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Effect of the interaction between *Anticarsia gemmatalis* multiple nucleopolyhedrovirus and *Epinotia aporema* granulovirus, on *A. gemmatalis* (Lepidoptera: Noctuidae) larvae

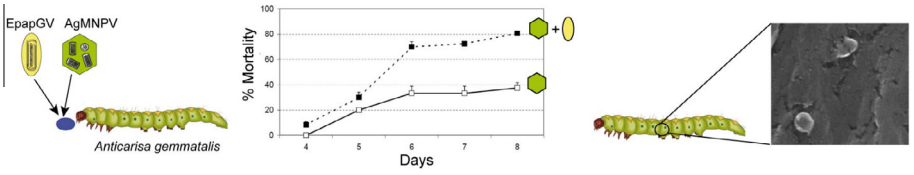
Marina E. Biedma [a,1], Ricardo Salvador [a,b], M. Leticia Ferrelli [a,b], Alicia Sciocco-Cap [b], Víctor Romanowski [a,*]

[a] Instituto de Biotecnología y Biología Molecular (UNLP – CONICET), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 115 y 49, 1900 La Plata, Buenos Aires, Argentina
[b] IMYZA-CICVyA, Instituto Nacional de Tecnología Agropecuaria (INTA), CC 25, B1712WAA Castelar, Buenos Aires, Argentina

HIGHLIGHTS

- The interaction between EpapGV and AgMNPV on *Anticarsia gemmatalis* larvae was evaluated.
- The addition of EpapGV OBs increased the virulence of AgMNPV.
- Damage to the peritrophic membrane was observed, although no enhacin gene was found in EpapGV.
- Damage might be associated with spheroidal inclusions present in preparations of EpapGV OBs.

GRAPHICAL ABSTRACT



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ABSTRACT

The bean shoot borer *Epinotia aporema* Wals. (Lepidoptera: Tortricidae) and the velvet bean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) are key pests of soybean and other legume crops in South America. They are often found simultaneously in certain regions. *A. gemmatalis* nucleopolyhedrovirus (AgMNPV) is widely used to control *A. gemmatalis*. More recently, *E. aporema* granulovirus (EpapGV) has been characterized and evaluated as a bioinsecticide for *E. aporema*. In order to increase its potential use and to design optimized strategies for the management of lepidopteran pests, we evaluated the interaction between EpapGV and AgMNPV on third instar *A. gemmatalis* larvae. Larvae fed with 50 AgMNPV OBs/larva showed an increase in the mortality rates (from 42% to 81%) and a decrease in the median survival time (from 7.7 days to 5.7 days) when these OBs were mixed with 6000 EpapGV OBs/larva. When 300 AgMNPV OBs/larva were used alone or in combination with EpapGV OBs no changes in biological parameters were observed. No mortality was detected in *A. gemmatalis* larvae treated with EpapGV alone. In larvae fed with the viral mixtures, only AgMNPV DNA was detected by PCR. *A. gemmatalis* peritrophic membranes (PMs) examined by SDS–PAGE and scanning electron microscopy showed signs of damage. Notably, we found the presence of spheroidal bodies associated with damaged areas in the PMs of larvae fed with EpapGV but not in those that were given AgMNPV alone. These results show that EpapGV increases the viral potency of AgMNPV, and thus the insecticidal efficiency, suggesting that the use of formulations including both viruses might be a valuable tool for pest management.

* Corresponding author at: IBBM (UNLP – CONICET), Facultad de Ciencias Exactas, calle 115 y 49, 1900 La Plata, Argentina.
E-mail address: victor@biol.unlp.edu.ar (V. Romanowski).
[1] Current address: Instituto de Estudios en Inmunología y Fisiopatología (IIFP), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, 47 y 115, 1900 La Plata, Argentina.

