

APPLICATION OF BIOTECHNOLOGY FOR IMPROVEMENT
OF *BACILLUS THURINGIENSIS* BASED PRODUCTS AND
THEIR USE FOR CONTROL OF LEPIDOPTERAN PESTS
IN THE CARIBBEAN

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ABSTRACT

Increased use of products based on *Bacillus thuringiensis* (*Bt*) in the Caribbean has led to increased awareness of the advantages, as well as the limitations of these biological insecticides. To improve the performance of *Bt* based products, recombinant DNA technology has been utilized to transfer the *Bt* delta endotoxin gene to microbes and plants. When the *Bt* protein is expressed in these recombinant organisms, improved delivery, persistence and insecticidal activity have been demonstrated. In this paper, the status of current research efforts to improve *Bt* will be reviewed, and future prospects for the development of products based on genetically engineered organisms will be discussed.

RESUMEN

El incremento en el uso de productos con *Bacillus thuringiensis* (*Bt*) en el Caribe, ha conducido a un incremento de las ventajas y desventajas de estos insecticidas biológicos. Para mejorar la actuación de los productos con base de *Bt*, la tecnología de DNA recombinado ha sido utilizada con el fin de transferir el gene de la endotoxina *Bt* delta a microbios y plantas. Cuando la proteína *BT* se expresa en estos organismos recombinados, se ha demostrado un aumento de persistencia y actividad insecticida. En este manuscrito, se discuten y revisan el estado de los esfuerzos de investigación actuales para mejorar *Bt* y se discuten los futuros proyectos para el desarrollo de productos basados en organismos mejorados genéticamente.

The use of insecticide products based on the naturally occurring bacterium, *Bacillus thuringiensis* (*Bt*) has increased rapidly during the last decade in the Caribbean region and worldwide. This is largely due to international demand for products, such as those based on *Bt*, that are effective but are also safe for consumers and workers, that complement rather than destroy natural enemy complexes, and that effectively control insects that have developed resistance to synthetic chemical insecticides. This demand has accelerated the adoption of *Bacillus thuringiensis* products, particularly in high value or environmentally sensitive "niche" markets such fresh market vegetables or forestry. Yet *Bt* based products also have several important limitations including a highly specific host range, short residual activity, and a unique mode of action which requires ingestion by the target insect. These limitations appear to outweigh the advantages of *Bt* in the majority of markets—particularly where profitability is lower and/or there is less demand for environmentally compatible products. To expand the use of *Bt* based products in agriculture, several projects are currently underway that utilize recombinant DNA technology, or biotechnology to improve the delivery, persistence and insecticidal activity of naturally occurring *Bt*.

In this paper, *Bt* biology and current use patterns for *Bt* based products in the Caribbean will be described. In addition, a review of progress in the development of

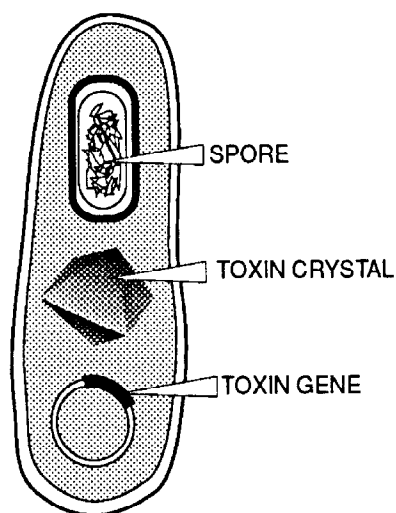


Fig. 1. Schematic diagram of a mature *Bacillus thuringiensis* cell. Contents of the cell are not drawn to scale.

improved recombinant *Bt* products, and a discussion of issues surrounding their use in the Caribbean against lepidopteran pests will be presented.

