FIELD TESTING A GENETICALLY MODIFIED BACULOVIRUS

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ABSTRACT

Baculoviruses (NPVs) are invertebrate-specific pathogens of which over 500 isolates/species have been documented to date. Their invertebrate specificity offers a clear advantage toward the development of specific environmentally compatible insecticides. Toward this end, several wild-type NPVs have been registered and used commercially with limited success. The key disadvantage to their broader application is the time required to kill the target species which in some cases may take a week or more. A promising approach to remedy this problem is the insertion of genes encoding insect-specific toxins into the NPV genome. Scientists at American Cyanamid successfully inserted the insect-specific toxin gene from the scorpion *Androctonus australis* Hector (i.e., AaHIT) into the EGT gene deleted NPVs of *Autographa californica* Speyer (AcNPV) and *Helicoverpa zea* (Boddie) (i.e., HzNPV). Laboratory and greenhouse studies show that incorporation of the gene changes the viral mode-of-action enabling it to kill its host 2-3 times faster than the wild-type. However, the true test of commercial potential is efficacy in the field. Releasing a genetically engineered microorganism in the field presents special concerns from both a public perception and a scientific aspect. American Cyanamid scientists were successful in meeting both challenges and conducted field trials in the U.S. with the Acal-AaHIT construct in both 1995 and 1996.

INTRODUCTION

Baculoviruses are invertebrate-specific pathogens usually from insects with the majority of isolates found in members of the order Lepidoptera. Numerous clinical tests have been conducted on various baculoviruses against mammalian species, including rats, mice, dogs, guinea pigs, monkeys, and humans. These studies have shown no deleterious effects on any of these vertebrates (Burges *et al.,* 1980; Doller, 1985; Ignoffo & Heimpel, 1965; Ignoffo, 1973). The baculoviruses tested against these vertebrate systems were administered orally, topically, and via injection into brain, muscle, and blood. Studies have also shown that ingested baculoviruses can pass through the digestive tracts of numerous mammalian and avian species including shrews, raccoons, squirrels, and chickens without causing any harmful effects to these vertebrates (Groner, 1990; Lautenschlager & Podgwaite, 1979). The specificity of

these viruses make them ideal candidates for developing insect-specific insecticides. However, it also presents a problem when competing with present day commercial broad spectrum insecticides in that the market for a highly selective product is much more limited.

Several wild-type baculoviruses have been registered for row crop use. These include the *Helicoverpa zea* NPV [Elcar™ (Sandoz) and Gemstar™ (Biosys)], *Anagrapha falcifera* NPV (cabbage looper), *Spodoptera exigua* NPV [Spod-X™ (Biosys)], and *Autographa californica* NPV [Gusano™ (Biosys)]. The latter three viruses have only been registered in recent years. Even though baculoviruses have been investigated and utilized as insecticides for more than 20 years, their widespread use and acceptance has never been achieved due to the very slow kill which is characteristic of wild-type viruses. The advent of biotechnology provided a new opportunity to make these organisms efficient insecticides by using them to deliver a foreign gene encoding for a protein which would have a lethal impact against the infected insect. Scientists at American Cyanamid used both deletion [deletion of non-essential genes in the virus that enable it to kill more quickly, i.e., the EGT gene (O'Reilly & Miller, 1991; Treacy *et al.,* in press)] and insertion (addition of foreign genes) technologies to create a recombinant NPV. The AaHIT virus referred to throughout this paper has the EGT gene deleted. The most promising approach based on preliminary studies was the incorporation of an insect-specific toxin gene from the scorpion *Androctonus australis* (AaHIT) (Treacy & All, 1996; Hammock *et al.,* 1993; Possee *et al.,* 1993; Miller, 1995). This toxin gene was incorporated into the *A. californica* NPV (AcNPV) and was tested for efficacy in laboratory and greenhouse studies. These studies showed that the construct killed the target insect species 2-3 times faster than the wild-type, making the insect unable to cling to the plant and feed during the time in which it succumbs to the toxin. This effect of nerve toxicity and eventual death provided excellent plant protection in greenhouse and laboratory studies. The obvious final step to determine commercial potential of such a genetically modified baculovirus is field evaluation. Black *et al.* (in press) summarizes the commercialization process. However, this presented numerous unique challenges since no baculovirus containing a foreign insect-specific toxin gene had ever been field released to evaluate the commercial potential. Any such release would require regulatory approval.

