Combination effects of *Spodoptera littoralis* nuclear polyhedrosis and granulous virus against larvae of the cotton leafworm

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Abstract: In the laboratory, the median lethal doses (LD50s), the median survival times (ST50s) and the median lethal times (LT50s) were measured for 3th instars larvae (L3) of *Spodoptera littoralis* (Boisd.) infected with *S. littoralis* nuclear polyhedrosis virus (SpliNPV), *S. littoralis* granulovirus (SpliGV) or both. The results revealed that interactions between SpliNPV and SpliGV were dose dependent. The larval mortalities in combined treatments of SpliNPV (2.556 OB/larvae and 63.900 OB/larvae) and SpliGV ($5.6x10^6$ OB / larvae and $1.4x10^8$ OB / larvae) were higher than those obtained in each single virus treatment. The LD50s was increased significantly in combined treatment than in single treatments. The median lethal doses (LD50s) of the SpliNPV were increased when combined with one dose SpliGV, which indicates a loss of virulence of SpliNPV. The ST50s for single and combined treatments with SpliNPV and SpliGV was significantly higher in combined treatment than in single SpliNPV treatment with 3-6 days, but the ST50s was reduced significantly in combined treatment with low doses of SpliGV. The ST50s in the treatments with SpliNPV doses and constant dose of SpliGV was decreased significantly with the increasing of the SpliNPV doses. The treatments of SpliGV doses and constant dose of SpliNPV the ST50s was increased respect to single treatments without significant differences. The LT50s in combined treatments was increased respectively to single doses of SpliNPV. The SpliGV LT50s were affected by neither the single treatments nor the combined treatments. As conclusion the combination of SpliNPV and SpliGV showed antagonism, so that it is not recommended to use the mixture of both viruses as bioinsecticides.

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1. Introduction:

There are now four genera: the Alphabaculoviruses (lepidopteron-specific NPV), Betabaculoviruses (lepidopteron specific GV), Gammabaculoviruses (hymenopteron-specific NPV), and Deltabaculoviruses (dipteron-specific baculovirus) (Jehle *et al.,* 2006).

The GVs possess a large circular double-stranded DNA genome that is packaged in a rod-shaped nucleocapsid surrounded by an envelope. The viriones are embedded in granule-shaped proteinaceous occlusion body (OB) containing a single virion (Blissard *et al.* 2000; Federici, 1997). The insecticidal activity of baculovirus was increased mortality results when combining a nucleopolyhedrovirus and granulovirus in larvae of *Pseudaletia unipuncta*, with respect to the independent action of each one (Tanada, 1956; Tanada and Hukuhara, 1971), for *Trichoplusia ni* (Lowe and Paschke, 1968), for *Spodoptera litura* F. (Guo *et al.,* 2007), and for *S. littoralis* (Hatem, *et al.,* 2011).

Efficacy of baculoviruses has been evaluated by median lethal dose (LD50), median lethal concentration (LC50), median lethal time (LT50) or median survival time (ST50) of infected larvae (Hughes *et al.,* 1983). Strategies to counteract some of the limitations of NPVs, especially their slow killing activity, have been investigated Moscardi, (1999). These strategies include the use of viral enhancin from GVs to improve the potency of NPVs. In 1959, Tanada first found that *Pseudaletia unipuncta* GV (PuGV) could improve the effectiveness of PuNPV; many GV species, including *Trichoplusia ni* GV (TnGV), Xestia c-nigrum GV (XcGV), *Helicoverpa armigera* GV (HaGV), and *Spodoptera frugiperda* GV (SfGV) have been found to improve the insecticidal activity and killing speed of some NPVs significantly (Derksen *et al.,* 1988; Goto, 1990; Gallo *et al.,* 1991; Shapiro, 2000).

The cotton leafworm, *Spodoptera littoralis* (Boisduval) is considered the most serious pest of cotton in Egypt (Ahmad, 1988; Hatem *et al.,* 2009a). It damages a wide range of vegetable crop plants, ornamentales and orchard trees (Belda *et al.,* 1994; Hatem, 2006). Indeed, *S. littoralis* is known to infest more than 112 plants belonging to 44 families (Brown and Dewhrst, 1975; Hatem *et al.,* 2009b) in

many geographical regions including Southern Spain, Middle East, and northern to central Africa (Gómez and Arroyo, 1994; Hatem, 2006). Intensive application of broad-spectrum insecticides has generated populations of *S. littoralis* that are resistant to organophosphate, carbamate and pyrethroid pesticides (El-Zemaity *et al.,* 2003). Moreover, the commercial insecticides based on *Bacillus thuringiensis* Berliner fail to adequately control *S. littoralis* (El-Zemaity *et al.,* 2003).

