IMPACT OF INSECT-SPECIFIC AaHIT GENE INSERTION ON INHERENT BIOACTIVITY OF BACULOVIRUS AGAINST TOBACCO BUDWORM, *HELIOTHIS VIRESCENS,* AND CABBAGE LOOPER, *TRICHOPLUSIA NI*

M.F. Treacy, Senior Research Entomologist
Insecticide Discovery,
American Cyanamid Company
Princeton, NJ
J.N. All, Professor
Department of Entomology,
University of Georgia
Athens, GA

Abstract

A series of laboratory, greenhouse and field studies were conducted to characterize the biological activity of a recombinant form of *Autographa californica* nuclear polyhedrosis virus (AcNPV). The recombinant NPV (vEGTDEL/AaIT) had a deletion in the ecdysteroid UDP-glucosyltransferase gene and carried a synthetic copy of a gene encoding expression of an insect-selective neurotoxin, AaHIT, which was isolated from the scorpion *Androctonus australis* Hector. Based on LT_{50} values obtained in treated artificial diet assays, vEGTDEL/AaIT controlled larvae of *Heliothis virescens, Trichoplusia ni* and *Helicoverpa zea* at rates of 96%, 51% and 2.6-fold faster than AcNPV, respectively. Results from a greenhouse study conducted against *H. virescens* on cotton showed that hastened speed of action exhibited by the gene-inserted NPV does indeed lead to improved plant protection. For example, following six foliar applications and artificial pest infestation sessions, cotton treated with equal doses of AcNPV or vEGTDEL/ AaIT averaged 46.9 and 18.9% damaged flower buds, respectively (untreated cotton had 68.9% damaged buds). When applied to field-grown cotton at equivalent rates of 2×10^{12} polyhedra/ha, vEGTDEL/AaIT controlled both *H. virescens* and *H. zea* significantly faster than a non-AaIT form of AcNPV. At three days posttreatment, vEGTDEL/AaIT and non-AaIT AcNPV caused 94.5 and 58.2% mortality in *H. virescens*, and 53.5 and 2.0% mortality in *H. zea*, respectively. Surveys of cotton plots over the duration of this field study showed that weekly applications of vEGTDEL/AaIT had no adverse effects on population densities of non-target arthorpods, with species representing 18 different non-lepidopteran families being found at the test site.

Introduction

Use of microbial based technology as an alternative or adjunctive strategy to synthetic insecticides for control of lepidopterous pests has been primarily limited to the bacteria, *Bacillus thuringiensis*. Over-reliance on *B. thuringiensis* as both foliar-applied products and via endotoxin expression in genetically engineered crops is of concern, since resistance to *B. thuringiensis* by certain lepidopteran species has been demonstrated in laboratory selection experiments (McGaughey 1985) and field scenarios (Tabashnik *et al.* 1990). An additional microbial approach to pest control is the use of baculoviruses.

Baculoviridae is the most common of the eight families of insect viruses, with over 500 species isolated to date (Martignoni and Iwai 1986). One such species is the nuclear polyhedrosis virus of alfalfa looper, *Autographa californica* (i.e., AcNPV). Extensive research conducted with over 20 different species of baculoviruses has shown that these naturally occurring pathogens cuase no deleterious effects in vertebrates (Ignoffo 1973, Burges *et al.* 1980, Doller 1985). Baculoviruses are not known to infect mammals, birds, fish, reptiles or plants. There is even specificity exhibited by baculoviruses within Insecta. For example, AcNPV only infects larvae of species within the order Lepidoptera, with the most sensitive being members of the noctuid family. Two pestiferous species which are highly sensitive, or permisive, to infection by AcNPV are cabbage looper, *Trichoplusia ni* (Hubner), and tobacco budworm, *Heliothis virescens* (Fabricius) (Vail *et al.* 1978, Possee *et al.* 1993). Relative to *H. virescens* and *T. ni*, other noctuids such as cotton bollworm, *Helicoverpa zea* (Boddie), are only moderately sensitive, or semipermissive, to infection by AcNPV (i.e., based on numbers of polyhedral inclusion bodies [PIBs] required to induce a lethal infection) (Vail *et al.* 1978).

Target-specific characteristics of NPVs make them good candidates for use in integrated pest management systems. However, although several baculoviruses have been registered as commercial products in the U.S. (e.g., NPVs of *H. zea, A. californica* and beet armyworm, *Spodoptera exigua*), they have not gained widespread use. One key limitation of NPVs restricting their acceptance by pest managers and growers is the length of time it takes to kill an infected insect. Depending on the virus and pest species, it may take nearly a week or longer before an infected insect dies. Further, an NPV-infected insect continues to feed, sometimes at a greater rate than a non-infected individual, until time of death (Subrahmanyam and Ramakrishnan 1981). Instead of providing the rapid curative action normally achieved with applications of commercial synthetic insecticides, a foliar application of an NPV may allow too much crop damage to occur before the pest population is brought under control.

Recently, genetic engineering has become a vehicle by which pesticidal properties of NPVs can be enhanced (via shortened speed of kill) while maintaining their desirable pest-specific characteristics. NPVs have double stranded DNA genomes which can be easily modified (Maeda and Hammock 1993). Two approaches for engineering

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:911-917 (1996)
National Cotton Council, Memphis TN

baculoviruses as biological insecticides are (a) deletion of genes which promote viral replication and hence prolong host survival and (b) utilization of the virus as a vector for carrying and expressing genes into to insect body cavity (Maeda and Hammock 1993).