TECHNOLOGY COMMUNICATION

Public Perception of Biotechnology

Biotechnology has come under public scrutiny and certain environmental activist groups have attacked biotechnology from scientific, ethical, and personal viewpoints. Although scientists have long been known to be credible sources of information, the public sector has begun to ask for more than just a scientist expressing a viewpoint based on data and hypotheses. Congressman George Brown (1987) suggested principles for dealing with the public sector which have great applicability in communicating biotechnology to the lay public. These principles are: (1) scientists

dealing with the public should avoid arrogantly dismissing lay hypotheses; (2) scientists should not ignore non-scientific points about biotechnology; and (3) never underestimate the intelligence or the power of the public. The Office of Technology Assessment (1987) further corroborated the concern the public had for biotechnology. In its report, a survey indicated that 8 of 10 Americans felt that small-scale field tests of genetically altered organisms should be permitted; however, the converse occurred when considering large-scale field trials or release -- where a majority of the people (53%) indicated that this type of release should not be permitted. Other surveys (Hoban & Kendall, 1992; Lacey *et al.*, 1991; Weber *et al.*, 1995) revealed that public perception of biotechnology is a very complex issue which included economic, moral, environmental, and health issues.

American Cyanamid was involved in earlier efforts in biotechnology to commercialize bovine somatotrophin. This issue very clearly raised the level of concern and a controversy ensued about the ethical, moral, and economic issues of a biotechnology-type product. Cyanamid scientists and management decided early on that this type of controversy had to be avoided if there was going to be any successful field testing and introduction of a recombinant NPV insecticide. Very clearly, a dialog had to be developed with those individuals and organizations which could have a positive or negative impact on the project, and that it was imperative that Cyanamid be proactive in informing the public and these organizations about the technology. Cyanamid personnel involved in the project expended significant effort in evaluating and understanding all aspects of the risk communications which might be involved which would enable them to communicate in the most effective way with the public.

Public Communication

The first steps in the communication process obviously were to identify those groups to whom the technology should be presented. Five key groups were identified -- regulatory personnel, general public, environmentalist organizations, academia personnel, and the media.

After identification of the key groups to address, the next step was to identify what information should be developed and communicated to each respective group. Numerous interviews were conducted via focus groups consisting of a cross section of individuals from the American Cyanamid Agricultural Research Center. The technology was presented, and participating individuals were asked to relay any questions or concerns as to how they viewed this technology. From these focus groups, a list of key questions was developed and answers assembled to address them. Questions for which answers were not available were set-up as research issues to be further addressed. In addition to answering questions and concerns, Cyanamid developed a video tape and brochure containing detailed information about the baculovirus project, the AaHIT toxin gene, and the engineering process, and discussed the potential benefits of this technology to agriculture and society.

Once the information was available, communication lines were opened among American Cyanamid, the U.S. Environmental Protection Agency (EPA), key scientists working on baculoviruses worldwide, environmental groups, and the public sector. This educational dialog process established a strong credibility with each of the different communities and paved the way for the first successful field testing in the U.S. of a recombinant NPV containing a toxin gene insertion. Once approval for field testing was secured from the U.S. EPA, Cyanamid scientists proceeded to inform the local public in the area where a field test was to be scheduled. The importance of this consideration was borne out by the field studies conducted in the U.K. (Cory *et al.*, 1994) where attempts were met with strong local opposition even though all legal obligations had been satisfied. A concept referred to as local Citizen's Advisory Panels was used to accomplish this local public information process. Before any recombinant NPV field test was initiated, key individuals from the area where the test was to be conducted were invited to meet and hear a presentation on the technology. At these meetings, a clear and complete presentation was made followed by an open question and answer session. The baculovirus video tape and brochure were made available to those desiring more information. This format received a very positive review from those individuals attending the meetings. The success of the program was confirmed in establishing field trials which met with virtually no resistance.

