EFFECTS OF MIXED INFECTIONS WITH GV AND NPV ON THE BIOLOGY OF THE EGYPTIAN COTTON LEAFWORM *SPODOPTERA LITTORALIS*

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ABSTRACT

The effects of infecting larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* with a mixture of GV and NPV were studied. The viruses were presented simultaneously to late third-early fourth instar larvae at the minimum concentration of each known to cause 100% mortality. The mixture of GV and NPV caused a hybrid infection; both viruses multiplying within the same larvae. Survival of larvae following mixed infection was longer than that following infection with NPV alone, and shorter than that following infection with GV alone. Although some of the larvae infected with both viruses survived for up to 17 days longer than it took for control larvae to pupate, the mean food consumption of the former was slightly less than for the latter. Also, ecdysis was delayed in infected larvae as compared to uninfected controls; 79% failed to reach larval stage VI (final instar) and 10% died in larval stage IV.

INTRODUCTION

The work presented here resulted from the isolation and identification of a granulosis virus (GV) infecting larvae of the Egyptian cotton leafworm, *Spodoptera littoralis* (Hunter-Fujita *et al.*, 1990). This virus, which had not been described previously, was isolated from field collected larvae. Some of the sites from which the GV was isolated had been sprayed with *S. littoralis* MNPV in field trials to determine the efficacy of the latter as a biopesticide (Jones *et al.*, 1994). Others had not been sprayed and were far removed from the vicinity of those that had. Subsequent examination of the spray formulation revealed that certain batches were contaminated with GV.

Although mixed infections are not uncommon in nature, the implications of their combination on the infection process are still unclear.

As both viruses used in the present study readily infect *S. littoralis* it was imperative to determine whether the presence of the GV was affecting the outcome of infection with the NPV and, if so, whether this was beneficial or deleterious. Tanada (1959), for example, described synergism between an NPV and the Hawaiian Strain of a GV of *Pseudaletia unipuncta*, but failed to observe the same phenomenon with the Oregon Strain of GV (1968).

Consequently a series of experiments was initiated to compare the effects of single and mixed infections with *S.* littoralis NPV and GV on larval growth, food consumption and survival.

MATERIALS AND METHODS

Virus

Both viruses, namely *Spodoptera littoralis* MNPV and *S. littoralis* GV, were derived from virus passaged from field isolates. They were purified by sucrose density gradient centrifugation and suspended in Nanopure water (NH_20). The NPV had been cloned in larvae by limiting dilution.

Rearing of insects

Larvae of *S. littoralis* were reared at 25-26°C, first on fine diet and then, from late third instar, on coarse diet (MCKINLEY *et al.*, 1984).

Inoculation of insects

Following a 24 hour starvation period, larvae weighing between 35-50 mg (third instar) were each presented with a diet plug impregnated with 2 μl of virus (NPV, GV, or NPV+GV) at a dilution of each just sufficient to cause 100% mortality. Control larvae received diet plugs impregnated with sterile NH_20. Larvae that consumed the entire food plug within 24 hours were transferred to individual jampots each containing a cube of uninfected coarse diet. They were monitored daily for signs of infection, until death or emergence as moths. Daily measurements were made of head capsule size, larval weight, frass production and food consumption.

Identification of virus

Dead insects were harvested individually and homogenised in NH_20. Samples were examined by scanning electron microscopy and the viruses identified by their morphology. The use of restriction endonuclease (RE) analysis of DNA extracts of larval homogenates was investigated as an alternative method for identifying type of virus.

RESULTS

Identification of virus in mixed infections

As observed by electron microscopy, both viruses were identifiable in all but one larva receiving the mixed inoculum. In most cases GV predominated but in one sample, that derived from one of the earliest larvae to die (8 days post infection), NPV only, was detected. Regardless of time of death and significant signs of GV infection, all other samples contained some NPV. The ratios of GV:NPV varied, with generally more NPV in samples derived from larvae dying within 8 days, and more GV in those derived from larvae dying after this period (Table 1).