AcNPV contains a gene which encodes the enzyme ecdysteroid UDP-glucosyltransferase (egt) (O'Reilly and Miller 1991). Expression of egt, which conjugates sugar molecules to ecdysone, enables the virus to inhibit or delay molting by the infected host. Since an insect stops feeding during the molting process, egt essentially functions to prolong the length of time the insect feeds after viral infection, and thus causes increase in weight gain by the insect host and replicaiton by the virus. O'Reilly and Miller (1991) showed that a deletion in the egt gene of AcNPV caused infected fall armyworm, *Spodoptera frugiperda,* to die approx 30% faster than larvae infected with wild-type AcNPV. In a series of vegetables and cotton field trials conducted in the U.S. and Brazil during 1993 and 1994, foliar sprays of egt-deleted AcNPV tended to provide more consistent control of *T. ni* and *H. virescens* than wild-type AcNPV (unpublished data, M. Treacy).

Insertion of synthetic copies of foreign genes into baculovirus genomes probably holds the greatest promise for hastening speed of kill by these pathogens against the insect host. Recombinant baculoviruses carrying foreign genes which encode proteins deleterious to insects have been successfully constructed. Examples of polypeptides which have shown increased insecticidal activity when expressed in lepidopteran larvae by a recombinant baculovirus, include (a) silkworm, *Bombyx mori*, NPV carrying a diuretic hormone gene (Maeda 1989), (b) AcNPV carrying cDNA encoding juvenile hormone esterase of *H. virescens* (Hammock *et al.* 1990) and (c) AcNPV carrying a toxin gene from the straw itch mite, *Pyemotes tritici* (Xang *et al.* 1991).

A recombinant baculovirus which has recently garnered much basic and applied research is AcNPV carrying a toxin gene from the scorpion *Androctonus australis* Hector (i.e., AaHIT gene). AaHIT is an insect-specific toxin which acts as a sodium channel agonist causing repetitive firing of the insect's motor nerves and overstimulation of skeletal muscle (Walther *et al.* 1976, Zlotkin *et al.* 1985, Zlotkin *et al.* 1991). Symptoms of AaHIT toxicity include cessation of feeding, paralysis and eventual death of the insect larva. Numerous studies conducted with muscle tissue, nerve fibers and synaptosomes have clearly demonstrated that AaHIT only exerts its impact on insect species and not on members of Crustacea, Arachnida or mammals (Tintpulver *et al.* 1976, Rathmayer *et al.* 1977, Ruhland *et al.* 1977, Teitelbaum *et al.* 1979, Zlotkin 1983, Gordon *et al.* 1985). Additionally, following oral and nasal routes of exposure, AaHIT was found to have no adverse effects on adult mice (Possee *et al.* 1993). Thus, in addition to the selectivity of the AcNPV vector, AaHIT expressed by the recombinant baculovirus is also highly insect selective. To further this point, Possee *et al.* (1993) exposed rats and guinea pigs to an AaHIT gene-inserted AcNPV via oral, dermal and subcutaneous routes, and found that this recombinant AcNPV caused no harm to these test animals.

A recombinant AcNPV containing the following gene cassette was recently evaluated in a caged-plot field study in England: AaHIT toxin gene under the control of an NPV p10 gene promoter and fused with a copy of the AcNPV gp67 signal peptide coding sequence, to facilitate secretion of the toxin from virus-infected cells (Cory *et al.* 1994). Results from this study conducted on cabbage showed that the recombinant virus killed *T. ni* larvae at a faster rate and reduced plant defoliation by up to 87% when compared with cabbage treated with wild-type AcNPV.

The purpose of the study described herein was to characterize the biological properties of a recombinant AcNPV containing a different AaHIT (or AaIT) cassette (i.e., promoter and signal sequence) than that utilized by Cory *et al.* (1994). Laboratory bioassays were conducted to establish LC_{50} and LT_{50} values for this recombinant AcNPV against *H. virescens, H. zea* and *T. ni* larvae. A greenhouse assay was conducted against *H. virescens* on cotton to determine if hastened speed of kill exhibited by the gene-inserted baculovirus could indeed translate to improved plant protection vs wild-type AcNPV. Finally, a field study was conducted on cotton to further evaluate pesticidal properties of this particular AcNPV recombinant, as well as its impact on non-target arthorpods.

Material and Methods

Baculoviruses

Wild-type and genetically altered baculoviruses evaluated in studies described herein were (a) V8 strain of AcNPV [i.e., AcNPV(V8)], (b) AcNPV(V8) with approx a 1 kilobase internal deletion in the ecdysteroid UDP-glucosyl-transferase gene [i.e., vEGTDEL(V8)] and (c) vEGTDEL(V8) into which was inserted a 0.8 kilobase fragment encoding AaIT [i.e., vEGTDEL (V8)/AaIT]. Each of the baculoviruses was produced *in vitro* using a *Spodoptera frugiperda* ovarian cell line (i.e., Sf9 cell line). For purposes of greenhouse and field evaluations, baculoviruses were formulated as two different wettable powders (designated WP(I) and WP(II)), each differing slightly in inert ingredients.

Laboratory Study

Laboratory assays were conducted to compare AcNPV(V8), vEGTDEL(V8) and vEGTDEL(V8)/AaIT for inherent activity against larvae of *H. virescens, T. ni* and *H. zea*. Insect population responses to aforementioned wild-type and recombinant baculoviruses were quantified by generation of LC_{50} and LT_{50} values (SAS Institute, 1989). For testing purposes, 10-fold serial dilutions (1×10^1 - 1×10^8 PIBs/ml, depending on insect species) were made for

each baculovirus treatment in deionized water and 0.01% SDS.