Regulatory Affairs Communication

To conduct small-scale field trials (< 10 acres) in the U.S. with a genetically manipulated microorganism containing an exogenous gene, a Notification of Intent to Field Test must be filed with the U.S. EPA. This Notification document must include information as follows: (1) identity of the microorganism; (2) description of the natural habitat of the parental strain; (3) information on host-range with an assessment of infectivity and pathogenicity to non-target organisms; (4) information on the survival and ability of the microbial pesticide to replicate in the environment; (5) identity of possible transmission vectors; (6) data on relative environmental competitiveness compared to the parental strain; (7) description of the genetic modification methods; (8) data on the potential for genetic exchange and on genetic stability of inserted sequences; (9) description of the proposed field program; and (10) a Statement of Composition for the formula to be tested. Some of the above required data were available in the voluminous database available on baculoviruses. However, numerous hours of research and data development were required to evaluate the effect of the recombinant baculovirus against non-target insects, environmental fitness, soil persistence, and host-range. For large-scale field trials (10-5000 acres), the U.S. EPA requires an Experimental Use Permit which necessitates more extensive toxicology and ecotoxicology information.

U.S. EPA officials voiced numerous concerns, especially on non-target species effects, specificity of the AaHIT toxin, competitiveness of the AaHIT containing baculovirus versus the wild-type parent, environmental persistence of the AaHIT form, and possible genetic exchange of the AaHIT gene with other viruses. To help alleviate these concerns, numerous meetings were held with EPA officials of the Biopesticides

& Pollution Prevention Division of the U.S. EPA Office of Pesticide Programs. Cyanamid scientists proceeded to address these concerns using the extensive literature database and by developing and carrying out experiments to complete voids in the database.

The effect on non-targets was addressed in the literature by Possee *et al.* (1993), McNitt *et al.* (1995) and McCutchen *et al.* (1996). Specific studies were also carried out by Cyanamid scientists to further elaborate the effect on non-targets in numerous other orders of insects as well as the earthworm (Treacy *et al.*, in press). All data in these studies clearly showed that the AaHIT recombinant baculovirus had no impact on non-permissive species.

Zlotkin *et al.* (1985, 1991, 1993) did an excellent job of demonstrating the insect specificity of the AaHIT toxin. His data clearly showed that the toxin was insect-specific, which produced two levels of safety in the rNPV (i.e., toxin specificity and baculovirus specificity).

Since the baculovirus kills its host so much more quickly via the toxin mode-of-action, fewer polyhedron inclusion bodies (PIBs) are produced. This indicated that the baculovirus would be non-competitive with its wild-type parental strain. American Cyanamid scientists designed a specific experiment to demonstrate this concept (Dierks *et al.*, in preparation). In this experiment, permissive insects were exposed to a mixture of AaHIT baculovirus and wild-type baculovirus (ratio of 10:1) on their food source. All dead and infected larvae from the first treated cohort were harvested and fed to a succeeding cohort of permissive insects. Dead insects from the second cohort were fed to a third, etc. After doing this for six generations, the AaHIT gene was undetectable in the population either by phenotype or by PCR analysis (a technique that detects the AaHIT gene). The recombinant produces 75%-95% fewer polyhedra than the wild-type. The recombinant-killed insects also do not liquify and lyse. This enables the wild-type to out-compete the recombinant. This experiment represents a worst-case scenario and would not occur in nature.

Persistence in the environment of the rNPV is an issue that is difficult to address. Baculoviruses are clearly very sensitive to UV degradation and, if left unprotected, persist only a few hours when exposed to direct sunlight. However, the polyhedra can persist for some time in the soil, which is believed to serve as a reservoir. Experiments conducted by Cyanamid scientists (Treacy *et al.*, in preparation) showed the persistence to be high, shortly after soil application, but that the persistence decreases over time to undetectable levels using the detection method of Wood *et al.* (1994).

One of the most difficult issues to answer was genetic exchange. Factually, genetic exchange does occur in nature, but barriers do arise to maintain the genetic integrity of the species. Probably the major barrier is the degree of homology of the donor to the recipient (i.e., one would expect genetic exchange to occur more frequently between genetically homologous organisms than between genetically heterologous organisms). Therefore, genetic exchange of the AaHIT gene would theoretically occur between

very closely related baculoviruses more likely than between distantly related baculoviruses, or even less likely, between baculoviruses and any other arthropod virus (e.g., granulosis virus). Bishop *et al.* (1995) reported studies which support this premise. Even if the highly unlikely successful genetic exchange with a heterologous virus did occur and produced viral progeny, the new microorganism would be genetically disadvantaged due to its reduced competitiveness. It is very clear that the AaHIT gene confers a clear competitive disadvantage upon the organism containing it.