To decrease the environmental impact of crop protection measures, there is a recognized need to find alternatives for the control of *S. littoralis* that are compatible with integrated pest management (IPM) practices (Adán *et al.,* 1996). Baculovirus-based insecticides are considered to be effective and enviromentaly safe control agents (Burges *et al.,* 1980; Doller, 1985).

The present study aims to explore the influence of co-infection of SpliNPV and SpliGV on their insecticidal activity on *S. littoralis* larvae.

2. Materials and Methods

1. Insect

The laboratory colony of *S. littoralis* was established in 2002 from larvae collected on alfalfa (*Medicago sativa* L.) in Córdoba province, Southern Spain, and maintained at 25±2° C, 65±5% RH and a photoperiod of 16h:8h (L:D). The population is renewed annually with individuals taken from the same areas. Larvae were reared according to the method of Poitout and Bues, (1974) and fed on artificial diet containing alfalfa Vargas-Osuna, (1985).

2. The baculovirus

Two baculovirus isolates have been used baculovirus:

a- The SpliNPV was a Moroccan isolate provided by the Station Biologique of Minière Lutte (INRA) in France and obtained by multiplication in larvae of *S. littoralis*, as an aqueous suspension containing $1.7x10^9$ OB/ml.

b- The SpliGV was obtained in 1991 from *S. littoralis* larvae collected in field populations of Egypt. The inoculum was supplied as a purified aqueous suspension of OBs by the Natural Resources Institute (Kent, UK) via Prof. Dr. J. Smith. The inoculum concentration ($3.5x10^{11}$ OB/ml) was determined by 10 independent counts under phase contrast microscopy in a Hawksley counting chamber, and the suspension was stored at 4º C.

3. Bioassay

To test the enhancement of SpliNPV by SpliGV, the median lethal dose (LD50s) and median lethal times (LT50s) of SpliNPV and SpliNPV combined with SpliGV against third instar of *S. littoralis* larvae were compared. SpliNPV or SpliGV was diluted with distilled water to 0.1% Agral **(**a wetting agent), third instars were placed singly in plastic cups (32 mm diameter and 12 mm high) and fed on alfalfa leaf disc (5 mm diameter), contaminated with 3µl of SpliNPV suspension with concentrations of 511.2, 2.556, 12.780, and 63.900 OB/larvae combined with SpliGV at a constant concentration of (kSpliGV) 140.000 OB/larvae and larvae treated with water and 0.1% Agral as control. In addition, each treatment was replicated three times using 30 larvae for each replicate, thus 770 larvae were used. The alfalfa leaf discs were replaced after 48 hours by untreated diet. The larvae that did not completely eat the treated discs were discarded Vargas-Osuna *et al.* (1994). Larval mortality was recorded daily and the cause of death was determined by examination of their tissues with a phase contrast microscope.

On the other hand to test the enhancement of SpliGV by SpliNPV, the LD50s and LT50s of SpliGV and SpliGV combined with SpliNPV against third instars of *S. littoralis* larvae were compared. SpliGV or SpliNPV was diluted with distilled water to 0.1% Agral **(**a wetting agent) third instars were placed singly in plastic cups (32 mm diameter and 12 mm high) and fed on alfalfa leaf disc (5 mm diameter), contaminated with 3µl of SpliGV suspension with concentrations of $1.12x10^4$, $5.6x10^4$, $2.8x10^5$, and $1.4x10^6$ OB/larvae combined with SpliNPV at a constant concentration of (kSpliNPV) 511.2 OB/larvae and larvae treated with water and 0.1% Agral as control with three replicates, at least 30 larvae each, thus 780 larvae were used for the full experiments. The bioassay procedure was same as described above.

4. Data Analysis

The dose-mortality data were processed and analyzed with a computer program, POLO-PC Russell *et al.* (1977) based on the probit analysis method described by Finney, (1971). Median lethal doses (LD50s) were determined by linear regression analysis. The regression line equations were compared by χ^2 test for parallelism and relative potencies were estimated. The Median lethal times (LT50s) were calculated according to the method of Biever and Hostetter, (1971). Analysis of the interactions, the method used for studying the interactions between the two baculoviruses was described by Harper, (1986) for microbial products. The test χ^2 to 5% of significance is used to detect differences between observed and expected.