Virulence of each baculovirus to each lepidopterous species was evaluated via a diet-overlay technique. Plastic bioassay trays, which were manufactured by C-D International, Inc. (Pitman, NJ), were utilized as test arenas in this study. Each tray contained 32 open-faced wells; dimensions of each well were 4.0 x 4.0 x 2.5 cm (L x W x H). A 5-ml quantity of wheat germ and soybean flour based artificial diet was poured into each tray-well. After the diet hardened, 0.4 ml of a virus solution was pipetted onto the diet surface in each well. Virus solutions were evenly spread over the surfaces of diet by picking up the tray and gently tilting it from side to side. Following application and spreading of treatments, trays were held in a vented area for approx. 2 hrs. until water was no longer pooled on diet surfaces. A single larva was then placed on the surface of diet in each tray-well. After larval infestation, each cell was covered with an adhesive, vented clear plastic sheet (C-D International).

All test arenas were held under constant fluorescent light and a temperature of approx 27°C throughout the post-infestation period. At each mortality rating period, a larva was considered to be dead if it exhibited no movement, even after shaking the diet-tray. Additional symptoms of larval death were liquification of the body (mostly evident in AcNPV(V8) and vEGTDEL(V8) infected larvae) and contraction or shrinkage of the body (evident in vEGTDEL(V8)/AaIT infected larvae). Larval mortality was rated on a daily basis over the first five days posttreatment, with a final rating taken at 10 days.

Greenhouse Study

A test was conducted in the greenhouse at the University of Georgia at Athens to (1) compare formulations of AcNPV(V8), vEGTDEL(V8)/AaIT, *Bacillus thuringiensis* (DIPEL™, Abbott Labs) and esfenvalerate (ASANA™, DuPont) for efficacy against *H. virescens* on cotton and (2) determine impact of spray nozzle orientation on performance of aforemen-tioned insecticides. This greenhouse test was conducted with potted cotton plants (*var* Delta Pine 90) using procedures developed to evaluate formulations of microbial pathogens in a manner comparable to field environments (All and Guillebeau, 1991). Briefly, plants were grown in 0.25 m diam pots in commercial potting soil. At time of initial treatment application session, plants were in early squaring stage of development. Plants were sprayed in a 1.3 x 1.3 x 1.6 m (L x W x H) chamber equipped with a rotating spray boom mounted with conventional hollow cone spray nozzles (TX4, Spraying Systems). Two different boom designs were evaluated in this study (1) two nozzles/plant positioned on boom as one over plant terminal and two spaced 37.5 cm either side of center (i.e., broadcast design) and (2) three nozzles/plant positioned on boom as one over plant terminal and two mounted on 10 cm drop-tubes angled at 45° toward each side of plant (i.e., directed design). Plants were sprayed with a volume equivalent to 189 l/ha. Insecticide formulations were mixed in dechlorinated water and combined with a feeding stimulant (COAX™ at 3.5 l/ha). Treatments were applied to cotton six times at approx. six-day intervals. Plants were arranged in a randomized complete block design with five replications in stainless steel pans flooded with 2 cm water to prevent larval migration between plants.

Plants were artificially infested with neonate *H. virescens* approx 1 hr after each spraying. With the use of a small paint brush, five larvae were placed in each cotton terminal on each infestation date. Efficacy of treatments was evaluated three and five days after the fourth and sixth application sessions, respectively. Percentage of flower buds (i.e., squares) damaged by *H. virescens* was determined for each treatment; damage levels were compared among treatments by analysis of variance and test of least significant difference.

Field Study

A small plot field study was conducted on a University of Georgia research farm located near Watkinsville, GA to evaluate several baculovirus treatments for bioactivity against AcNPV-permissive, -semipermissive and -non-permissive arthropod species. Seven treatments and an untreated check were compared in 3-row by 6.1 m plots of cotton (*var* Chembred 1135) which was planted as seed in late-May 1995 on 96-cm row spacing; only the middle row of each plot received foliar applications of treatments throughout the study. Treatments and untreated check were replicated 4-fold in a randomized complete block design. As recommended by the U.S. Environmental Protection Agency, procedures such as use of ground cover, establishment of plant-free buffer zones, crop destruction and liming of soil were implemented at the test site.

The ten plants in the middle row of each plot were artificially infested with neonate *H. virescens* approx 2 hrs prior to each treatment application session. Larvae used in this study were supplied (via shipment of eggs) from a laboratory colony maintained at the Cyanamid Agricultural Research Center in Princeton, NJ. Using a small paint brush, five larvae were placed in the terminal of each of 10 plants/plot. Treatment applications were initiated on August 4, 1995. Additional treatment applications were made on August 11, 18 and 25, 1995. Treatments were mixed with water and applied with a CO_2-powered backpack sprayer which was calibrated to deliver 188 l/ha through three Tee-Jet® 3X hollow-cone nozzles/row (2.8 kg/cm^2). Spray boom design consisted of one nozzle over top of cotton row and one nozzle directed at each of the two sides of the cotton row (dropped from main boom and angled inward to cover upper one-third of plant canopy).

On August 10, 17, 24 and 31, 1995, each of the plants in the middle row of each plot was visually inspected (i.e.,

plant terminals, squares, flowers, bolls and foliage) for presence of live and dead arthropods, as well as damage from feeding by *H. virescens* or other phytophagous arthropods. Arthropod and plant-injury data were subjected to analysis of variance, and if found to be significant (P=0.05), treatment means were compared via Duncan's multiple range test.

Additionally, to measure speed of pesticidal activity caused by foliar sprays of selected treatments, leaves were collected from plots and assayed against *H. virescens* and *H. zea* larvae. Approx 1 hr after application of following treatments on August 18, 1995, six cotton leaves (located near terminal portion of each plant) were randomly harvested from each plot and individually paced into plastic petri dishes containing water-moistened filter paper: vEGTDEL(V8)/AaIT WP(I) at 2.0 x 10^{12} PIBs/ha, vEGTDEL(V8)/ AaIT WP(II) at 2.0 x 10^{12} PIBs/ha, vEGTDEL(V8) WP(I) at 2.0 x 10^{12} PIBs/ha and DIPEL™ WP at 0.56 kg/ha. Each petri dish arena was then infested with either five neonate *H. virescens* or five neonate *H. zea* (three arenas/species). Percent larval mortality was assessed at one, two, three and four days post-infestation. This assay process was repeated with leaves collected two days after the August 18, 1995 application session in order to measure residual bioactivity of treatments. Larval mortality data from each assay were subjected to analysis of variance and Duncan's multiple range test.