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1. Introduction

The bean shoot borer *Epinotia aporema* Wals. (Lepidoptera: Tortricidae) and the velvet bean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) are key pests of soybean and other legume crops in South America. In Argentina both pests are frequently found simultaneously in soybean crops (Aragón et al., 1998). At present, broad spectrum chemical insecticides are used to control these pests, with the consequent detrimental effects on the environment. Several members of the family *Baculoviridae* (Carstens and Ball, 2009; Jehle et al., 2006) have been successfully used as bioinsecticides (Szewczyk et al., 2006). Among naturally occurring baculoviruses, the *E. aporema* granulovirus (EpapGV; Genus *Betabaculovirus*) and the *A. gemmatalis* multicapsid nucleopolyhedrovirus (AgMNPV; Genus *Alphabaculovirus*), are alternative tools for the biological control of the above mentioned pests. AgMNPV has been used as a microbial insecticide in Brazil since early 1980's and, on a lower scale, in Argentina, Paraguay and Bolivia (Moscardi, 1999; Moscardi and Sosa Gómez, 2007; Szewczyk et al., 2006). In addition, EpapGV has been characterized and evaluated under controlled conditions (Ferrelli et al., 2012; Goldberg et al., 2002; Parola et al., 2002; Sciocco-Cap et al., 2001). These studies indicated that EpapGV is a good candidate for the control of *E. aporema* and a new bioinsecticide based on this virus is in the process of registration in Argentina.

At least three types of interactions in baculovirus infections have been described, in which one or both interacting viruses result in a neutral, inhibiting or enhanced activity due to the presence of the other (Cheng and Lynn, 2009). The enhanced activity is the most studied because of its potential use in biocontrol strategies. Several studies have shown that this effect was due to synergistic factors present in occlusion bodies (OBs) of granuloviruses (Derksen and Granados, 1988; Goto, 1989; Hashimoto et al., 1991; Hukuhara et al., 1987; Mukawa and Goto, 2007). It was demonstrated that a granulovirus-encoded protein, enhancin, causes the degradation of mucin II, the major protein component of the peritrophic membrane (PM), thereby allowing a greater access of the virions to the midgut epithelial cells, and resulting in the insect's increased susceptibility to the viral infection (Derksen and Granados, 1988; Lepore et al., 1996; Wang and Granados, 1997). More recently, another protein encoded in several baculovirus genomes (GP37) has been described and shown to exhibit an enhancing activity on nucleopolyhedroviruses (NPVs) and binding capacity to chitin, a component of the PM. It is presumed that the binding of this protein to chitin produces the disruption of the PM contributing to enhanced NPVs infection (Liu et al., 2011).

In order to increase the potential use of EpapGV and to design optimized strategies for the management of lepidopteran pests in soybean crops, we tested its interaction with AgMNPV. In this study we report the enhanced viral potency of AgMNPV when applied in combination with EpapGV on *A. gemmatalis* larvae.

2. Materials and methods

2.1. Sources of insects and viruses

The experiments were conducted with *A. gemmatalis* and *E. aporema* larvae from established colonies of insects collected in the main soybean area (Córdoba and Santa Fe Provinces), and maintained at the IMYZA (INTA-Castelar). The insects were reared on artificial diet at 25 ± 1 °C, 14:10 h light–darkness cycle, and 60% relative humidity (Greene et al., 1976). The indigenous AgMNPV isolate, (AgMNPV-SF, obtained from J. Claus, Universidad Nacional del Litoral, Santa Fe, Argentina and S. Lorenzatti, INTA-Oliveros, Argentina) and EpapGV (Sciocco-Cap et al., 2001) were propagated on their original hosts, respectively. OBs were purified as previously described (Garcia-Maruniak et al., 2004; Goldberg et al., 2002).