AMERICAN CYANAMID U.S. FIELD TRIALS

American Cyanamid received approval from the U.S. EPA to conduct field studies with the Acal-AaHIT baculovirus for 1995 and 1996. The 1995 field trials consisted of two studies, one located in Georgia and one located in Texas. These were the first U.S. field trials of a toxin expressing baculovirus. Twenty trials were approved in 1996.

Materials and Methods

One of the 1995 studies was carried out in a small plot field study on the University of Georgia research farm located near Watkinsville, Georgia (Treacy & All, 1996). The plot consisted of four treatments and an untreated check compared in 3 row by 6.1 meter plots of cotton. Only the middle row of each plot received foliar application of treatments throughout the study. Treatments and untreated checks were replicated 4-fold in a randomized complete block design. The 10 plants of the middle row of each plot were artificially infested with neonate *H. virescens* larvae about 2 hours prior to each treatment. Five larvae were placed on the terminal of each of the 10 plants per plot. Treatment applications were initiated on 4-August-1995. A total of 4 weekly applications were made. All treatments were applied in water with a CO_2-powered backpack sprayer calibrated to deliver 188 liters per hectare through 3 Tee-Jet 3X hollow cone nozzles per row. The spray boom was designed to have 1 nozzle over top of the cotton row, and 1 nozzle directed at each of the 2 sides of the cotton row, dropped from the main boom and angled inward.

Each evaluation was made 6 days post-application on each of the plants in the middle row of each plot. Plant terminals, squares, flowers, bolls, and foliage were examined for the presence of live and dead arthropods as well as for any feeding damage produced 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 (DMRT).

The U.S. EPA approved up to 20 trials on 2.96 hectares in 12 states in 1996. The final count was 13 trials conducted (6 in cotton, 3 in tobacco, and 4 in lettuce/cabbage). Four of these studies have been selected as representative and represent studies conducted in cotton (Louisiana and Georgia), cabbage (New Jersey), and tobacco (North Carolina). All studies were small plot (4 rows x 22.9 meters long) with only

the center 2 rows being treated. Treatments and the untreated check were replicated 4-fold in a randomized complete block design. A gustatory stimulant [Coax™ (Lobel Chemical Corp.)] was added to the biological insecticide treatments at a 2% concentration (v/v). The minimum spray volume was 56.8 liters per hectare. Ratings consisted of plant damage and/or live insect counts. 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 (DMRT).

Field Trials Results and Discussion

In 1995, plots were artificially infested with neonate *H. virescens* on a weekly basis. However, a low density natural infestation of the test site by *H. virescens* and *H. zea* also occurred (3:1, *H. virescens:H. zea*). Statistically significant differences were found on only 1 of the 4 posttreatment sample dates (10-August-1995, Table 1). At 6 days following the first application, the Acal-AaHIT WP(I) at $2x10^{12}$ PIBs per hectare averaged significantly less *H. virescens* damaged squares, blooms, and bolls than the untreated cotton. There was a strong numerical trend indicating that the baculovirus and *Bacillus thuringiensis* (Bt) treatments reduced crop damage versus untreated throughout the study. Surveys of the field plots over the duration of the study showed that weekly applications at the high rate of Acal-AaHIT had no adverse effect on the densities of non-target arthropods. Insect families represented in this survey included Acrididae, Anthocoridae, Apidae, Braconidae, Chrysomelidae, Cicadellidae, Coccinellidae, Chrysopidae, Formicidae, Halictinae, Ichneumonidae, Lygaeidae, Miridae, Psyllidae, Reduviidae, Sarcophagidae, Tephritidae, and Vespidae. Various spiders were also observed at the test site.

Following is a summary of the key field trials conducted in 1996. These trials represent a cross section covering all crops tested.