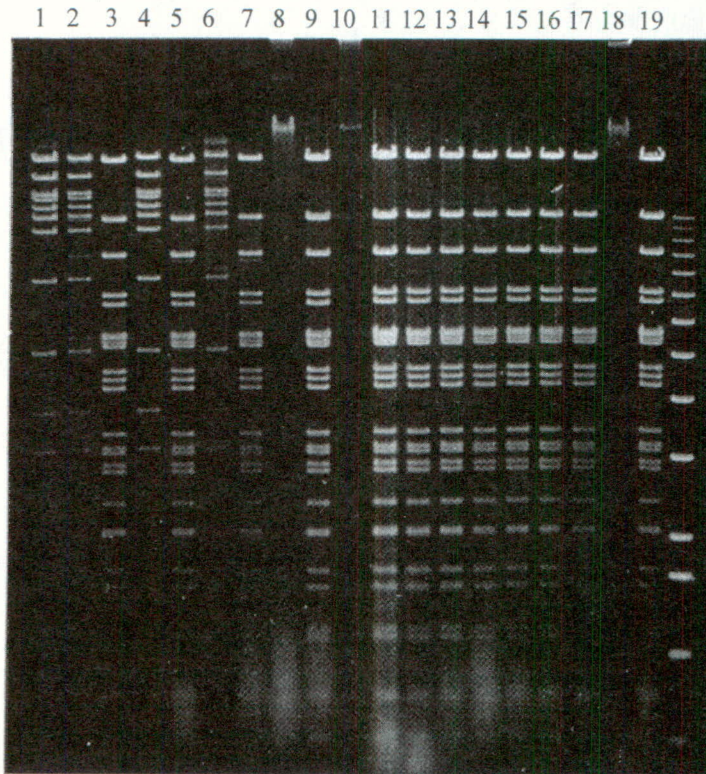
Table 1. Identification by scanning electron microscopy of type of virus in individual larvae following inoculation with GV and NPV

Day of death (post inoculation)	Type of virus NPV	Type of virus GV	Relative proportion of each virus
5	+	+	NPV > GV
6	+	+	NPV = GV
7	+	-	NPV only
7	+	+	NPV = GV
7	+	+	NPV = GV
8	+	+	NPV < GV
8	+	+	NPV < GV
9	+	+	NPV < GV
12	+	+	NPV < GV
16	+	+	NPV < GV
16	+	+	NPV < GV
17	+	+	NPV < GV
17	+	+	NPV < GV
18	+	+	NPV < GV
19	+	+	NPV < GV
20	+	+	NPV < GV
23	+	+	NPV < GV
23	+	+	NPV < GV
24	+	+	NPV < GV

RE profiles of virus DNA extracted from larvae receiving a mixture of NPV and GV are shown in Fig. 1. Profiles characteristic of the NPV DNA were only detected in the first few larvae to die. Of these, only one appeared to contain a higher proportion of NPV to

GV. The RE profiles of virus extracted from larvae surviving for longer periods post infection were exclusively those of the GV.

Fig. 1. Restriction endonuclease (*Hin* dIII) profiles of virus DNA extracted from individual larvae inoculated with a mixture of NPV and GV (lanes 2 - 7, 9, 11 - 17), NPV alone (lane 1) or GV alone (lane 19). Larvae died on days 5 (lane 2); 6 (lane 3); 7 (lanes 4, 5); 8 (lanes 6,7); 9 (lane 9); 12 (lane 11); 16 (lane 14); 17 (lane 15); 18 (lane 16); 19 (lane 17). Lane 2 contains the profiles of both viruses.