2.2. Bioassays

Third instar larvae of *A. gemmatalis* were starved for 24 h, and individually inoculated using the droplet feeding technique (Hughes and Wood, 1981). Briefly, the larvae were allowed to ingest viral suspensions made in 1% sucrose and 0.1% Coomassie Brilliant Blue (fagostimulant solution). Larvae that had consumed the suspension within 5 min, were transferred to 30 ml plastic cups containing fresh virus-free diet, and maintained at 25 °C. Control larvae were treated similarly, using water instead of viral suspension. The concentration of OBs for each viral suspension was adjusted on the basis of the mean ingested volume per larva (Kunimi and Fuxa, 1996). *A. gemmatalis* larvae were treated with two doses of AgMNPV, *i.e.* 50 and 300 OBs/larva tested alone and in combination with EpapGV (6000 OBs/larva). In addition, EpapGV alone was also used as a negative control. The doses and treatments were selected on the basis of the previously estimated median lethal dose (LD_{50}) of AgMNPV-SF (168 OBs/larva) (Biedma, 2009) and a dose of EpapGV (6000 OBs/larva) that caused 100% mortality of last instar larvae of *E. aporema* (Goldberg et al., 2002). Three replicate assays of thirty larvae each were performed. Mortality was recorded every 12 h until death or pupation. Mortality was estimated using the time-mortality response model of Vistat software (R.P. Hughes, Cornell University, Ithaca, NY) and

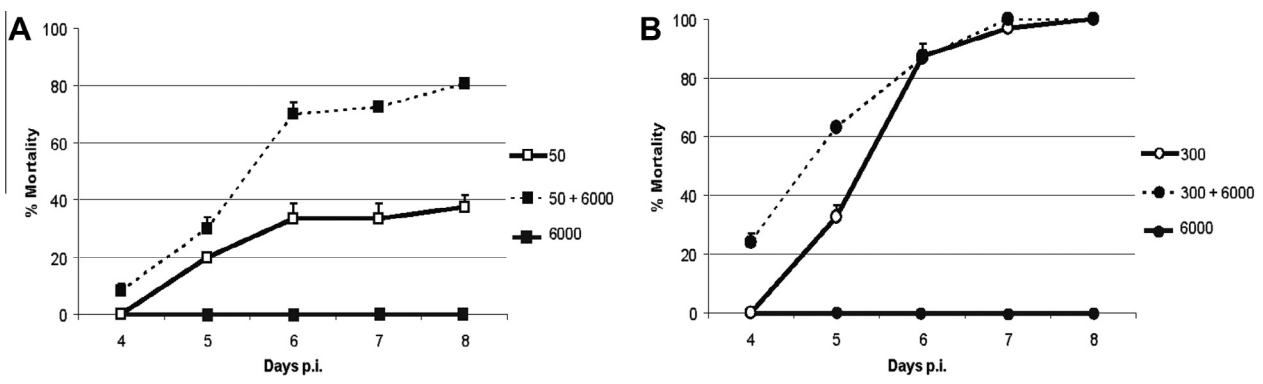

Fig. 1. Mortality of *A. gemmatalis* larvae treated with AgMNPV and EpapGV. (A) The larvae were fed 300 OBs of AgMNPV alone (white squares, solid line), with the addition of 6000 OBs of EpapGV (black squares, square dot line) or with 6000 OBs of EpapGV alone (black squares, solid line). (B) The larvae were fed 50 OBs of AgMNPV alone (white circles, solid line), 50 OBs of AgMNPV with the addition of 6000 OBs of EpapGV (black circles, square dot line) or 6000 OBs of EpapGV alone (black circles, solid line).

subjected to one-way analysis of variance (ANOVA) and Tukey's test ($p < 0.01$) using the InfoStat software (2009 version; FCA, Universidad Nacional de Córdoba, Argentina). The median survival times (ST_{50}) for the treatments were estimated using the Kaplan–Meyer Product-Limit estimator method in MedCalc Statistical Software version 15.2.2 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2015), and were compared using log-rank test.

2.3. PCR analysis

DNA samples were prepared as described by Manzan et al. (2008) from virus-killed larvae or control larvae. The presence of AgMNPV-SF and/or EpapGV viral DNA in the treated larvae, was determined using specific primers designed to amplify the AgMNPV *polyhedrin* ORF (Arana et al., 2001) and EpapGV *granulin* ORF (Parola et al., 2002).

2.4. Peritrophic membrane extraction and SDS–PAGE

A total of ten peritrophic membranes (PMs) were isolated from ten larvae 18 h after treatment (300 OBs AgMNPV and 6000 OBs EpapGV) as described by Wang and Granados (1997). The PMs were washed in distilled water, eliminating intestinal content and quickly homogenized in sample buffer. Proteins were separated by SDS–PAGE (Laemmli, 1970) and analyzed after silver staining.

