Potentiation by a Granulosis Virus of Gypchek, the Gypsy Moth (Lepidoptera: Lymantriidae) Nuclear Polyhedrosis Virus Product[1]

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Abstract Gypchek and a granulosis virus were applied in various combinations against several gypsy moth instars under field conditions, and a Blankophor BBH + Gypchek treatment was included as a comparison of virus enhancers. The residual effects of the treatments were determined over a 3-wk period. The addition of *Helicoverpa armigera* granulosis virus at a 1:100 dilution to Gypchek resulted in an approximate 10-fold increase in observed mortality, while the addition of Blankophor BBH at 1% resulted in an approximate 100-fold increase in observed mortality. The addition of *Helicoverpa armigera* granulosis virus at a 1:1000 dilution resulted in no consistent increase in recorded mortality, and the 1:100 granulosis virus dilution applied alone was inactive against gypsy moth. The residual activity of Gypchek was little enhanced by the addition of the granulosis virus at either dose.

Key Words *Lymantria dispar,* baculovirus, adjuvant, Blankophor BBH, virus-enhancement, forest pest

Gypchek® (USDA Forest Service, Washington, DC), a product with the *Lymantria dispar* multienveloped nuclear polyhedrosis virus as the active ingredient, is registered by the USDA Forest Service with the U. S. Environmental Protection Agency as a general use bioinsecticide for aerial and ground application against the gypsy moth, *Lymantria dispar* (L.) (Reardon et al. 1996). Successful field trials with Gypchek incorporated with the commercially-produced Carrier 038 (Novo Nordisk, Franklinton, NC) (Reardon et al. 1996, Webb et al. 1998b,c) and environmental concerns over the effects of non-specific insecticides applied to forest ecosystems have stimulated interest in the use of Gypchek (Reardon et al. 1996). The addition of certain stilbene-derived optical brighteners enhanced the performance of this virus in the laboratory (Shapiro and Robertson 1992) and field (Webb et al. 1994a,b). Additional field work in 1996 (Webb et al. 1998a) compared properties of this virus with standard insecticides using the "bugs-in-bags" approach developed by D'Amico and Elkinton (1995) and found that the optical-brightener-enhanced virus gave control statistically equiva-

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lent to the best standard insecticides for at least 7 d after treatment. A different approach to enhancing the performance of baculoviruses involves the use of certain proteins coded for by various granulosis viruses. Tanada (1959) established that the activity of a nuclear polyhedrosis virus against an armyworm can be synergized by the co-application of the *Pseudaletia unipuncta* granulosis virus. It was later established that such synergism is due to enhancin proteins present in the granules of granulosis viruses (Gijzen et al. 1995), including the granulosis virus of the cotton bollworm, *Helicoverpa armigera* (Hübner) (Granados 1990). Shapiro (in press) found in laboratory studies that the co-application of the *H. armigera* granulosis virus resulted in the potentiation of the *Lymantria dispar* nuclear polyhedrosis virus against the gypsy moth.

In the present study, we used a bugs-in-bags approach to determine the extent that co-application of *H. armigera* granulosis virus and Gypchek would induce viral death of gypsy moths under field conditions. We applied Gypchek and the granulosis virus in various combinations against several gypsy moth instars, and included a Blankophor BBH + Gypchek treatment as a comparison of virus potentiators. The residual effects of the treatment combinations were determined over a 3-wk period.

Materials and Methods

Insect colony and virus. Gypsy moth larvae from Newark, DE (USDA-ARS Newark stock culture) were reared in 230-ml paper cups on standard gypsy moth artificial diet (Bell et al. 1981). Larvae were reared 100 per cup for second instars, 50 per cup for third instars, and 25 per cup for fourth instars. The Gypchek virus-inoculum used was the Hamden isolate LPD-226, with dosages calculated from polyhedral inclusion body counts quantified by visual counting in a hemacytometer. The cotton bollworm granulosis virus, obtained initially from J. J. Hamm (USDA-ARS, Tifton, GA), was produced at Beltsville from corn earworm, *Helicoverpa zea* (Boddie), larvae and was extracted from virus-infected and virus-killed larvae by the method of Shapiro et al. (1981). Corn earworm larvae were blended separately as a 1:10 stock suspension (1 g larval tissue per 10 ml water). Granulosis viral inclusion bodies were not counted due to their small size. The 1:10 stock suspension was diluted in distilled water to produce virus dilutions of 1:100 and 1:1000 (wt/wt).