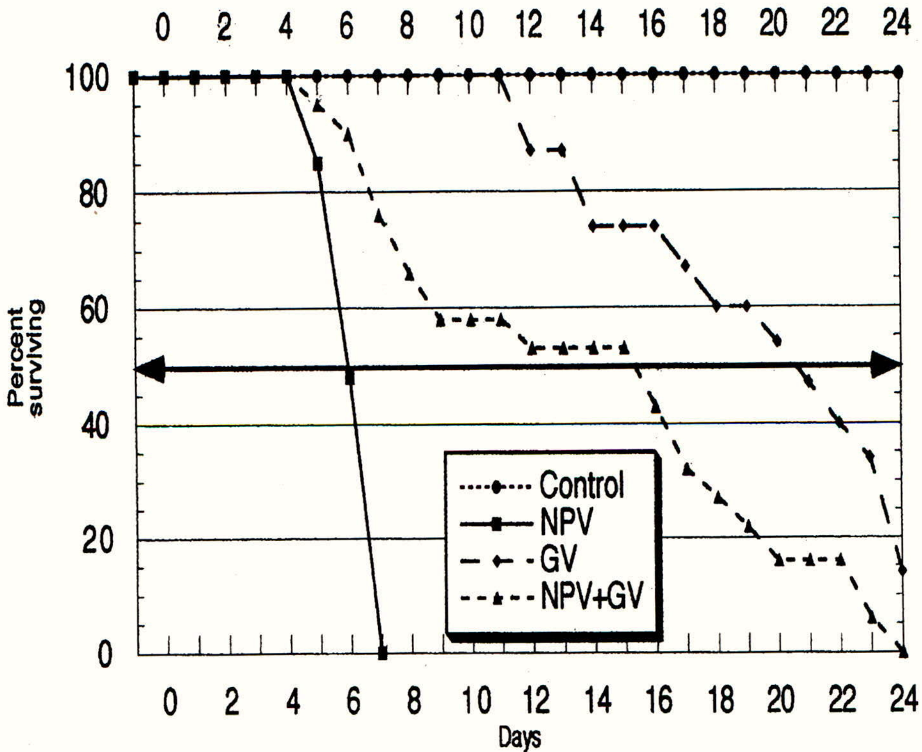
Signs of infection

Signs of infection differed depending on whether larvae were infected with NPV or GV, either alone or in combination. These included differences in colour, number of moults and behaviour patterns. Larvae infected by both viruses exhibited some characteristics of each type of single infection. Their size at death tended to be similar to that of larvae infected with NPV (small) while their behaviour was similar to that of larvae infected with GV. They neither climbed to the top of their containers before death nor liquefied shortly afterwards.

Effect of virus infection on larval survival

For mixed infections the duration of larval survival was longer than for NPV-infected individuals (4-7 days post infection) but shorter (6-24 days post infection) than for those infected with GV (12-25 days post infection). The majority of the doubly-infected larvae died between 10 and 20 days after inoculation with 50% surviving up to day 15 (Fig. 2).

Fig. 2. Comparison of percentage larval survival following infection with NPV. GV, or NPV + GV. Approximately 20 larvae were used for each treatment.