2.5. Scanning electron microscopy (SEM)

Third instar larvae of *A. gemmatalis* treated as described in Section 2.4 were processed for the isolation of PMs following the methodology described by Mitsuhashi et al. (2007). The PMs were washed in 0.1% PBS and fixed at room temperature in 1% glutaraldehyde, followed by dehydration in an ethanol series (50%, 70%, 90%, and 100%). The resulting samples were critical point dried (BAL-TEC Model CP-30), metallized with gold (JEOL mod. Fine Coat Ion Sputter) and examined individually by SEM (JEOL, model JSM-6360 LV).

Additionally, OBs from EpapGV were resuspended in distilled water. Five microliter samples containing of 5×10^7 OBs were deposited on copper grids, dried at room temperature, metallized with gold and examined individually by SEM.

Table 1
Administration of AgMNPV and EpapGV OBs to *x* 3rd instar larvae.

AgMNPV (OBs/larva)	EpapGV (OBs/larva)	ST_{50} (days)*
50	–	7.7[b]
50	6000	5.7[a]
300	–	5.6[a]
300	6000	5.1[a]

The median survival time (ST_{50}) values were determined by the Kaplan–Meier estimator. For each treatment, ST_{50} (*) values with different letters are significantly different at $p = 0.05$ as tested by log-rank comparison.

3. Results and discussion

In order to evaluate the effect of EpapGV OBs and AgMNPV OBs mixtures on third instar *A. gemmatalis* larvae bioassays were conducted using different viral OBs dose combinations. The comparisons of cumulative mortalities caused by AgMNPV alone and in combination with EpapGV are shown in Fig. 1. The highest mortality rates were observed when *A. gemmatalis* were treated with 300 OBs/larva (Fig. 1A). In this case the mortality curves obtained were not significantly affected by the addition of EpapGV OBs, in both treatments the mortality reached 100%. However, when the lower dose of AgMNPV was used (50 OBs), the results were clearly different (Fig. 1B) and the mortalities scored were significantly different ($F = 6.79$, $df = 3$, $p < 0.01$). The treatment with AgMNPV only gave lower mortality (41.66% ± 2.88%) than when combined with EpapGV (80.66% ± 1.16%). No mortality or infection signs were detected in larvae treated with EpapGV alone. Table 1 shows the ST_{50} calculated for the different treatments. ST_{50} for larvae infected with AgMNPV alone was 7.7 and 5.6 days for 50 and 300 OBs treatments, respectively. When AgMNPV infection was conducted in the presence of EpapGV OBs a significant decrease (Log Rank $\chi^2 = 9.6231$; $df = 1$; $p = 0.0019$) of the ST_{50} was observed with 50 OBs AgMNPV treatment. However, no differences in ST_{50} were observed between single and combined treatment with 300 OBs/larvae (Log Rank $\chi^2 = 0.01404$; $df = 1$; $p = 0.9057$). Strikingly, the addition of EpapGV to 50 AgMNPV OBs shortened the ST_{50} to values similar to those obtained by infection with 300 OBs AgMNPV alone (5.7 and 5.6 days, respectively). At the end of the bioassay total DNA was extracted from treated larvae and subjected to PCR to detect AgMNPV-SF and/or EpapGV genomic DNA using specific primers as described in Section 2. Only AgMNPV DNA was detected in the dead larvae (Fig. 2) while EpapGV DNA was not, confirming that EpapGV does not infect *A. gemmatalis* as previously reported by Sciocco-Cap et al. (2001).