Results and Discussion

Laboratory Study

Based on final mortality raings at 10 days posttreatment and resultant LC_{50} values, wild-type AcNPV(V8) and the two genetically altered baculoviruses, vEGTDEL(V8) and vEGTDEL(V8)/AaIT, were equally effective against *H. virescens* larvae (Table 1). LC_{50} values among the three baculoviruses averaged approx 1 x 10^2 PIBs/diet-well (i.e., 16 cm^2 surface area). However, vEGTDEL(V8) and particularly vEGTDEL(V8)/AaIT caused *H. virescens* mortality at rates significantly faster than that of AcNPV(V8). When exposed to larvae at approx LC_{99} doses (i.e., 4 x 10^3 PIBs/16 cm^2), resultant LT_{50} values for AcNPV(V8), vEGTDEL(V8) and vEGTDEL(V8)/AaIT were 5.1, 3.8 and 2.6 days, respectively.

Although *T. ni* larvae were, as expected, highly permissive to both genetically altered baculoviruses, there was a trend for vEGTDEL(V8)/AaIT to be slightly less virulent than AcNPV(V8) or vEGTDEL(V8) (Table 2). vEGTDEL(V8) and AcNPV(V8) possessed LC_{50} values averaging 0.8 x 10^2 PIBs/16 cm^2, whereas *T. ni* LC_{50} value for vEGTDEL(V8)/AaIT was 2.8 x 10^2 PIBs/16 cm^2. Interestingly, when exposed to *T. ni* at doses lower than that required to kill at least 80-90% of the test population, vEGTDEL(V8)/AaIT caused contractile paralysis in only a small portion of the dead larvae; most of the larvae appeared to have succumbed to normal viral infection (i.e., liquification of body). However, when *T. ni* were exposed to doses of vEGTDEL(V8)/AaIT which caused at least 85-90% population mortality, rapid larval death was clearly caused by the expressed insect-specific toxin (i.e., all dead larvae exhibited contractile paralysis). For example, when exposed to *T. ni* larvae at approx LC_{99} doses (i.e., 4 x 10^3 PIBs/16 cm^2), resultant LT_{50} values for AcNPV(V8), vEGTDEL(V8) and vEGTDEL (V8)/AaIT were 4.1, 3.2 and 2.7 days, respectively.

As mentioned earlier, the recombinant AcNPV evaluated by Cory *et al.* (1994) contained a cassette consisting of the AaIT gene under control of the p10 gene promoter and an AcNPV gp67 signal sequence. This particular recombinant was reported to control *T. ni* larvae approx 25-30% faster than wild-type AcNPV (Stewart *et al.* 1991). However, vEGTDEL(V8)/AaIT (with a different promoter and signal sequence) evaluated herein killed *T. ni* larvae 50% faster than wild-type AcNPV. These findings may suggest that changes to portions of the cassette, such as the promoter and/or signal sequence, may allow for enhanced toxin expression and transport within the infected insect, and thus improved pesticide performance.

Not surprisingly, results from these diet-overlay assays showed *H. zea* to be semi-permissive to AcNPV(V8) and its two genetically altered counterparts (i.e., LC_{50} values 100- to 1000-fold higher than against *H. virescens* or *T. ni*). However, based on 10-day LC_{50} values (and non-overlapping CLs), vEGTDEL(V8)/AaIT was approx 10- to 20-fold more active against *H. zea* than either vEGTDEL(V8) or AcNPV(V8) (Table 3). Additionally, when exposed to *H. zea* at doses causing approx 90% population mortality, vEGTDEL(V8)/AaIT induced 2.5-fold faster larval mortality than vEGTDEL (V8) or AcNPV(V8) (i.e., LT_{50} values of 2.8, 7.3 and 6.4 days, respectively). The efficient action of vEGTDEL(V8)/AaIT against *H. zea* (based on LC and LT_{50} values relative to AcNPV(V8)) may have resulted from factors such as (1) larva's ability to express toxin, (2) ability of toxin to be transported to action site after expression and (3) susceptibility of this insect species to the toxin. These data suggest that if a lepidopteran species possesses at least some degree of sensitivity to AcNPV (i.e., allows viral infection and replication), it could produce a pharmacologically active amount of the insect-specific toxin coded for in the vEGTDEL(V8)/AaIT construct. The pharmaco-dynamics of the toxin may, therefore, effectively lower the LC_{50} value (based on no of PIBs) of such a recombinant baculovirus vs. its wild-type counterpart. From an applied standpoint, it could be possible for foliar applications of a recombinant baculovirus to provide crop protection from a semipermissive pest species at some level better than that provided by applications of its wild-type counterpart (as long as the pest species responds to the internally expressed toxin).

Greenhouse Study

In this field simulation study, vEGTDEL(V8)/AaIT provided better control of *H. virescens* in cotton than wild-type AcNPV(V8) (Table 4). When treatments were applied to cotton via directed spray method, average damage to squares over the two posttreatment rating dates (i.e., May 18 and 29, 1995) was as follows: AcNPV(V8) at 2.5 x 10^{12} PIBs/ha = 31.8%, vEGTDEL (V8)/AaIT at 2.5 x 10^{12} PIBs/ha = 11.4%, DIPEL™ 2X at 0.56 kg/ha = 21.8% and untreated = 57.8%. Broadcasted applications of the pyrethroid, esfenvalerate, at 0.03 kg/ha allowed an average of 7.7% damage to squares over the two rating dates.