BIOLOGY OF *BACILLUS THURINGIENSIS*

Bacillus thuringiensis is a naturally occurring, Gram positive bacterium, which is most frequently found in soil. Upon maturation, each *Bt* cell produces a spore and a proteinaceous crystal or crystals that are stomach poisons for specific insects (Fig. 1). When *Bt* is commercially produced, it is grown in submerged liquid culture in large-scale fermentors where, at the completion of its growth cycle, the cells lyse or burst and release spores and protein crystals into the liquid medium. These spores and crystals are harvested and concentrated, and serve as the active ingredient of commercial *Bt* formulations (Rowe & Margaritis 1987).

In most interactions between *Bt* and insects, it is the protein crystal which is responsible for *Bt* insecticidal activity. Classified as stomach poisons, these *Bt* proteins or delta endotoxins are highly potent, with as little as 25 grams of protein required per hectare to achieve acceptable levels of insect control (Mycogen Corporation, unpublished data). When insects ingest foliage treated with *Bt*, the delta endotoxin crystal is rapidly dissolved in the insect midgut and delta endotoxin molecules bind to specific receptors on the microvillar membranes of midgut epithelial cells. The first gross symptom observed is an almost immediate (within one hour) feeding inhibition response due to paralysis of the gut. Susceptible insects rarely recover their appetites, and starvation is certainly a contributing factor in *Bt* induced mortality. However, it is the gradual disintegration of the midgut epithelium, followed by a lethal mixing of hemocoel and gut contents, that is ultimately responsible for the death of the target insect, usually 1-7 days after ingestion. (Heimpel & Angus 1959, Knowles & Ellar 1987). Although ingestion of the delta endotoxin crystals is a requirement for insect mortality, the presence of *Bt* spores may also be required for death to occur in certain insects (Heimpel & Angus 1959).

When *Bt* was first described in 1901, it was isolated from diseased silkworm (*Bombyx mori*) larvae. Since that time, thousands of *Bt* isolates have been described, with the majority having specific activity towards lepidopteran larvae such as the cabbage looper

(*Trichoplusia ni*), the diamondback moth (*Plutella xylostella*) and the imported cabbageworm (*Pieris rapae*). All *Bt* isolates are currently grouped into over 30 different varieties in a classification system based on serotyping of *Bt* flagellar proteins, insect host range, and biochemistry (Dulmage 1982). Commercial products available today are most commonly based on the lepidopteran active *Bt* variety *kurstaki*, although products based on the mosquitocidal *Bt* variety *israelensis* and on the beetle active *Bt* varieties *san diego* and *tenebrionis* are also available worldwide. In recent years, *Bt* isolates with specific activity for unique hosts such as nematodes (Edwards et al. 1990) have been described, and there is every reason to believe that many more isolates, with even more diverse activities, remain to be discovered.

An important feature of each *Bt* isolate is its relatively narrow and specific host range. Thus, *Bt* variety *kurstaki* is highly active against larvae of several noctuid pests including the cabbage looper, but has very limited activity against the closely related corn earworm, *Helicoverpa zea*. By the same token, *Bt* varieties *san diego* and *tenebrionis* are quite active against chrysomelid beetles such as the Colorado potato beetle, *Leptinotarsa decemlineata*, but have no effect on other chrysomelids such as the corn rootworm, *Diabrotica longicornis*. The basis for the specificity exhibited by each *Bt* isolate has long eluded insect pathologists. The most recent hypotheses rely primarily on the structure of the delta endotoxin and its relationship to the type and number of specific binding sites on the surface of the insect midgut epithelium. Other factors involved in *Bt* specificity include the internal environment of the insect gut, which may influence the activation of *Bt* protein crystals to delta endotoxin molecules (Johnson et al. 1990), as well as the interactions between multiple delta endotoxin molecules and between the delta endotoxin and *Bt* spores (Heimpel & Angus 1959).

USE OF COMMERCIAL *Bt* PRODUCTS IN THE CARIBBEAN

Commercial formulations of *Bacillus thuringiensis* have been available since the 1950's, and represent the most successfully commercialized group of biological insecticides available today. Relatively high levels of potency for specific pest insects, coupled with environmental, mammalian and non-target safety, have recently led to dramatic increases in sales of *Bt* based products throughout the world (McKemy 1990).