The increased susceptibility of *A. gemmatalis* to AgMNPV infection could be due to proteins associated with EpapGV OBs. Some baculoviruses are known to encode proteins responsible for similar effects in interaction studies (Derksen and Granados, 1988; Mukawa and Goto, 2010). The EpapGV genome analysis revealed the absence of an *enhancin* homolog but a *gp37* gene was detected and characterized (Ferrelli et al., 2012; Salvador et al., 2012). A recent study demonstrated that this protein is involved in

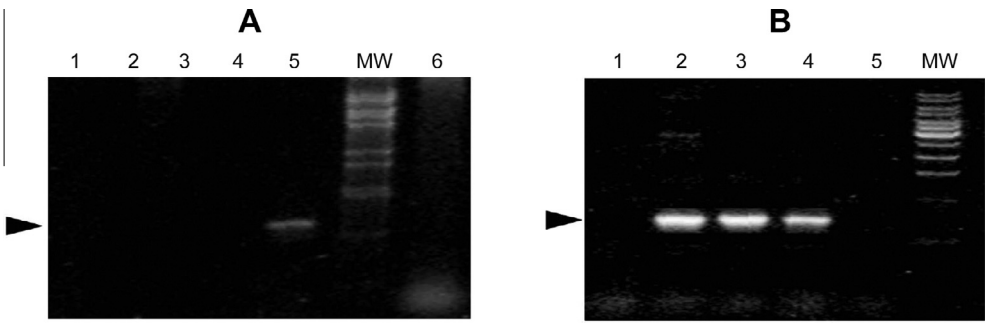

Fig. 2. PCR products obtained using primers for *granulin* (Gr-up and Gr-dw) (A) and *polyhedrin* (orf-i and orf-f) (B). In A and B, lane 1: EpapGV treated larvae; lane 2: AgMNPV/EpapGV (50/6000 OBs/larva); lane 3: AgMNPV alone; lane 4: AgMNPV DNA (control); lane 5: EpapGV DNA (control). In A, lane 6: control larvae (negative control); MW markers: lambda DNA/*Bst*E II; arrow: *granulin* amplicon (830 bp). In B, arrow: *polyhedrin* amplicon (750 bp); MW: ladder 500 bp.