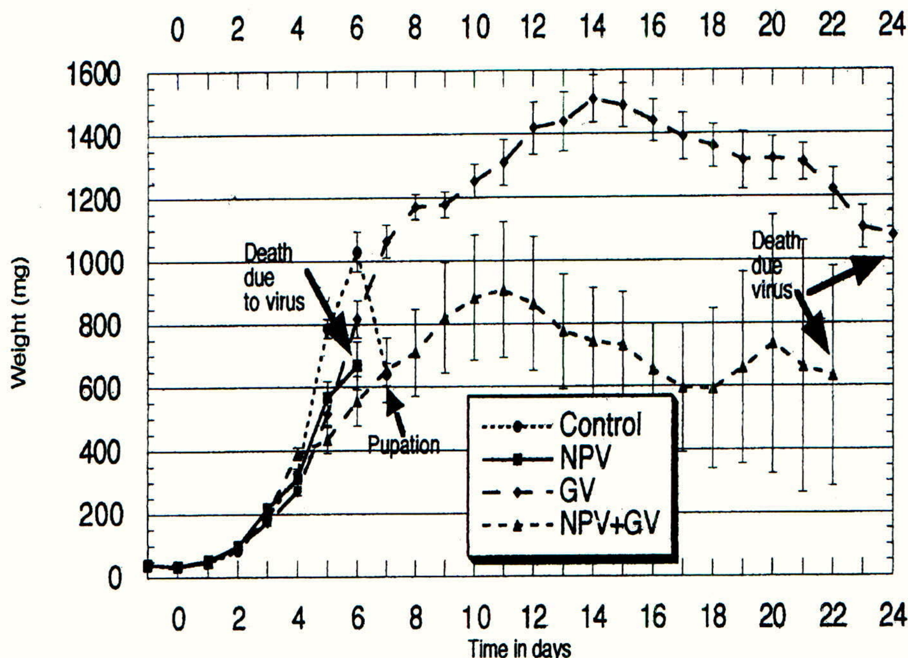


Effect of virus infection on weight gain

The weights of test and control larvae were almost identical up to 4 days post infection. By 7 days post infection, however, control larvae weighed more than test larvae and then lost weight prior to pupation. Insects infected with GV or the mixture of viruses gained weight for a further 14 days. The maximum mean weight of larvae infected with NPV was 668 mg ± 76(SE) as compared to 1500 mg ± 63(SE) for those infected with GV. The equivalent value for dually infected larvae was 917 mg ± 215 (SE) and for control larvae

was 1003 mg ± 63 (SE) (Fig. 3). Larvae infected with GV or the mixture of GV and NPV ceased feeding two to three days before death.

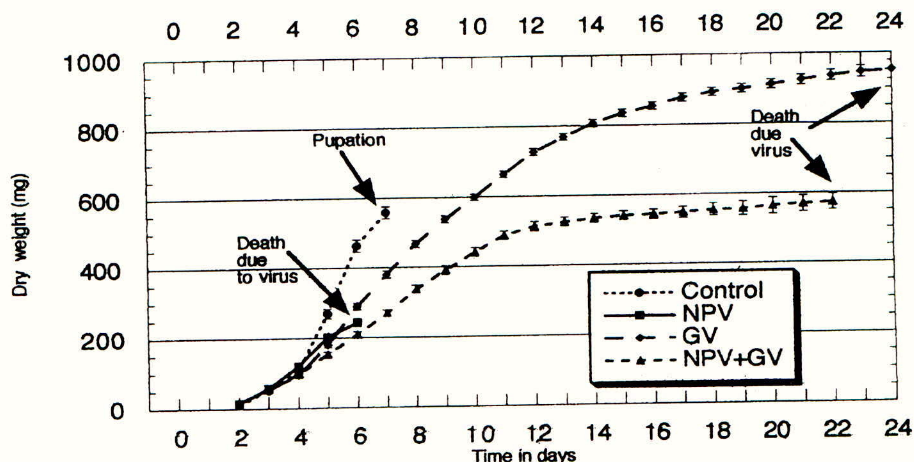
Fig. 3. Comparison of mean weights of larvae following infection with NPV, GV or NPV+GV. Vertical bars indicate standard errors.



Effect of virus infection on food consumption

Duplicate experiments were done. In the first, wet weights of food consumed were measured (results not shown). In the second, dry weights were recorded. Although absolute values differed, the ratios of food consumed by infected larvae to those of uninfected controls were similar. Control and test larvae consumed the same amount of food up to 3 days post infection but by day 5 control larvae were eating at a greater rate than test larvae and had consumed twice as much (Fig. 4). The maximum food consumption of NPV infected larvae was 82.5 mg ± 13.1(SE). The daily intake of food was greatest (112 mg ± 17.3(SE)) on day 8 post infection for larvae given a mixed infection and on day 6 (195 mg ± 11.29) for control larvae.

Fig. 4. Comparison of mean cumulative food consumption per larva following infection with NPV, GV or NPV + GV. Vertical bars indicate standard errors.



Effect of virus infection on moulting

By 2 days post infection all control larvae and most of the test larvae had moulted once (third to fourth instar). Ten % of the control larvae moulted (4th to 5th instar) at day 3, with the remainder completing ecdysis by day 4. At day 6 post infection all control larvae had reached larval stage 6 and would eventually develop into pupae. Test larvae infected with GV alone underwent the same number of moults as controls. They went through ecdysis and reached the 6th instar a day earlier than control larvae. Extra moults were observed in 26% of the population moulting. Not all larvae infected with NPV alone underwent ecdysis in the first few days post infection. Those larvae that did (42%), only reached the 4th instar before death intervened.