3. Results and Discussion

1. Combination of SpliNPV doses and constant dose of SpliGV

To determine the synergistic effect of SpliGV on biological activity of SpliNPV against third instars of *S. littoralis*, a dose-response bioassay with a constant SpliGV dose of 140.000 OB/larvae and SpliNPV concentration varying from 511.2 to 63.900 OB/larvae was conducted.

1.1. Larval mortality

In either single or combined treatment we have observed increasing in mortality with increased doses. In combined treatments, the mortality was less than in the single treatments. (Table1).

1.2. Mortality by instars

When compared every single dose with the combined of SpliNPV was observed a slight delay in the instar dead, except with the high doses.

1.3. Probit regression line and LD50s

Analysis by probit regression line revealed the following equations and LD50s (with 95% confidence limits): y=1.11x+1.88, χ^2=2.05 (2df) and 654.24 OB/larvae (102.56±1503.64) for SpliNPV; y=0.62x+2.87, χ^2=5.54 (2df) and 2662.93 OB/larvae) for SpliNPV+kSpliGV. The adjustment was acceptable using the test χ2.

1.4. Median survival time (ST50s)

In either single or combined treatments the ST50s was decreased with increasing the doses of SpliNPV with significant differences (p=0.0016). The ST50s was less in the single dose than combined dose without significant difference (Table 2).

1.5. Median lethal time (LT50s)

The LT50s was significantly affected (p=0.0043) by the doses of single and combined viruses. The LT50s for variation doses of SpliNPV was (LT50=6.80, 6.66 and 6.19 days at 2556, 12780 and 63900 OB/larvae respectively), but with combined doses (SpliNPV+kSpliGV) were (LT50=7.96 days at 12780+140000 OB/larvae and 6.97 days at 63900+140000 OB/larvae) (Table 3).

2. Combination of SpliGV doses and constant dose of SpliNPV

To determine the synergistic effect of SpliNPV on biological activity of SpliGV against third instars of *S. littoralis*, a dose-response bioassay with a constant SpliNPV dose of 511.2 OB/larvae and SpliGV concentration varying from $1.12x10^4$ to $1.4x10^6$ OB/larvae was conducted.

2.1. Larval mortality

In either single or combined treatments, mortality increased with increasing the doses. When comparing each single treatment with corresponding combined treatments, total mortality did not vary significantly, except when $5.6x10^4$ OB/larvae dose of combined treatment mortality was approximately 20% higher compared to the single. The mortality percentage of infected larvae with SpliNPV in all combined treatments were reduced by more than half compared to single treatment.The mortality of infected larval with SpliGV decreased slightly in almost combined treatments with the single treatments, except with $5.6x10^4$ OB/larvae dose (Table 4).

2.2. Mortality by instars

There was no significant difference in the mortality for different larval instars infected neither by single doses nor by combined doses of SpliGV.

2.3. Probit regression line and LD50s

Analysis by probit regression line revealed the following equations and LD50s (with 95% confidence limits): y=0.80x+1.2, χ^2=7.16 (2df) and 54838 OB/larvae for SpliGV; y=0.65x+1.67, χ^2=1.02 (2df) and 130490 OB/larvae) (72459±228740) for SpliGV+kSpliNPV. The adjustment was not acceptable using the χ^2 test.

2.4. Median survival time (ST50s)

The ST50s in all combined treatments was increased respect to their corresponding single treatments, without significant differences (p=0.8723).

2.5. Median lethal time (LT50s)

The LT50s were not significantly affected in both single and combined treatments (p=0.6428). The LT50s for SpliGV doses were (LT50=12.29 and 17.55 days at 2.8×10^5 and $1.4x10^6$ OB/larvae respectively), but with combined doses (SpliGV+kSpliNPV) were (LT50=17.25 days at $2.8x10^5$+511.2 OB/larvae and 18.60 days at $1.4x10^6$ +511.2 OB/larvae).

Several GVs were found to enhance NPV infection, and more and more NPV-insect systems were found to be improved by the same GVs (Tanada, 1959; Derksen *et al.,* 1988; Goto, 1990; Gallo *et al.,* 1991; Shapiro, 2000). In general, LD50 in our work for SpliNPV and SpliGV of *S. littoralis* larvae treated in third instar agreed with Granados, (1998) and Fernandez Vilchez, (2000). All single treatments with SpliNPV were higher than the combined treatments (SpliNPV+kSpliGV), and this showed the inhibitory effect on SpliNPV by SpliGV. The mortality refers only to the dead larvae infected with SpliNPV the decrease was very clear in all doses, with a slight delay in the development larval instar.