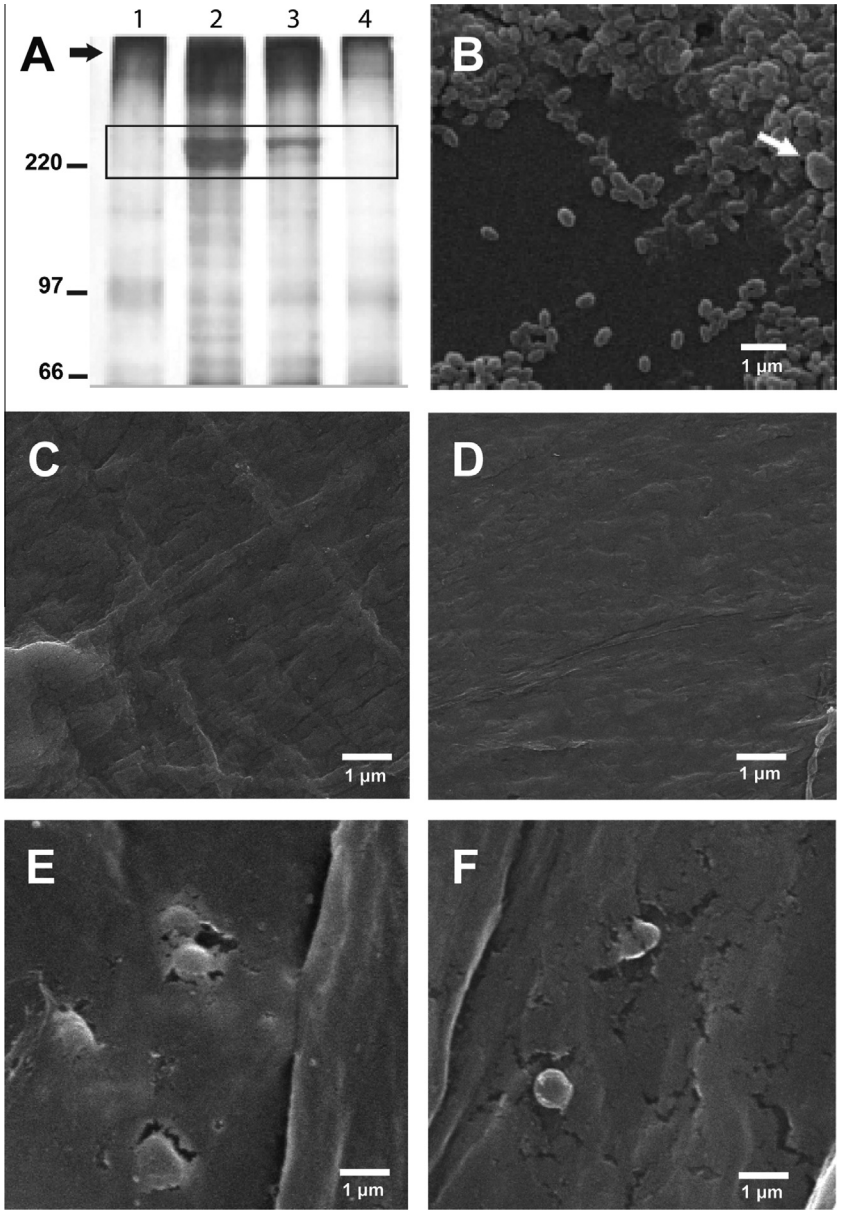

Fig. 3. Effect of EpapGV OBs on *A. gemmatalis* peritrophic membrane (PM). (A) SDS–PAGE of PM extracted 18 h after treatment. Treatments: 1. Control larvae; 2. 300 AgMNPV OBs + 6000 EpapGV OBs; 3. 6000 EpapGV OBs; 4. 300 AgMNPV OBs. Arrow indicates high molecular weight PM proteins; putative degradation protein products are enclosed in a box in lanes 2 and 3. (B–F) Scanning electron microscopy. (B) EpapGV OBs (the arrow shows an atypical structure); (C) PM of control larvae; (D) PM of larvae treated with 300 AgMNPV OBs; (E) PM of larvae treated with 300 AgMNPV OBs + 6000 EpapGV OBs; (F) PM of larvae treated with 6000 EpapGV OBs.

enhanced infectivity of NPVs by PM alteration related to its chitin-binding properties (Liu et al., 2011). Other reports showed that some nucleopolyhedrovirus GP37 homologs are physically associated with OBs (Gross et al., 1993; Li et al., 2003). In order to evaluate the effect of EpapGV and AgMNPV OBs mixtures on *A. gemmatalis* larvae, PMs were extracted 18 h after treatment and analyzed by SDS–PAGE and SEM (Fig 3). SDS–PAGE (Fig 3A) shows the degradation of high molecular weight protein(s) in PMs isolated from *A. gemmatalis* larvae subjected to treatment with the mixture of both viruses (lane 2) and in those treated with EpapGV alone (lane 3). The PMs of control larvae (lane 2) as well as those treated with AgMNPV (lane 4), did not show this protein degradation pattern. In the SEM analysis no modification of the PM structure was detected in larvae treated with AgMNPV OBs (Fig. 3C and D). In contrast, a clear alteration of the PM structural organization was apparent in samples obtained from larvae treated with EpapGV alone or in combination with AgMNPV. In addition, spheroidal bodies with an approximate average diameter of 1 µm were observed in the damaged areas of the PM (Fig. 3E and F). Similar structures were also observed in EpapGV OBs preparation by SEM analysis (Fig 3B). Further studies are necessary to characterize and confirm the identity of these structures.

Summarizing, the bioassay and PCR results suggest that the decrease in the speed of kill and increase of mortality of *A. gemmatalis* treated with viral mixtures was not due to a co-infection. This might be the result of the structural change observed in the PM owing to components present in the OB preparations of EpapGV. In conclusion, the study presented here shows the potential use of formulations that include both viruses as a valuable tool for pest management in areas with temperate climates (mean temperature: 20 °C), where high incidence of *E. aporema* on legume crops is observed and AgMNPV has a slow speed of action on its

host (Moscardi, 1999). This can be regarded as an environmentally friendly alternative to increasing the viral potency of AgMNPV with no chemical enhancers/additives. Finally, the formulation of biopesticides containing both AgMNPV and EpapGV OBs is certainly of value since *A. gemmatalis* and *E. aporema* are frequently found as simultaneous pests in legume cultivated fields and it would permit decreasing the amount of AgMNPV in the final product used with the consequent impact on the production costs.

Acknowledgments

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