One cotton trial (Louisiana) showed that the Acal-AaHIT alone at the high rate produced control of *H. virescens* equivalent to the full rate of the chemical standard, chlorfenapyr and was significantly better than the untreated control. The combination of Acal-AaHIT plus chlorfenapyr produced control equivalent to the full rate of chlorfenapyr and on the 9-September-1996 rating date was equivalent to the low rate of chlorfenapyr (Table 2). A second cotton study (Georgia) showed that the Acal-AaHIT alone and in combination with cypermethrin produced results comparable to the cypermethrin standard alone. Again, all treatments were significantly better than the untreated, with the exception of the low rate of the recombinant baculovirus (Table 3). Both of these studies clearly indicate that the Acal-AaHIT baculovirus alone and in combination has the potential to reduce crop damage and control the permissive insect species (*H. virescens*). Interestingly, both studies had a significant level of *H. zea* as part of the population. The data indicated that there was significant suppression of this species, even though it is known to be semi-permissive to the Acal baculovirus.

Tobacco was chosen as a target crop where the tobacco budworm, *H. virescens,* occurs as a solitary pest. It was felt at the time that the field trial protocols were

designed so that it would be necessary to have at least one crop where all target pests would be highly susceptible to the virus. This would enable the development of data without the compounding problem of crop damage caused by semi-permissive or non-permissive hosts to the virus. In the tobacco study (North Carolina), the Acal-AaHIT material at the rate of 5 x 10^{11} PIBs per hectare gave control equivalent to the commercial rates of both Bt and acephate (Table 4). These data clearly show that if the pest is susceptible to the virus, the Acal-AaHIT gives commercial control equivalent to the best standards, be they chemical or biological.

The vegetable testing presented some very challenging situations in that the pest complex that can be present at any one time can be numerous and in several situations present insects which are not susceptible to infection by the Acal-AaHIT virus (infection by the virus is essential to enable the production of toxin gene and quick kill of the pest species). In the cabbage study conducted in New Jersey, the treatments of Acal-AaHIT alone at 2 rates, Bt at the commercial rate, and an alternation of Acal-AaHIT with Bt at full rates, were evaluated against the untreated check. Very clearly, the Acal-AaHIT did not control the insect spectrum as well as did the Bt or Acal-AaHIT/Bt alternation, as indicated by the percent defoliation ratings. However, all treatments produced control significantly better than the untreated check. Control of the target species (*Trichoplusia ni*) was confounded by the occurrence of *Pieris rapae, Plutella xylostella,* and *Spodoptera* spp. which are non-permissive or at best semi-permissive to infection by the baculovirus. The baculovirus must possess a broad enough host spectrum to infect the economically important insects appearing on the crop. With the technology available today, this could be accomplished by mixing other insect viruses engineered with a toxin gene which will infect each of the respective target species. In the future, we may be able to actually engineer into one baculovirus the ability to infect non-permissive or semi-permissive species more effectively. It may be possible to actually design an insecticide to control a pest spectrum in any crop situation, still retaining the specificity of the insecticide to the target species.

CONCLUSION

Baculoviral insecticides today constitute a very small component of the global insecticide usage but the promise that genetic engineering offers to elevate these insect diseases to a world class category of insecticides is obviously on the brink of discovery. The data collected from the American Cyanamid field studies show that against a permissive insect species, the genetically engineered baculovirus is able to compete directly with chemical standards. The data also show that even against semi-permissive species the toxin producing construct shows improved activity due to the altered mode-of-action of the baculovirus insecticide. The first generation of recombinant baculovirus insecticides will likely only be able to occupy niche markets due to their limited host spectrum. However, one would anticipate there will be improvements through the isolation of baculoviruses with wider host-ranges and eventually with the manipulation of host-range possibly to be customized to control all lepidoptera in any cropping scenario. Also, more effective insect-specific toxins will be

isolated. There are numerous efforts going on in the world today to identify new toxins from the myriad of venomous arthropods that successfully use venom to capture and kill their prey. Also, improved promoter, signal sequence genes to effectively complete the toxin gene construct will be discovered which will enable quicker production and movement of the toxin to the target site within the insect host. Sufficient progress has been made to demonstrate the benefit of biotechnology in producing effective baculoviral insecticides. Use of this technology will redefine the role of baculoviruses in agriculture and provide a desirable non-chemical alternative for insect control. It is imperative that other issues such as public education, acceptance, safety concerns, and regulatory issues continually be addressed in the current and future research in this area. With the safer pesticide policies being embraced around the world, the demand for new and more effective biological insecticides will increase drastically. Once these products are available, the governmental mandates to lower chemical inputs to control pests will be achievable, and it is foreseen that regulatory bodies will provide regulatory advantages to these newer, safer products.