Mixed infection caused a range of effects on ecdysis. In general ecdysis was delayed compared with control larvae and the last ecdysis was observed 10 days post infection. Most larvae (69%) reached the 5th instar but 10% died in the previous instar. Only 21% of larvae infected with the mixture of GV and NPV underwent ecdysis from 5th to 6th instar even though they survived for up to 22 days post infection.

DISCUSSION

Much is known concerning mixed infections with NPV and GV that are favourable to the former (Tanada 1959), but less is known about interactions that are not. From the experimental findings presented in this work, it is evident that the presence of the GV in the mixture was influencing the outcome of the infection in a way that was not advantageous to the NPV. Simultaneous presentation of the two viruses resulted in a variable period of extended survival and greater mean food consumption than for larvae

infected with NPV alone. Surprisingly, perhaps, mean food consumption was less for larvae infected with both viruses than for those that received none. Larvae infected with GV alone ate significantly more food (almost twice as much) than the controls. In terms of control, application of NPV alone is preferable to applying a mixture and application of either is preferable to applying GV alone. Some control will be afforded, however, by application of the latter since death will be the final outcome.

Scanning electron microscopy was superior to RE analysis for detecting small quantities of virus. The latter method is clearly unsatisfactory for screening batches of seed virus where even small amounts of contaminating virus may have serious consequences for the efficacy of the final product. For this, DNA hybridisation should be used.

These laboratory-based findings may have significance for the use of virus in the field. It is likely that the presence of the GV in any field formulation will delay control. Furthermore, even if the spray preparation lacks GV, the presence of GV in the field may affect the efficiency of any NPV that is applied. The effect of non-synchronous infection requires investigation in order to more accurately predict the efficacy of virus in control programmes. The exact nature of the relationship between GV and NPV in mixed infections, and mechanism by which GV prolongs survival over that of NPV alone, have yet to be determined.

ACKNOWLEDGEMENTS

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STUDY ON THE BIOLOGICAL PROPERTIES OF A NOVEL RECOMBINANT BACULOVIRUS

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ABSTRACT

Black widow spider venom contains a number of high molecular mass proteins that act on vertebrate and invertebrate synapses. Two of these have been identified as single proteins of Mr 130 kDa which are referred to as alpha-latrotoxin (LT) and alpha-latroinsectotoxin (LIT). These neurotoxins act selectively on presynaptic nerve endings of vertebrates and insects, respectively, promoting exocytosis of synaptic vesicles and extensive neurotransmitter release, although the precise mechanism of action of LT and LIT is unknown. In a recent study (Kiyatkin *et al.*, 1995), LT and LIT were expressed in insect cells using recombinant, polyhedrin-negative baculoviruses. When insects (*Trichoplusia ni*) were injected with cell-culture derived LT or LIT, no toxic effects were observed. However, when the insects were infected with recombinant viruses, a strong toxic effect was observed with the virus expressing LIT.

In the present study the LIT is expressed in a polyhedrin-positive, recombinant baculovirus. Samples of the virus were produced *in vivo* in *Trichoplusia ni* and *in vitro* in an Sf21 cell line. The efficacy of the recombinant LIT viruses as biological control agents, compared to wild type *Autographa californica* nucleopolyhedrovirus (AcNPV) and a standard recombinant AcNPV was evaluated versus neonate *Trichoplusia ni* and neonate and second instar *Heliothis virescens*. Infected insects demonstrated a progressive rigid paralysis from which they did not recover. There were indications that the *in vitro* produced AcNPV LIT had an improved speed of action over the wild type AcNPV versus neonate *Trichoplusia* and second instar *Heliothis*.