To better understand specific trends in the use of *Bt* in the Caribbean region, a survey was developed and distributed to 53 key researchers, growers, distributors, and extension agents in 15 countries. Twenty six responses were received (from Belize, Columbia, Costa Rica, Dominican Republic, Guatemala, Haiti, Honduras, Jamaica, Martinique, Panama, Puerto Rico, Trinidad, Venezuela, Virgin Islands and South Florida U.S.), and form the basis of the information presented in this section.

The large majority of *Bt* products utilized in the Caribbean are targeted for lepidopterous pests, and are based on *Bt* variety *kurstaki* according to survey participants. Products most commonly marketed in the Caribbean include Dipel® (Abbott Labs), Thuricide® and Javelin® (Sandoz Corporation), and Bactospeine® (Duphar). Among survey participants, there was little or no familiarity with mosquito and black fly active products such as Vectobac® (Abbott Laboratories), Skeetal® (Novo Laboratories), and Teknar® (Sandoz Corporation) or with beetle specific products such as M-One® (Mycogen Corporation) and Trident® (Sandoz Corporation).

Survey participants indicated that of all biological control methods available (including fungi, nematodes, beneficial insects, and baculoviruses), *Bt* based products had the most potential for success in the Caribbean. In their opinion, the most attractive features of *Bt* include (in priority order):

- 1) can be used to avoid development of resistance to synthetic insecticides
- 2) increased food, worker and environmental safety

TABLE 1. CARIBBEAN CROPS AND INSECTS, MOST COMMONLY TREATED WITH *Bt* BASED PRODUCTS. INSECTS ARE RANKED ACCORDING TO THE FREQUENCY WITH WHICH THEY ARE TREATED WITH *Bt* BASED PRODUCTS BY SURVEY PARTICIPANTS.

Crop	Insect (common name)	Insect (scientific name)
cucurbit crops (melon, cucumber, squash pumpkin)	melonworm	<i>Diaphania hyalinata</i>
	armyworms	<i>Spodoptera</i> spp.
	squash vine borer	<i>Melittia cucurbitae</i>
	cabbage looper	<i>Trichoplusia ni</i>
	<i>Heliothis, Helicoverpa</i> spp.	<i>Heliothis, Helicoverpa</i> spp.
cole crops (cabbage, broccoli, cauliflower, greens, Chinese vegetables)	diamondback moth	<i>Plutella xylostella</i>
	cabbage looper	<i>Trichoplusia ni</i>
	imported cabbageworm	<i>Pieris rapae</i>
	beet armyworm	<i>Spodoptera exigua</i>
	fall armyworm	<i>Spodoptera frugiperda</i>
tomatoes	tomato fruitworm	<i>Helicoverpa zea</i>
	armyworms	<i>Spodoptera</i> spp.
	leaf caterpillar	<i>Mocis</i> spp.
	cabbage looper	<i>Trichoplusia ni</i>
corn	corn earworm	<i>Helicoverpa zea</i>
	fall armyworm	<i>Spodoptera frugiperda</i>
	beet armyworm	<i>Spodoptera exigua</i>
ornamentals	banana moth	<i>Opogona sacchari</i>
cassava	cassava horn worm	<i>Erinnyis ello</i>

- 3) lack of toxic pesticide residues
- 4) can be used to preserve beneficial insects

Consumer demand and restrictive government regulations were not considered important factors in the adoption of *Bt* products.

When asked to list insects and crops where *Bt* based products are used, insects resistant to synthetic chemical insecticides (e.g., the diamondback moth, the beet armyworm, *Spodoptera exigua*, the cabbage looper and *Heliothis* and *Helicoverpa* species) on fresh market produce were most frequently mentioned (Table 1). It is interesting to note that although these resistant pests occur on a variety of crops including cotton, sorghum and grains, *Bt* products were most frequently used on higher value crops such as fresh market produce and ornamentals.