Field plots. One hundred groups of 5 oak branch tips, primarily pin oak, *Quercus palustris* Muenchh., accessible from the ground, were marked along a forest edge abutting an open field in the Cedar Swamp Wildlife Management Area northeast of Smyrna, DE, in the spring of 1999. Each group of tips was separated by at least 2 m. There was no evident natural gypsy moth population in this forest. A randomized complete block design was used consisting of 10 blocks, each with all 10 treatments randomly assigned (=100 sites).

Host plant phenology. Leaf expansion was determined at the end of each evaluation period by removing all leaves from the branch tips taken from five of the plots (the same plots were used for all time periods), as the gypsy moth larvae were removed from the bags, and measuring the areas of these leaves using a Li-cor LI-3100 area meter (Li-cor, Inc., Lincoln, NE). The leaves from the 2-wk evaluation averaged (±SEM) 36.6 ± 11.6 cm^2 and were considered fully expanded. Leaf area averaged (±SEM) 9.3 ± 3.9 cm^2 at the time of treatment, indicating 25% leaf expansion based on the 3-wk measure. Leaf expansion 1 wk after treatment averaged 54%.

Table 1. Composition of the treatments evaluated in this study

Treatment no.	Gypchek PIBs*	GV** dilution	Bond sticker	Blankophor BBH
1	1×10^{12}	—	2%	—
2	1×10^{11}	—	2%	—
3	1×10^{11}	1:100	2%	—
4	1×10^{11}	1:1000	2%	—
5	1×10^{10}	—	2%	—
6	1×10^{10}	1:100	2%	—
7	1×10^{10}	1:1000	2%	—
8	—	1:100	2%	—
9	1×10^{10}	—	2%	0.5%
10	—	—	2%	—

* Number of polyhedral inclusion bodies per 378 liters final tank mixture (100 gal).
** Cotton bollworm granulosis virus.

Treatments. Treatments 1 through 10, given in Table 1, consisted of various mixtures and dilutions of Gypchek, cotton bollworm granulosis virus, and Blankophor BBH, with all treatments containing 2% Bond (Loveland Industries, Greeley, CO) sticker. All treatments were sprayed to runoff on 1 May 1999. Treatments were applied using 373-ml hand-held trigger-pump sprayers (Delta Industries, Philadelphia, PA) to the *in situ* branch tips and were allowed to dry for approximately 1 h prior to the encagement of the first cohort of larvae. Each branch tip received a bag consisting of 60 × 60 cm squares of organza cloth seamed to make a bag as per Webb et al. (1998a). Three bags were placed over branch tips at each treatment site for the 1-h evaluation (cohorts 1-3), one cohort for each of the second, third, and fourth instars (=1 bag per site for each cohort, 300 bags total). One additional bag containing second instars was added to each site 1 wk after treatment (cohort 4), and an additional bag containing second instars was added 2 wks after treatment (cohort 5) for a total of 5 bags per site, or 500 bags total for the experiment. Ten gypsy moth larvae were placed in each bag, which was then tied off. Larvae were left in the bags for 1 wk, after which the bagged tips were removed and taken to the laboratory, where all larvae were removed from the bags and placed on artificial diet (Bell et al. 1981) in 30-ml plastic cups with paper lids, one larva per cup. The rearing cups were held on shelves in a wooden outdoor insectary (368 cm long, 215 cm wide, 92 cm deep, with hardware cloth covering the front to allow natural conditions of light, temperature, and humidity but not rain) at the Beltsville Agricultural Research Center, Beltsville, MD. All larvae in the insectary were observed every 2 to 3 d for mortality until death, pupation, or for 52 d. Dead larvae were labeled by date-of-death and frozen to await necropsy. Wet mounts of tissue samples from all cadavers were examined under 400X for the presence of nuclear polyhedrosis virus inclusion bodies. If determinations could not be made with certainty using the above procedure, smears of tissue

were fixed over a flame and dilute Giemsa solution was added to stain the polyhedral inclusion bodies (Glaser 1915) and then examined for inclusion bodies under oil emersion at 1000×.