There was a strong trend which indicated that all three biological agents evaluated in this study provided better control of *H. virescens* when applied as directed sprays (three nozzles/plant) vs. broadcasted sprays (two nozzles/ plant). Compared to the broadcasted applications, the directed sprays may have deposited a greater number of particles on each plant, as well as on both upper and lower surfaces of leaves. Optimization of plant coverage may be more critical for insect control agents which must be ingested by the pest vs. those agents (e.g., pyrethroids) which exert their lethal effects on pests via both oral and cuticular-contact routes of exposure. For orally active materials, it is desirable to have the phytophagous pest acquire a lethal amount of the control agent in as few bites as possible, and a higher density of active particles per unit area of plant tissue can hasten this dose acquisition process.

Field Study

Results from leaf bioassays clearly showed that, dose-for-dose, foliar applications of vEGTDEL(V8)/AaIT to cotton subdued AcNPV-permissive neonate *H. virescens* at a faster rate than vEGTDEL(V8) (Table 5). For example, two days after being placed on freshly treated cotton foliage (i.e., one- to two-hr-old residues), larval mortality averaged 74, 88 and 4% for vEGTDEL(V8)/AaIT WP(I), WP(II) and vEGTDEL(V8) WP(I), respectively. Further, both formulations of the gene-inserted baculovirus caused greater than 90% larval mortality by three days post-infestation, whereas larval mortality on leaves treated with vEGTDEL(V8) reached only 83% out to four days post-infestation. Unlike the baculovirus treatments, DIPEL™ caused some larval mortality one day post-infestation (36%). However, by post-infestation day two, gene-inserted baculovirus was as active as DIPEL™.

As shown in previously described diet-overlay assays, although *H. zea* is only semipermissive (vs. *H. virescens*) to infection by each of the AcNPV-based baculoviruses described herein, it is significantly more sensitive to AaIT gene-inserted AcNPV than to EGT gene-deleted or wild-type AcNPV (Tables 1 and 3). Bioassay of leaves collected from field plots demonstrated that foliar applications of vEGTDEL(V8)/AaIT were significantly faster in controlling neonate *H. zea* than vEGTDEL(V8) (Table 6). At four days after being placed on freshly treated foliage, *H. zea* population mortality levels averaged 80, 81 and 17% for vEGTDEL(V8)/AaIT WP(I), WP(II) and vEGTDEL(V8) WP(I), respectively. Data from diet-overlay and foliar assays suggest that, although vEGTDEL(V8)/AaIT possesses better activity against *H. virescens* than against *H. zea*, this particular baculovirus construct likely has a better chance of controlling/suppressing both species in cotton than its wild-type or EGT gene-deleted forms.

Bioactivity of baculovirus treatments and DIPEL™ was found to be short-lived in this study. When cotton foliage was removed from the field and bioassayed two days after application, mortality of *H. virescens* and *H. zea* averaged 15% or less among the treatments.

In addition to leaf assays described above, cotton field plots were evaluated for damage by pest species and surveyed for densities of nontarget arthropods. Although plots were artificially infested with neonate *H. virescens* on a weekly basis, a low density natural infestation of the test site by *H. virescens* and *H. zea* also occurred (approx 3:1, *H. virescens:H. zea*, based on moth trap catches). Statistically significant differences in crop damage were found on only one of the four posttreatment sample dates, i.e., August 10, 1995 (Table 7). At six days following the first application session, vEGTDEL(V8)/AaIT WP(I) at 2 x 10^{12} PIBs/ha, vEGTDEL(V8) WP(I) at 2 x 10^{12} PIBs/ha and DIPEL™ at 0.56 kg/ha each averaged significantly less *Heliothis*-damaged squares, blooms and bolls (combined fruiting bodies) than untreated cotton; with damage rates of 6.1, 7.6, 8.4 and 19.0%, respectively. There was a strong numerical trend indicating that the baculovirus and DIPEL™ treatments reduced crop damage vs untreated throughout the study.

Surveys of field plots over the duration of this study showed that weekly applications of vEGTDEL(V8)/AaIT, at rates as high as 2 x 10^{12} PIBs/ha, had no adverse effects on population densities of nontarget arthropods (Table 8). Insect families represented in this survey were Acrididae, Anthocoridae, Apidae, Braconidae, Chrysomelidae, Cicadellidae, Coccinellidae, Chrysopidae, Formicidae, Halictinae, Ichneumonidae, Lygaeidae, Miridae, Psyllidae, Reduviidae, Sarcophagidae, Tephritidae and Vespidae. Spiders were also observed at the test site.

Non-target data gathered from the cotton field trial support findings in numerous laboratory assays. Heinz *et al.* (1995) showed that predatory green lacewing larvae, *Chrysopa carnea* Stephens, and insidious flower bug adults, *Orius insidiosus* (Say), were not adversely affected after feeding on larvae of *H. virescens* infected with AaIT-inserted AcNPV. Additionally, these researchers demonstrated that body injections with budded AcNPV-AaIT virus particles caused no harm to adult honeybees, *Apis mellifera* L. In a study reported by Possee *et al.* (1993), predatory adult carabid beetles, *Pterostichus madidus*, survived and behaved normally for two weeks (i.e., duration of study)

after feeding on a diet of AcNPV-AaIT infected *T. ni* larvae. A series of laboratory assays recently conducted at American Cyanamid (Princeton, NJ; unpublished data) showed that the following species were unaffected by constant exposure to vEGTDEL(V8)/AaIT via their food-source (e.g., infected larvae, treated foliage, etc.) and/or habitat (e.g., soil): Japanese beetle grubs, *Popillia japonica*, fruitfly larvae, *Drosophila melanogaster*, German cockroach adults, *Blatella germanica*, boll weevil adults, *Anthonomus grandis*, twospotted spider mite nymphs, *Tetranychus urticae*, western corn rootworm larvae, *Diabrotica virgifera*, Chinese mantid nymphs, *Tenodera aridifolia sinsensis*, and funnel web spider adults, *Ixeuticus* spp.