INTRODUCTION

The biological properties of a recombinant AcNPV expressing alpha-latroinsectotoxin constructed at Oxford Brookes University, Oxford, UK have been investigated by ZENECA Agrochemicals, Jealott's Hill, UK. The main objective was to evaluate and

compare the intrinsic activity and speed of action of the virus against key lepidopteran pest species.

Virus strains

AcNPV wt - Wild type AcNPV strain. Produced in Sf21 cell line.

AcNPV Tox34#4 (Zeneca recombinant baculovirus standard) - Recombinant pol+ AcNPV expressing a gene encoding an RT-PCR generated clone derived from *Pyemotes tritici* mites (Firemites™, Biofac) which codes for a protein with a high level of homology (94% identity) to the original TxP-1 itch mite (*Pyemotes tritici*) toxin described by Tomalski & Miller (1991), (unpublished results, M-M Suner & J D Windass, Zeneca Agrochemicals). Produced in Sf21 cell line.

AcNPV LIT - Recombinant pol+ AcNPV expressing a gene encoding alpha-latroinsectotoxin. Produced in Sf21 cell line and also *in vivo* in larvae of *Trichoplusia ni*.

MATERIALS AND METHODS

A range of diet-based assays were used to evaluate the biological activity of the viruses against larvae of the tobacco budworm, *Heliothis virescens* (Lepidoptera:Noctuidae) and the cabbage looper, *Trichoplusia ni* (Lepidoptera:Noctuidae).

Droplet feeding assay

A modified version of the Hughes & Wood (1981) droplet feeding assay was used to determine the intrinsic efficacy and speed of action of virus preparations against first instar larvae.

Serial dilutions of virus/water suspensions were prepared incorporating Brilliant Cresyl Blue dye (Sigma Chemicals ref.B5388) at 1% w/v. Neonate larvae were allowed to feed from small droplets of the test suspensions. Treated larvae were readily distinguished by their blue coloration.

Thirty "blue" larvae from each treatment were transferred to individual pots containing artificial insect diet. These pots were incubated for 7 days at 27°C/24°C and a 16h/8h light/dark photoperiod. Symptomology, response to stimulation and mortality were assessed daily from 2-7 days post treatment.

A standard logit dose response procedure (Ashton 1972) was used to estimate LC_{50}/LC_{90} values. Graphical techniques were used to generate LT_{50} values. Lethal time ratios were calculated for selected rates using the following formula:-

$$\text{Lethal ratio for time (LRT)} = \frac{LT_{50} \text{ for test virus}}{LT_{50} \text{ for wild type control}}$$

(Bonning & Hammock 1992)

Surface dosing assay

This is an alternative technique for the evaluation of intrinsic efficacy and speed of action of virus preparations against first instar larvae.

Larvae were allowed to feed for 24 hours on the surface of diet dosed with serial dilutions of virus/water suspension. After 24 hours feeding 20 larvae for each treatment were transferred to individual pots containing artificial insect diet, incubated & assessed as described for the droplet feeding technique. Data were analysed using the procedures described earlier.

Diet plug assay

The intrinsic efficacy and speed of action of virus preparations against second instar larvae were evaluated using a diet plug assay.

Larvae were allowed to feed for 24 hours on small plugs of artificial diet that had been dosed with 1µl of virus/water suspension. Larvae that consumed the entire plug and hence the entire dose, were transferred to individual pots containing artificial diet, incubated and assessed as described earlier. Data were analysed as previously described.

RESULTS

Symptomology

AcNPV wild type - Typical NPV infection symptoms observed. Heavily infected larvae appear paler than controls with a swollen, bloated appearance. Cadavers were fragile and frequently ruptured.

AcNPV Tox34#4 - Affected larvae demonstrated a characteristic rigid paralysis. Larvae were unable to stand, reinvert or walk and were capable of only very feeble movements of mouthparts in response to stimulation. The paralysis was irreversible and death typically occurred within twelve hours of the onset. Cadavers remained intact and were often melanised.