Statistical methods. Mortality data from the 1-h residue study were analyzed by a mixed-model analysis of variance (ANOVA) (SAS 1996; PROC MIXED). When treatment effects were significant, means were separated at a comparison-wise error rate of 0.05 using the least significant differences (LSD) procedure (SAS Institute 1996). A log ($x + 1$) transformation was used to normalize and stabilize the variance. The 1- and 2-wk larval placements were analyzed by ANOVA using the General Linear Models (GLM) procedure (SPSS, Inc. 1997). When treatment effects were significant, means were separated at a comparison-wise error rate of 0.05 using the least significant differences (LSD) procedure (SPSS, Inc. 1997). An arcsine-square root transformation was used on all percentage data for the 1- and 2-wk larval placements. All values that were analyzed using transformations are presented in the tables back-transformed. For the days-to-death data, attempts to normalize and stabilize the variance by transformations were unsuccessful, so nonparametric methods were used in lieu of a parametric ANOVA. The data were subjected to two series of Kruskal-Wallis tests, treatment by instar and instar by treatment. Dunn's method was used to isolate the group or groups of treatments and instars that differed from the others (SPSS, Inc. 1997).

Results

Virus-induced mortality of larvae fed on 1-h residues. Mortality caused by virus varied by treatments and by instar (Table 2). The effects were significant for treatment ($F = 79.88$; df = 9,270; $P < 0.0001$) and instar ($F = 6.82$; df = 2,270; $P = 0.0013$), with the treatment*instar interaction non-significant ($F = 0.89$; df = 18,270; $P = 0.60$). The 3 Gypchek treatments exhibited an appropriate dose-response, with mortality across instars ranging from 30% for Treatment 5 (10^{10} Gypchek) to 87% for Treatment 1 (10^{12} Gypchek). The addition of the granulosis virus at the 1:100 dilution led to a 10-fold increase in virus-induced mortality, with 10^{11} Gypchek co-applied with the granulosis virus at 1:100 statistically equivalent to 10^{12} Gypchek, and 10^{10} Gypchek co-applied with the granulosis virus at 1:100 statistically equivalent to 10^{11} Gypchek. However, 10^{10} Gypchek co-applied with Blankophor BBH was also statistically equivalent to 10^{12} Gypchek, for an apparent 100-fold increase in effectiveness. The co-application of Gypchek with 1:1000 dilution granulosis virus gave no apparent boost to Gypchek efficacy, and Treatment 8 (1:100 dilution granulosis applied alone) was statistically equal to the surfactant control (Treatment 10). Separation of instar means by LSD revealed that third-instar larvae averaged 44% mortality, which was statistically less than fourth-instar larvae (=53%), or second-instar larvae (=51%). Second and fourth instar mortality were statistically equal.

Virus-induced mortality of larvae fed 1- or 2-wk residues. There were significant differences among the different levels of treatments for 1-wk residues ($F = 15.55$; df = 9,81; $P < 0.001$) but not 2-wk residues ($F = 0.90$; df = 9,81; $P = 0.5$) (Table 3). Only the mortality seen for the 3 most potent treatments (Treatments 1, 3 and 9) were significantly higher than controls. Treatment 9 (10^{10} polyhedral inclusion bodies + 1% BBH) was significantly higher than the other treatments, but still averaged only 38%. The mortality seen for treatments, Treatments 1 and 3 gave 8% and 7% virus-induced mortality, respectively. Only a few scattered larvae died from virus in the remaining

Table 2. One hour effectiveness: Transformed least square means for mortality of gypsy moth larvae (all instars) placed in bags on virus-treated oak foliage 1 hr post treatment in the Cedar Swamp Wildlife Management Area, DE, in 1999. Gypchek tank mixed with granulosis virus (GV) or Blankophor BBH (BBH) compared with Gypchek treatments lacking GV or BBH; data for the three combined instars (column 2) with observed mortality (SEM) for each instar (columns 3-5)