The LD50 of SpliNPV for *S. littoralis* larvae treated in third instar was 654 OB/larvae, this value was lower than that obtained by Granados, (1998) for the same species and baculovirus, but the colony was not the same. As known that populations came from different origins or conditions differ in susceptibility to baculovirus. The larvae were treated with doses of SpliNPV and constant SpliGV dose showed poor probit regression line. The worse adjustment of mortality data was probably due to effect of the combined, as happened with baculovirus and

adjuvants synergistic from the group optical brighteners Granados, (1998).

The LD50 of combined treatment was 2663 OB/larvae, greater than the single treatment with SpliNPV, which indicated that the larvae were less susceptible to infection by SpliNPV when treated with both viruses. Also the slope of the regression line was lower in the combined treatment than in the single SpliNPV treatment, which means a greater heterogeneity in the larval response. This makes the interference of the SpliGV on the SpliNPV infection, and differs depending on the SpliNPV dose. Thus, from the dose equivalent 105 OB/larvae (value well below the LD50) the interaction grows with increasing dose.

Both the ST50s and LT50s of dead larvae by SpliNPV infected were higher in combined treatments than in the single treatments, with significant differences in the case of the LT50s, with about 1 day difference. The results confirmed the antagonism of SpliNPV by SpliGV that indicated the interaction should be related to competition during the infectious process of both viruses. Probably the existence of SpliGV OBs interfered with the SpliNPV infection, for that the antagonism had not been directly related to the SpliNPV dose.

In other investigation with the NPV and GV of *Pseudaletia unipuncta* found that a chemical factor associated with the PsunGV OBs, acted as a synergistic factor to improve the infection of PsunNPV Tanada and Hukuhara (1971). In this case the level of synergism was independent on the NPV dose, but only on the dose of GV (Tanada *et al.,* 1980; Shapiro, 2000). Our results showed that the infection by the SpliGV cause significant interference in the infections process of SpliNPV, although the SpliNPV infection have a shorter time than the SpliGV. Therefore, the interference happened during the early stages of infections process, probably during primary infection of midgut columnar cells Harper, (1986).

For explain the mechanism of interference in infections combined two baculovirus, Maracajá, (1995) suggested that the OBs virions released from GV was smaller than the virions of NPV, for that it is easily overcome the barrier of the peritrophic membrane in the midgut and (Ritter and Tanada, 1978) reported that once the nucleocapsids of the GV in the midgut columnar cells can transform the structure, so that the larvae were not susceptible to infection by the NPV. Another possibility was that primary infection by both viruses in the midgut columnar cells occurring in the same time, but once it is inside, the nucleocapsids of GV compete favourably to reach the nucleus and initiate DNA replication phase.

The treatment with single doses of SpliGV gave higher mortality than in combined doses. Our results were agree with that obtained by (Calderon, 1990; Fernandez Vilchez, 1998; Granados, 1998). The infection rate of SpliGV was lower than SpliNPV, due to the fewer infected tissues, allowing a longer survival of the larvae to avoid affecting other vital tissues. There was no available histological study of this virus, but it is likely to be a Type 1 GV Federici, (1997). Type 1 GV infect the midgut columnar cells and subsequently the body fat, without affecting the tracheal matrix, hemocytes or epidermis, tissues themselves were susceptible to infection by the SpliNPV, as has been demonstrated by Maracajá, (1995).

The larval mortalities in the combined treatments (SpliGV+kSpliNPV) did not change significantly from the corresponding single SpliGV dose. Nevertheless, if we consider only the dead larvae infected with the SpliGV, there were reduction in the mortality rates for all doses in comparison with their single treatments.

Neither ST50s nor LT50s were variations in the dead larval instars. Our resulted was agree with Guo *et al.* (2007) who reported that the LT50s in the single treatments was not significant shortened in the combined treatment for *Xestia c-nigrum* Granulovirus (XcGV) and *Spodoptera litura* Nucleopolyhedrovirus (SlNPV) in *Spodoptera litura* larvae.

