

To determine whether the lack of elevated pathogenicity in plaque purified variants (and their mixtures) was due to selection of variants during the cell culture process,  $LC_{50}$ s were determined for the mixture of 30 field isolates and 52 plaque variant mixture. The  $LC_{50}$  of the mixture of 30 field isolates was estimated at  $9.6 \times 10^4$  occlusion bodies per mL (95% C.I.:  $5.0 \times 10^4$  to  $1.8 \times 10^5$  occlusion bodies per mL, slope  $\pm$  SE =  $0.5800 \pm 0.881$ ) and was significantly greater ( $\chi^2 = 4.99$ ,  $df = 1$ ,  $P = 0.03$ ) than the 52 plaque mixture at  $LC_{50}$   $5.6 \times 10^4$  occlusion bodies per mL (95% C.I.:  $3.8 \times 10^4$  to  $7.9 \times 10^4$  occlusion bodies per mL,  $0.8466 \pm 0.0807$ ). A test for parallelism indicated that the slopes of each regression differed significantly ( $\chi^2 = 7.09$ ;  $df = 1$ ;  $P < 0.01$ ).

## Discussion

A total of 30 field isolates of AgMNPV were obtained from *A. gemmatalis* larvae. Between 9 and 20% of field collected larvae died from

polyhedrosis during laboratory rearing, indicating that the virus is a natural mortality factor in this region. The mortality levels in our study attributed to natural virus infection were similar to those observed in the United States by Beach et al. (1984), Young & Yearian (1986), Boucias et al. (1987), and Fuxa & Richter (1999).

Five genotypic variants were identified in our study, of which genotype 3 was the most prevalent. Similar variation has been reported previously in AgMNPV field populations from Brazil from field-collected *A. gemmatalis* larvae (Maruniak 1989) and plaque purified variants (Garcia-Maruniak et al. 1996; Ribeiro et al. 1997; Maruniak et al. 1999). Indeed, the reference isolate AgMNPV-2D has been identified previously as the most frequent plaque-purified variant from a natural *A. gemmatalis* population originally isolated in Brazil in 1972 (Maruniak et al. 1999).

Most nucleopolyhedrovirus populations harbor high levels of genetic diversity, which is reflected in variation of phenotypic traits such as infectivity, speed of kill, and occlusion body production, among others (Erlandson 2009). This is clearly the case for AgMNPV, in which diversity is maintained by recombination among variants during coinfection (Croizier & Ribeiro 1992), loss or duplication of genomic sequences (Hayakawa et al. 2000), or other evolutionary processes (de Brito et al. 2016) and ecological factors, as observed in other nucleopolyhedrovirus pathosystems (Hodgson et al. 2004; Hitchman et al. 2007).

The fact that occlusion-derived virions and occlusion bodies are genotypically diverse favors the transmission of genetic diversity from insect to insect (Clavijo et al. 2010), and is likely to be crucial to the survival of nucleopolyhedroviruses in host populations (Sanjuán 2017). The genetic diversity present within occlusion derived virions also would explain the prevalence of mixed genotype infections in amplified plaques in our study. Of the 52 amplified plaques we studied, 23 (44%) consisted of genotype mixtures that contained sub-molar restriction fragments. This suggested that these plaques originated from infection by 2 or more genetically distinct budded virions. Presumably, these virions independently infected cells that were in close proximity to each other and formed a single overlapping plaque, or may have adhered to each other during sample preparation as observed for other viruses (Aguilera et al. 2017; Drayman 2017).

Unfortunately, it was not possible to obtain original samples of the AgMNPV that was used initially as a biological insecticide for the control of *A. gemmatalis* in the Huastecas (Mexico) region. Nonetheless, comparison of restriction endonuclease profiles revealed that the AgMNPV isolates present in the Huastecas region clearly were related to the reference variant from Brazil (Johnson & Maruniak 1989). To determine if the AgMNPV isolates from Mexico are the result of displacement, or recombination among autochthonous and introduced strains from the Brazilian isolate, a comparative systematic phylogenetic analysis of field isolates from Mexico and the United States with the established pangenome sequences from Brazil would be required (de Brito et al. 2016).

Previous studies have reported high levels of variation among AgMNPV isolates from Brazil (Ribeiro et al. 1997), which led us to expect clear differences among the variants in our study. Similarly, none of the variant mixtures improved pathogenicity when compared with individual variants or the mixture of 30 field isolates. This contrasts with previous findings on other nucleopolyhedroviruses, where mixtures of variants demonstrated greater pathogenicity than individual variants or wild-type populations (Bernal et al. 2013; Arrizubieta et al. 2015).

We also examined the possibility that our plaque purification process may have selected for variants amenable to cell culture but less pathogenic to *A. gemmatalis* caterpillars. Although the 52-plaque mixture was slightly more pathogenic than the mixture of 30 field isolates, this data supports the belief that plaque purification was not a major

selection factor that eliminated highly pathogenic variants from the wild-type population. It is possible that reduced pathogenicity of this mixture may have resulted from the elimination of defective mutants in the virus population by complementation. Such defective variants in virus populations have been reported to adversely affect the pathogenicity of *Spodoptera exigua* (Lepidoptera: Noctuidae) nucleopolyhedrovirus (SeMNPV) (Muñoz & Caballero 2000). Conversely, López-Ferber et al. (2003) and Simón et al. (2013) demonstrated that a defective genotype was responsible for increased pathogenicity of a *Spodoptera frugiperda* nucleopolyhedrovirus (SfMNPV) isolate by modulating the concentration of a per os infection factor in the occlusion derived virion envelope.

In conclusion, the 5 genotypic variants isolated from field-collected *A. gemmatalis* larvae in our study varied in pathogenicity and speed of kill but not occlusion body production per larva. However, these individual variants did not possess greater pathogenicity when compared with the mixture of 30 field isolates. Therefore, the mixture of 30 field isolates appears promising for further development and field testing in order to relaunch the successful use of this nucleopolyhedrovirus as a biological insecticide against *A. gemmatalis* in the Huastecas soybean-producing region of Mexico.

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