In conclusion, results from laboratory, greenhouse and field studies described herein indicate that genetic modification of baculoviruses to hasten their speed of action is a very promising strategy for improving the pesticidal properties of these pathogens. In the case of vEGTDEL(V8)/AaIT, not only does this recombinant baculovirus have the potential to control AcNPV- permissive *T. ni* and *H. virescens* in crops such as cotton, tobacco and vegetables, but also provide some level of suppression of *H. zea* (a species against which wild-type AcNPV is not considered to be a highly effective pathogen).

Further, field and laboratory studies indicate that both the isolated AaIT toxin and the AaIT gene-inserted AcNPV are very restrictive in their impact on organisms, with the toxin exhibiting no effects on mammals, crustaceans or arachnids and the recombinant baculovirus causing no adverse impact on mammals or non-lepidopteran arthropod species. Further improvements in pest-spectrum and/or speed of action by recombinant baculoviruses may be achieved via (a) use of different baculovirus species as vectors, (b) insertion of genes coding for other insecticidal proteins and (c) construction of cassettes with different promoter and signal sequence genes. Also, duration of pest control by foliar sprays of recombinant baculoviruses may be lengthened through the use of improved formulation technology (e.g., agents which protect virus from sunlight).

Acknowledgements

We give special thanks to Berry Tanner, Thor Coutts, Chris Fartner, Sara Ranger, Theresa Singer (University of Georgia) and Ray Borysewicz (American Cyanamid Company) for their assistance in conducting laboratory, greenhouse and field trials described herein.

References

All, J.N. and L.P. Guillebeau. 1991. New ideas for screening insecticides on squaring cotton in the greenhouse and in field trials. Proc. Beltwide Cotton Prod. & Research Conf., National Cotton Council (Memphis, TN) pp. 756-757.

Burges, H.D., G. Crozier and J.A. Huber. 1980. A review of safety tests on baculoviruses. Entomaphaga 25:329-340.

Cory, J.S., M.L. Hirst, T. Williams, R.S. Hails, D. Goulson, B.M. Green, T.M. Carty, R.D. Possee, P.J. Cayley and D.H.L. Bishop. 1994. Field trial of a genetically improved baculovirus insecticide. Nature 370:138-140.

Doller, G. 1985. Safety of insect viruses as biological control agents, In Viral Insecticides for Biological Control (Academic Press, NY) pp. 399-439.

Gordon, D., E. Zlotkin and W.A. Catterall. 1985. Binding of insect-selective neurotoxin and saxitoxin to insect neuronal membranes. Biochem. Biophys. Acta 821:130-136.

Hammock, B.D., B.C. Bonning, R.D. Possee, T.N. Hanzlik and S. Maeda. 1990. Nature 344:458-461.

Heinz, K.M., B.F. McCuthen, R. Herrmann, M.P. Parrella and B.D. Hammock. 1995. Direct effects of recombinant nuclear polyhedrosis viruses on selected nontarget organisms. J. Econ. Entomol. 88:259-264.

Ignoffo, D.M. 1973. Effects of entomopathogens on vertebrates, In Annals of New York Academy of Sciences 217:141-164.

Maeda, S. 1989. Biochem. Biophys. Res. Commun. 165:1177-1183.

Maeda, S. and B.D. Hammock. 1993. Recombinant baculoviruses expressing foreign genes for insect pest control, In Pest Control with Enhanced Environmental Safety, American Chemical Society, 20:281-295.

Martignoni, M.E. and P.J. Iwai. 1986. General Technical Report PNW-159, 4th Edition, USDA, 50 pp.

McGaughey, W.H. 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science (Washington, DC) 229:193-195.

O'Reilly, D.R. and L.K. Miller. 1991. Improvement of a baculovirus pesticide by deletion of the *egt* gene. Biotechnology 9:1086-1089.

Possee, D., M. Hirst, L.D. Jones, D.H.L. Bishop and P.J. Cayley. 1993. Field tests of genetically engineered baculoviruses, In Opportunities for Molecular Biology in Crop Production. British Crop Protection Council, monograph 55, pp. 23-36.

Rathmayer, M., C. Walther and E. Zlotkin. 1977. Effect of different toxins from scorpion venom on neuromuscular transmission and nerve action potential in the crayfish. Comprehensive Bioch. and Physiol. 56:35-38.

Ruhland, M., E. Zlotkin and W. Rathmeyer. 1977. Effect of toxins from the venom of scorpion *Androctonus australis* on a spider nerve-muscle prepara-tion. Toxicon 15:157-160.

SAS Institute, Inc. 1989. SAS/STAT User's Guide, Version 6, Fourth Edition, SAS Institute, Inc. (Cary, NC) 943 pp.

Stewart, L.M.D., M. Hirst, M.L. Ferber, A.T. Merryweather, P.J. Cayley and R.D. Possee. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. Nature 352:85-88.

Subrahmanyan, B. and N. Ramakrishnan. 1981. Influence of a baculovirus infection on molting and food consumption by *Spodoptera littura*. J. Invert. Path. 38:161-168.

Tabashnik, B.E., N.L. Cushing, N. Finson and M.W. Johnson. 1990. Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 83:1671-1676.

Teitelbaum, Z., P. Lazarovici and E. Zlotkin. 1979. Selective binding of the scorpion venom insect toxin to insect nervous tissue. Insect Biochem. 9:343-346.