	Mortality (%)			
Treatment (per 378 liters)	All instars*	(2nd instar)**	(3rd instar)**	(4th instar)**
Trt 1, 10^{12} Gypchek	0.27 (87)a	90 (2.8)	83 (4.7)	89 (3.4)
Trt 2, 10^{11} Gypchek	0.20 (60)b	69 (8.8)	46 (8.3)	66 (9.9)
Trt 3, 10^{11} Gyp. + 1% GV	0.27 (87)a	87 (4.2)	85 (4.3)	90 (3.3)
Trt 4, 10^{11} Gyp. + 0.1% GV	0.21 (64)b	66 (8.2)	58 (8.4)	69 (6.4)
Trt 5, 10^{10} Gypchek	0.12 (34)c	33 (9.6)	22 (6.6)	48 (7.1)
Trt 6, 10^{10} Gyp. + 1% GV	0.18 (54)b	62 (9.6)	47 (9.8)	52 (10.0)
Trt 7, 10^{10} Gyp. + 0.1% GV	0.11 (30)c	35 (9.3)	20 (5.1)	35 (7.2)
Trt 9, 10^{10} Gyp. + 0.5% BBH	0.26 (82)a	86 (4.1)	75 (8.2)	84 (5.2)
Trt 8, 1% GV	0.02 (5)d	0 (0)	5 (1.7)	9 (4.0)
Trt 10, distilled water	0.001 (0.3)d	0 (0)	1 (1.0)	0 (0)
Std err of LS mean:	0.01			

* Two way analysis of variance, data for all instars combined, least square means. Values are transformed (log x + 1) (with average mortality for the 3 instars in parenthesis). Means within the same column followed by a different letter were significantly different at a comparison-wide error rate of 0.05 when analyzed by the GLM procedure followed by LSD separation of means (SAS 1996).

** Observed mortality (SEM) for the indicated instars.

treatments. Only scattered virus-induced mortality was seen for gypsy moth larvae fed on 2-wk residues.

Patterns of virus-induced larval mortality. Kruskal-Wallis one-way analysis of variance showed significant differences in days-to-death among instars across all treatments, with H-values ranging from 10.88 to 65.81 (df = 2; $P < 0.004$). Across all treatments, younger larvae died sooner than older larvae, and in nearly every treatment, there were stepwise increases in days-to-death with each later instar (Table 4). In addition, Kruskal-Wallis one-way analyses of variance showed significant differences in days-to-death among treatments across all instars, with H-values ranging from 38.44 to 45.23 (df = 7, $P < 0.001$). For second instars, only the extremes (Treatments 6 and 9) differed significantly. For later instars, those for treatments 1, 3 and 9 died sooner than those in treatments 2, 4 and 6, which in turn died sooner than those in treatments 5 and 7. Treatment 9 (containing Blankophor BBH) larvae consistently died fastest.

Discussion

Shapiro (in press) evaluated 2 granulosis viruses as enhancers for the gypsy moth nuclear polyhedrosis virus. He found that the *Helicoverpa armigera* granulosis virus

Table 3. Residual effectiveness: Mortality of second-instar gypsy moth larvae placed in bags 1 wk post treatment, and untransformed mortality values for larvae placed 2 wk, on treated oak foliage in the Cedar Swamp Wildlife Management Area, DE, in 1999. Gypchek mixed with granulosis virus (GV) or Blankophor BBH (BBH) compared with Gypchek treatments lacking GV or BBH

Treatment (per 378 liters)	Mortality (%)	
	1-wk residue*	2-wk residue**
Trt 1, 10^{12} Gypchek	13b (8)	2
Trt 2, 10^{11} Gypchek	4cd (2)	1
Trt 3, 10^{11} Gypchek + 1% GV	11bc (7)	2
Trt 4, 10^{11} Gypchek + 0.1% GV	2d (1)	1
Trt 5, 10^{10} Gypchek	0d (0)	0
Trt 6, 10^{10} Gypchek + 1% GV	0d (0)	1
Trt 7, 10^{10} Gypchek + 0.1% GV	0d (0)	0
Trt 9, 10^{10} Gypchek + 0.5% BBH	36a (38)	2
Trt 8, 1% GV	0e (0)	0
Trt 10, distilled water	0e (0)	0
Std err of LS mean:	8.1	

* Least square means of arcsine-square root transformed data (with backtransformed values). Means within the same column followed by a different letter were significantly different at a comparison-wise error rate of 0.05 when analyzed by the GLM procedure followed by LSD separation of means (SPSS Inc. 1997).