The LD50 of the SpliGV for *S. littoralis* L3 larvae was 54.838 OB/larvae, but the LD50 of SpliNPV was lower than that obtained by Granados, (1998) for the same species and baculovirus. The LD50 of the combined treatment (SpliGV+kSpliNPV) was 130.490 OB/larvae, slightly higher than the single treatment. The regression line refers to the single SpliGV treatment was a poor adjustment, which corresponds with other studies of biological activity of the baculovirus Fernandez Vilchez, (2000). The GV have characterized biological activity by heterogeneity in individual larval response the reason may be due to the long infection period. When the two regression lines were compared both treatments did not differ significantly in the larval response to the SpliGV. This confirmed the absence of interference of SpliNPV infection on SpliGV.

The interactions analyses of both baculovirus were antagonism to all treatment, regardless of the used doses, which showed a significant effect of interference between both viruses. Obtained data revealed that the SpliGV interfered significantly with SpliNPV infections, this antagonistic interference was greater when increasing the dose of SpliGV. On the other hand, the SpliNPV did not cause any significant change in infections caused by the SpliGV.

Table 1. Mortality of *S. littoralis* larvae treated in third instar with single treatments of SpliNPV and SpliNPV combined with constant dose of SpliGV.

Treatment	N	Mortality	
		%	% Abbott
Control	87	2.30	0
SpliNPV 1	89	49.44	48.25
SpliNPV 2	82	69.51	68.79
SpliNPV 3	88	94.32	94.18
SpliNPV 4	88	98.86	98.83
SpliNPV 1+kSpliGV	86	38.37	36.92
SpliNPV 2+kSpliGV	85	41.18	39.79
SpliNPV 3+kSpliGV	81	74.07	73.46
SpliNPV 4+kSpliGV	84	79.76	79.28

SpliNPV 1: 511.2 OB/larvae. SpliNPV 2: 2.556 OB/larvae. SpliNPV 3: 12.780 OB/larvae. SpliNPV 4: 63.900 OB/larvae. kSpliGV: 140.000 OB/larvae. N=Number of treated larvae.

Table 2. Survival time of *S. littoralis* larvae treated in third instar in single treatments with variation doses of SpliNPV and combined with a one dose of SpliGV.

Treatment	% Mortality	Median survival time (days)
SpliNPV 1	49.44	10.58 a
SpliNPV 2	69.51	7.40 b
SpliNPV 3	94.32	7.15 b
SpliNPV 4	98.86	6.59 b
SpliNPV 1+kSpliGV	38.37	11.69 a
SpliNPV 2+kSpliGV	41.18	8.13 b
SpliNPV 3+kSpliGV	74.07	7.88 b
SpliNPV 4+kSpliGV	79.69	6.98 b

SpliNPV 1: 511.2 OB/larvae. SpliNPV 2: 2.556 OB/larvae. SpliNPV 3: 12.780 OB/larvae. SpliNPV 4: 63.900 OB/larvae. kSpliGV: 140.000 OB/larvae. Means followed by the same letter are not significantly different (LSD, P=0.05).

Table 3. Lethal time of *S. littoralis* larvae treated in third instar in single treatments with variation doses of SpliNPV and combined with a one dose of SpliGV.

Treatment	% Mortality	Median lethal times (days)
SpliNPV 2	69.51	6.80 bc
SpliNPV 3	94.32	6.66 bc
SpliNPV 4	98.86	6.19 a
SpliNPV 3 + kSpliGV	74.07	7.96 c
SpliNPV 4 + kSpliGV	79.76	6.97 b

SpliNPV2: 2.556 OB/larvae. SpliNPV 3: 12.780 OB/larvae. SpliNPV 4: 63.900 OB/larvae. kSpliGV: 140.000 OB/larvae. Means followed by the same letter are not significantly different (LSD, P= 0.05).

Table 4. Mortality of S. littoralis larvae treated in third instar with single treatments of SpliGV and SpliGV combined with constant dose of SpliNPV.

Treatment	Nº	Mortality	
		%	% Abbott
Control	88	3.41	0
SpliGV 1	87	39.08	36.93
SpliGV 2	87	41.38	39.31
SpliGV 3	85	71.76	70.76
SpliGV 4	87	90.80	90.47
SpliGV 1 + kSpliNPV	86	27.90	25.35
SpliGV 2 + kSpliNPV	85	43.52	41.53
SpliGV 3 + kSpliNPV	88	55.68	54.12
SpliGV 4 + kSpliNPV	87	78.16	77.39

SpliGV1: $1.12x10^4$ OB/larvae. SpliGV2: $5.6x10^4$ OB/larvae. SpliGV3: $2.8x10^5$ OB/larvae. SpliGV4: $1.4x10^6$ OB/larvae. kSpliNPV: 511.2 OB/larvae. Nº=Number of treated larvae.

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