Tintpulver, M. T. Zerachia and E. Zlotkin. 1976. Actions of toxins derived from scorpion venom on the ileal smooth muscle preparation. Toxicon 14:371-377.

Vail, P.V., D.L. Jay, F.D. Stewart, A.J. Martinez and H.T. Dulmage. 1978. Comparative susceptibility of *Heliothis virescens* and *H. zea* to the nuclear polyhedrosis virus isolated from *Autographa californica*. J. Econ. Entomol. 71:293-296.

Walther, C., E. Zlotkin and W. Rathmeyer. 1976. Action of different toxins from scorpion *Androctonus australis* on a locust nerve-muscle preparation. J. Insect. Physiol. 22:1187-1194.

Xang, X. B.G. Ooi and L.K. Miller. 1991. Gene 100:131-137.

Zlotkin, E. 1983. Insect selective toxins derived from scorpion venoms: an approach to insect neuropharmocology. Insect Biochem. 13:219-236.

Zlotkin, E., D. Kadouri, D. Gordon, M. Palhate, M.F. Martin and H. Rochat. 1985. An excitatory and a depressant insect toxin from scorpion venom both affect sodium conductance and possess a common binding site. Archives of Biochem. and Biophys. 240:877-887.

Zlotkin, E., M. Eitan, V.P. Bindokas, M.E. Adams, M. Moyer, W. Burkart and E. Fowler. 1991. Functional duality and structural uniqueness of depressant insect-selective neurotixins. Biochemistry 30:4814-4820.

Table 1. Mortality of *H. virescens* larvae during chronic exposure to wild-type and recombinant baculoviruses.

Treatment[a]	***n***	**LC_{50} (95% CL)**[b]	**LT_{50} in days (95% CL)**[c]
AcNPV(V8)	512	0.57	5.1
		(0.02 - 5.08)	(4.7 - 5.4)
vEGTDEL(V8)	512	1.09	3.8
		(0.81 - 1.48)	(3.5 - 4.0)
vEGTDEL(V8) /AaIT	510	1.30	2.6
		(0.25 - 6.69)	(2.2 - 2.9)

[a] Four replicates per virus concentration, 30-32 insects per treatment replicate, 32 untreated insects per replicate, larvae were four to five days old at test initiation. Final mortality at 10 d.[b] Virus concentrations expressed as no PIBs x 10^2/16 cm^2 diet surface.[c] At virus dosage rate of 4 x 10^3 PIBs/16 cm^2 (i.e., approx LC_{99} dose).

Table 2. Mortality of *T. ni* larvae during chronic exposure to wild-type and recombinant baculoviruses.

Treatment[a]	***n***	**LC_{50} (95% CL)**[b]	**LT_{50} in days (95% CL)**[c]
AcNPV(V8)	508	0.99	4.1
		(0.73 - 1.33)	(3.7 - 4.2)
vEGTDEL(V8)	512	0.62	3.2
		(0.13 - 2.81)	(3.0 - 3.4)
vEGTDEL(V8)/AaIT	512	2.85	2.7
		(2.11 - 9.63)	(2.2 - 3.0)

[a] Four replicates per virus concentration, 30-32 insects per treatment replicate, 32 untreated insects per replicate, larvae were four to five days old at test initiation. Final mortality at 10 d.[b] Virus concentrations expressed as no PIBs x 10^2/16 cm^2 diet surface.[c] At virus dosage rate of 4 x 10^3 PIBs/16 cm^2 (i.e., approx LC_{99} dose).

Table 3. Mortality of *H. zea* larvae during chronic exposure to wild-type and recombinant baculoviruses.

Treatment[a]	***n***	**LC_{50} (95% CL)**[b]	**LT_{50} in days (95% CL)**[c]
AcNPV(V8)	509	1.96	7.3
		(1.12 - 3.52)	(6.8 - 7.9)
vEGTDEL(V8)	510	0.75	6.4
		(0.38 - 1.39)	(5.8 - 7.0)
vEGTDEL(V8)/AaIT	511	0.07	2.8
		(0.04 - 0.11)	(2.4 - 3.1)

[a] Four replicates per virus concentration, 30-32 insects per treatment replicate, 32 untreated insects per replicate, larvae were three days old at test initiation. Final mortality at 10 d.[b] Virus concentrations expressed as no PIBs x 10^6/16 cm^2 diet surface.[c] Dosage rates for AcNPV(V8) and vEGTDEL(V8) were 4 x 10_7 PIBs/16 cm^2 and dosage rate for vEGTDEL(V8)/AaIT was 4 x 10^6 PIBs/16 cm^2 (i.e., approx LC_{90}/LC_{99} doses).

Table 4. Control of *H. virescens* on cotton in a field simulation study conducted in the greenhouse (1995).

Treatment and application method[a]	Dosage per ha	Mean % damaged squares	
		18 May	29 May
"Directed Application"			
AcNPV(V8) WP(I)	2.5 x 10^{12}	16.8 bc	46.9 bc
vEGTDEL(V8)/AaIT WP(I)	1.2 x 10^{12}	18.8 bc	33.4 cd
vEGTDEL(V8)/AaIT WP(I)	2.5 x 10^{12}	4.0 c	18.9 de
DIPEL™ 2X	0.56 kg	14.8 bc	28.9 cde
"Broadcast Application"			
AcNPV(V8) WP(I)	2.5 x 10^{12}	33.8 ab	55.3 ab
vEGTDEL(V8)/AaIT WP(I)	1.2 x 10^{12}	21.8 bc	44.4 bc
vEGTDEL(V8)/AaIT WP(I)	2.5 x 10^{12}	17.2 bc	35.6 cd
DIPEL™ 2X	0.56 kg	19.2 bc	55.2 ab
Esfenvalerate	0.03 kg	2.4 c	13.0 e
Untreated	na	46.6 a	68.9 a

Within column, means followed by a common letter are not significantly different (P<0.05; for 18 May, LSD=22.9, for 29 May, LSD=18.5).[a] Treatments were applied on 28 April, 3, 9, 15, 19 and 24 May; plants were artificially infested with neonates 1 hr after each treatment application.