** Untransformed means. Treatment effects were not significantly different at $P = 0.05$ when analyzed by the GLM procedure which prevented LSD separation of means (SPSS Inc. 1997).

applied alone had no detrimental effect on gypsy moth but, in combination with the gypsy moth nuclear polyhedrosis virus, reduced both the LC_{50} (by as much as 300-fold) and the LT_{50} (by up to 18%) for the nuclear polyhedrosis virus. The purpose of this study was to determine the extent to which the enhancement of Gypchek by the co-application of *Helicoverpa armigera* granulosis virus reported by Shapiro (in press) would be expressed under field conditions, how this potentiation is affected by dose and instar, and how the potentiation induced by the granulosis virus compares with that seen for Blankophor BBH. Additionally, we elucidated the residual activity of Gypchek and *Helicoverpa armigera* granulosis virus combinations. The mode of action underlying the synergism of Gypchek, by either the granulosis virus or by Blankophor BBH, was not the subject of this field study, and is discussed elsewhere (Shapiro, in press). However, the effects caused by the two enhancers may well be due to unrelated mechanisms.

The addition of *Helicoverpa armigera* granulosis virus at 1% to Gypchek resulted in a 10-fold increase in observed mortality compared with a 100-fold increase in observed mortality recorded for Treatment 9 (10^{10} inclusions of Gypchek with 1% BBH). Webb et al. (1996) gave a favorable economic assessment for the tank-mixing

Table 4. Pattern of NPV-induced death: means ± SE for the time-to-death (days) due to virus infection of gypsy moth larvae placed in bags on treated oak foliage 1 hr post treatment in the Cedar Swamp Wildlife Management Area, DE, in 1999. Gypchek tank mixed with granulosis virus (GV) or Blankophor BBH (BBH) compared with Gypchek treatments lacking GV or BBH; for the indicated instars

Treatment (per 378 liters)	Days to death		
	(2nd instar)	(3rd instar)	(4th instar)
Trt 1, 10^{12} Gypchek	20.6 ± 0.8 ab,x	22.3 ± 1.0 b,y	24.3 ± 1.9 bc,z
Trt 2, 10^{11} Gypchek	21.0 ± 1.3 ab,x	23.7 ± 2.4 ab,y	26.1 ± 1.7 ab,z
Trt 3, 10^{11} Gypchek + 1% GV	20.6 ± 1.3 ab,x	21.8 ± 1.2 b,y	24.3 ± 1.8 bc,z
Trt 4, 10^{11} Gypchek + 0.1% GV	21.9 ± 3.4 ab,x	23.4 ± 2.0 ab,y	25.5 ± 1.8 abc,z
Trt 5, 10^{10} Gypchek	21.3 ± 2.8 ab,x	25.2 ± 2.9 a,y	27.4 ± 3.2 a,y
Trt 6, 10^{10} Gypchek + 1% GV	22.5 ± 2.2 a,x	25.4 ± 2.9 ab,y	27.4 ± 3.2 ab,z
Trt 7, 10^{10} Gypchek + 0.1% GV	22.1 ± 1.8 ab,x	23.9 ± 3.7 ab,xy	25.3 ± 3.6 abc,y
Trt 9, 10^{10} Gypchek + 0.5% BBH	19.8 ± 0.3 b,x	21.7 ± 2.0 b,y	23.6 ± 1.4 c,z

Means in the same column followed by the same letter (a-c) do not differ significantly; means in the same row followed by the same letter (x-z) do not differ significantly (Dunn's Method; $P < 0.05$) (SPSS Inc. 1997).

of Blankophor BBH with Gypchek for use by arborists. The order-of-magnitude superior enhancement effect recorded in the present study for Blankophor BBH compared to that seen for the granulosis virus indicates that Blankophor BBH should be a more economical enhancing agent for Gypchek than *Helicoverpa armigera* granulosis virus; however, the addition of *Helicoverpa armigera* granulosis virus to Gypchek may appeal to consumers seeking an "all natural" control system. A potentially more practical approach to utilizing the granulosis enhancing protein might be the use of the enhancing protein in the genetic improvement of Gypchek, an approach to baculovirus improvement suggested by Granados and Corsaro (1990). If the addition of the gene coding for the enhancen protein led to a 10-fold increase in field activity of Gypchek, without the addition of a costly adjuvant, Gypchek could be applied at a lower dosage without sacrificing efficacy. Our results, together with those of Shapiro (in press), suggest that *Lymantria dispar* nuclear polyhedrosis virus would be a prime candidate for genetic improvement by the insertion of a gene coding for an enhancin protein.

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