AcNPV LIT - Affected larvae demonstrated a progressive paralysis. The rear portion of the insect was affected first with serious adverse effects on co-ordination and locomotion. The whole insect eventually demonstrated a rigid paralysis. Total paralysis was irreversible, although a low frequency of slightly affected individuals recovered locomotion, but did not thrive. Cadavers were fragile and frequently ruptured.

Intrinsic efficacy/speed of action

There were no significant differences in the intrinsic efficacy values of the test viruses relative to the wild type when tested versus *Trichoplusia ni*. When tested versus *Heliothis virescens* only the AcNPV LIT (*in vivo*) sample differed significantly in activity from the wild type, demonstrating significantly lower activity. This sample was produced in *T. ni* and the results obtained in this study support the hypothesis that the species of insect used to passage a virus can influence its host range activity, with the virus performing as expected versus *T.ni* but with poorer activity versus *H.virescens*. Table 1 illustrates typical patterns of activity observed. These were consistent across all bioassay methods.

Table 1. Examples of Intrinsic Efficacy Data 7 Days Post Treatment

Species + Test Type	Virus	LC_{50} (PIB/ml)	95% confidence intervals
Heliothis virescens Surface Dosing	AcNPV wild type	2.2×10^4	1.5×10^4-3.2×10^4
	AcNPV Tox34#4	1.4×10^4	7.6×10^3-2.8×10^4
	AcNPV LIT (*in vitro*)	2.8×10^4	1.8×10^4-4.6×10^4
	AcNPV LIT (*in vivo*)	1.9×10^5	1.1×10^5-3.9×10^5
Trichoplusia ni Surface Dosing	AcNPV wild type	7.1×10^3	3.6×10^3-1.4×10^4
	AcNPV Tox34#4	6.0×10^3	2.9×10^3-1.2×10^4
	AcNPV LIT (*in vitro*)	7.6×10^3	4.7×10^3-1.2×10
	AcNPV LIT (*in vivo*)	1.7×10^3	$0 - \infty$

From Tables 2 & 3 it can be seen that the AcNPV LIT (*in vitro*) demonstrates an improved speed of action over the wild type virus when evaluated against first instar *T.ni* and second instar *H.virescens*. There is no evidence of improved speed of action against first instar *H.virescens* and the speed of action of AcNPV LIT is significantly slower than that of the AcNPV Tox34#4 recombinant standard in all bioassays performed.

Table 2. Summary of Lethal Time Ratios for *Heliothis virescens* Bioassays

Virus	Lethal Time Ratio (LRT)		
	Droplet Feeding	Surface Dosing	Diet Plug
AcNPV wild type	1.00	1.00	1.00
AcNPV Tox34#4	0.67	0.60	0.73
AcNPV LIT (*in vitro*	0.96	0.99	0.88
AcNPV LIT (*in vivo*)	-	1.01	-

Table 3. Summary of Lethal Time Ratios for *Trichoplusia ni* Surface Dosing Bioassays

Virus	Lethal Time Ratio (LRT)
AcNPV wild type	1.00
AcNPV Tox34#4	0.44
AcNPV LIT (*in vitro*)	0.88

Recent research on δ-latroinsectotoxin, a related toxin also from the venom of the black widow spider, has demonstrated that when the full length δ-LIT cDNA was expressed in bacteria the protein product was inactive, but expression of a C-terminally truncated cDNA produced a protein that caused massive neurotransmitter release at the locust neuromuscular junction at nanomolar concentrations (Dulubova *et al* 1996). It is possible that a similar truncation to the alpha-latroinsectotoxin expressed by the AcNPV may further enhance the neurotoxic effects and improved speed of action observed in this study.

CONCLUSIONS

- Expression of alpha-latroinsectotoxin by AcNPV resulted in clear neurotoxic symptomology in infected insects.
- There is some evidence that expression of alpha-latroinsectotoxin by AcNPV can significantly improve the speed of action over a wild type virus.
- Despite differences observed in speed of action, there was no significant difference between the *in vitro* produced viruses in intrinsic efficacy against either pest species.
- Choice of host insect species for *in vivo* production of AcNPV may influence the host range and activity of the progeny virus.

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