Table 5. Rate of *H. virescens* mortality on cotton foliage collected from field plots previously sprayed with selected biological insecticides; Oconee County, GA (1995).

Treatment[a,b]	Dosage per ha	Cumulative mean % larval mortality at selected days post-infestation			
		1 d	2 d	3 d	4 d
vEGTDEL(V8)/AaIT	2 x 10^{12}	0.0 b	74.2 a	94.5 a	98.0 a
vEGTDEL(V8)/AaIT	2 x 10^{12}	0.0 b	88.0 a	96.5 a	100.0 a
vEGTDEL(V8) WP(I)	2 x 10^{12}	0.0 b	3.5 b	58.2 b	82.5 b
DIPEL™ WP	0.56 kg	36.2 a	84.2 a	89.2 a	100.0 a
Untreated	na	0.0 b	0.0 b	0.0 c	0.0 c

Within column, means followed by a common letter are not significantly different (P<0.05, Duncan's multiple range test).[a] All treatments had COAX™ (CCT Corp.) at 3.5 l/ha added to the water diluent.[b] Foliage was collected and fed to larvae approx two hr after treatments were applied to cotton.

Table 6. Rate of *H. zea* mortality on cotton foliage collected from field plots previously sprayed with selected biological insecticides; Oconee County, GA (1995).

Treatment[a,b]	Dosage per ha	Cumulative mean % larval mortality at selected days post-infestation			
		1 d	2 d	3 d	4 d
vEGTDEL(V8)/AaIT WP(I)	2 x 10^{12}	0.0 b	0.0 c	53.5 b	80.2 a
vEGTDEL(V8)/AaIT WP(II)	2 x 10^{12}	0.0 b	17.5 b	70.5 ab	81.2 a
vEGTDEL(V8) WP(I)	2 x 10^{12}	0.0 b	1.8 c	2.0 c	17.2 b
DIPEL™ WP	0.56 kg	48.0 a	75.8 a	81.2 a	87.2 a
Untreated	na	0.0 b	0.0 c	6.2 c	6.2 b

Within column, means followed by a common letter are not significantly different (P<0.05, Duncan's multiple range test).[a] All treatments had COAX™ (CCT Corp.) at 3.5 l/ha added to the water diluent.[b] Foliage was collected and fed to larvae approx two hr after treatments were applied to cotton.

Table 7. Control of *H. virescens* and *H. zea* in cotton; Oconee County, GA (1995).

Treatment[a]	Dosage per ha	Mean % damaged squares, bolls and blooms			
		10 Aug	17 Aug	24 Aug	31 Aug
vEGTDEL(V8)/ AaIT WP(I)	5 x 10^{11}	13.0 ab	13.9 a	10.3 a	6.5 a
	2 x 10^{12}	6.1 b	12.7 a	6.3 a	15.3 a
vEGTDEL(V8)/ AaIT WP(II)	2 x 10^{12}	12.9 ab	14.3 a	9.2 a	11.8 a
vEGTDEL(V8) WP(I)	5 x 10^{11}	9.5 ab	12.9 a	9.2 a	11.7a
	2 x 10^{12}	7.6 b	13.3 a	8.9 a	6.9 a
vEGTDEL(V8) WP(II)	2 x 10^{12}	10.3 ab	14.8 a	7.6 a	10.6 a
DIPEL™ WP	0.56 kg	8.4 b	9.6 a	11.2 a	8.1 a
Untreated	na	19.0 a	23.4 a	12.5 a	21.0 a

Within column, means followed by a common letter are not significantly different (P<0.05, Duncan's multiple range test).[a] Treatment applications were made on 4, 11, 18 and 25 Aug; approx two hr before each application session, each plant terminal was artificially infested with five neonate *H. virescens* (adult trap catches indicated that plots could have also been naturally infested with *H. virescens* and *H. zea* at a ratio of 3:1).

Table 8. Impact of recombinant baculovirus on natural populations of nontarget arthropods in small plots of field-grown cotton; Oconee County, GA (1995).

Treatment[a]	Dosage per ha	Mean no. live arthropodsper 6.1 row-m[b]			
		10 Aug	17 Aug	24 Aug	31Aug
vEGTDEL(V8)/ AaIT WP(I)	5 x 10^{11}	31.7 a	26.3 a	25.7 a	32.2 a
	2 x 10^{12}	24.4 a	32.2 a	36.1 a	30.7 a
vEGTDEL(V8)/ AaIT WP(II)	2 x 10^{12}	27.3 a	32.5 a	30.4 a	29.1 a
vEGTDEL(V8) WP(I)	5 x 10^{11}	27.8 a	33.0 a	27.8 a	28.1 a
	2 x 10^{12}	28.3 a	26.3 a	28.1 a	27.1 a
vEGTDEL(V8) WP(II)	2 x 10^{12}	29.6 a	32.5 a	29.6 a	28.3 a
DIPEL™ WP	0.56 kg	27.6 a	29.4 a	33.8 a	34.1 a
Untreated	na	24.7 a	30.4 a	24.4 a	27.0 a

Within column, means followed by a common letter are not significantly different (P<0.05, Duncan's multiple range test).[a] Treatment applications were made on 4, 11, 18 and 25 Aug.[b] At least 20 different families, immatures and adults, representing Orthoptera, Hemiptera, Hymenoptera, Coleoptera, Homoptera, Neuroptera, Diptera and Arachnidae were identified in the field plots.