Table 1. Control of *H. virescens* and *H. zea* in Cotton; Oconee County, Georgia, USA (1995).

Treatment	Dosage per ha	Mean % damaged squares, bolls & blooms 10-Aug	17-Aug	24-Aug	31-Aug
Acal-AaHIT WP(I)	5×10^{11}	13.0 a b	13.9 a	10.3 a	6.5 a
	2×10^{12}	6.1 b	12.7 a	6.3 a	15.3 a
Acal-AaHIT WP(II)	2×10^{12}	12.9 a b	14.3 a	9.2 a	11.8 a
Bacillus thuringiensis 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). Approx. 2 hours before each application session, each plant terminal was artificially infested with 5 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 2. Control of Heliothine Complex in Cotton; Louisiana, USA (1996).

Treatment	Dosage per ha	Mean % damaged squares 3-Sept	9-Sept	16-Sept
Acal-AaHIT (PIBs)	5×10^{11}	27 a	36 b	46 b
	1.25×10^{12}	7 c	25 b c	20 c
Chlorfenapyr (kg)	0.23	27 a	24 b c	21 c d
	0.34	9 c	12 c	3 e
Acal-AaHIT	5×10^{11}			
+ Chlorfenapyr (PIBs+kg)	+0.23	13 b c	14 c	4 e
Untreated	na	31 a	49 a	63 a

Within column, means followed by a common letter are not significantly different ($P=0.05$, Duncan's multiple range test). Four applications at 3-5 day intervals; 70:30 ratio (*Heliothis virescens:Helicoverpa zea*).

Table 3. Control of Heliothine Complex in Cotton; Georgia, USA (1996).

Treatment	Dosage per ha	Mean % damaged squares 5-Aug	13-Aug	29-Aug
Acal-AaHIT (PIBs)	0.5×10^{12}	18 a b	28 a b	9 a b
	1.2×10^{12}	14 b	13 b c	11 a b
Cypermethrin (kg)	0.09	14 b	10 b c	8 a b
Acal-AaHIT	0.5×10^{12}			
+ Cypermethrin (PIBs+kg)	+0.09	5 b	6 c	0 b
Untreated	na	32 a	32 a	15 a

Within column, means followed by a common letter are not significantly different (P=0.05, Duncan's multiple range test). Six applications at 4-6 day intervals; 40:60 ratio (*Heliothis virescens:Helicoverpa zea*).

Table 4. Control of *H. virescens* in Tobacco; North Carolina, USA (1996).

Treatment	Dosage per ha	No. larvae/20 plants 1-July	5-July	% plants w/damage rating < 2
Acal-AaHIT (PIBs)	5×10^{11}	1.8 a	0.5 b c	90 a
Bacillus thuringiensis (kg)	1.13	1.2 b	2.5 b	97 a
Acephate (kg)	0.85	1.2 b	0.8 b c	92 a
Untreated	na	8.8 a	9.0 a	45 b

Within column, means followed by a common letter are not significantly different (P=0.05, Duncan's multiple range test). Application made 26-June-1996. "2" rating = moderate damage, many leaves w/0.5-1.0 inch holes.

Table 5. Control of Lepidopteran Complex in Cabbage; New Jersey, USA (1996).

Treatment	Dosage per ha	Season means T. ni/10 plants	No. misc. lep's./10 plants	% defoliation
Acal-AaHIT (PIBs)	1×10^{12}	8.6 b	10.0 a	13 b
	1.5×10^{12}	9.4 b	8.0 a	11 b
Bacillus thuringiensis (kg)	1.13	7.2 b	1.4 b	2 c
*Acal-AaHIT (PIBs) or	1.5×10^{12}			
Bacillus thuringiensis (kg)	or 1.13	6.2 b	3.4 b	2 c
Untreated	na	32.2 a	12.0 a	34 a

Within column, means followed by a common letter are not significantly different (P=0.05, Duncan's multiple range test). Five applications at 7 day intervals; 5 rating dates. Miscellaneous lepidoptera includes *Pieris rapae, Plutella xylostella,* and *Spodoptera* spp. "*" = the two active ingredients were alternated weekly.

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