Survey participants cited several reasons why *Bt* based products are not more widely adopted in the Caribbean including (in priority order):

- 1) narrow host range
- 2) relatively high cost of *Bt* based products compared to conventional insecticides
- 3) lack of efficacy compared to chemical insecticides
- 4) increased management inputs (scouting to determine correct timing of applications, improved application equipment to provide better foliar coverage) required
- 5) short residual activity
- 6) inability to target internally feeding (cryptic) insects

APPLICATION OF BIOTECHNOLOGY IN THE IMPROVEMENT OF
Bt BASED PRODUCTS

To resolve problems such as the above which are associated with the use of *Bt* based insecticides, several research programs have focused on the use of recombinant DNA technology, or biotechnology. For most isolates of *Bt*, the delta endotoxin gene is coded for on extrachromosomal plasmids, making it possible for genetic engineers to locate, manipulate and transfer the gene to other organisms including bacteria, viruses and plants. In this way, scientists hope to improve *Bt* delivery, persistence and insecticidal activity (Table 2).

Delivery

Because *Bt* based products must be ingested to be effective, insects that feed internally within the plant or the roots cannot be controlled by conventional *Bt* foliar applications. In addition, even those insects which feed on the outer plant surface are often difficult to control well because precise application timing (when the majority of the insects are newly hatched and therefore most sensitive) and excellent foliar coverage are required for optimum control with *Bt* products. Delivery of delta endotoxins can be improved, however, if microorganisms associated with the plant, or the plant itself, are engineered to produce *Bt* endotoxins. For example, larvae of the European corn borer, *Ostrinia nubilalis*, most commonly feed within the stalk, the ear or the stem of corn and other plants. Although these insects are quite susceptible to *Bt* variety *kurstaki*, they are impossible to target with foliar applications of *Bt* once they have bored inside the plant. To improve delivery of *Bt* toxins inside the plant, Crop Genetics International Corporation has developed the InCide™ biopesticide technology which is based on the endophytic (vascular system colonizing) bacterium, *Clavibacter xyli* subsp. *cynodontis*; this microorganism has been engineered to produce a *Bt* variety *kurstaki* endotoxin. When corn seed is treated with the engineered *C. xyli*, the bacteria multiply and produce the delta endotoxin inside the growing corn plant. Several outdoor field trials have been conducted with the InCide technology which demonstrate that corn plants inoculated with the recombinant *C. xyli* had significantly less damage by the European corn borer than untreated plants. Expanded field tests and attempts to increase delta endotoxin expression levels in *C. xyli* are continuing during 1991 (Beach 1990). By genetically modifying root colonizing bacteria such as *Pseudomonas fluorescens* to produce *Bt* variety *kurstaki* endotoxin, Monsanto scientists have developed a different delivery system—one that targets soil insects such as cutworms (Obucowicz et al. 1987). In another example of improved delivery systems, companies such as Monsanto and Plant Genetic Systems are currently developing recombinant plants that have been engineered to produce various *Bt* endotoxins in plant tissues. Successful outdoor field tests with transgenic tobacco and tomatoes have effectively targeted such cryptic pests as tomato pinworm, *Keiferia lycopersicella* and tobacco budworm, *Heliothis virescens* (Fischhoff et al. 1987, Gasser & Fraley 1989, Honee et al. 1989, Vaeck et al. 1987).

Persistence

For conventional *Bt* based products, the active ingredient or delta endotoxin is present as a "naked" or unprotected protein crystal. Not surprisingly, the endotoxin protein crystal breaks down rapidly on the surface of the crop plant—usually within 1-2 days—as the result of harsh or inactivating light, microbial and plant enzymes, pH extremes, moisture and high temperatures (Gelernter 1990). Because of their short residual activity, *Bt* products must be applied more frequently and with greater atten-

TABLE 2. USE OF GENETIC ENGINEERING TO IMPROVE PERFORMANCE OF *Bt* DELTA ENDOTOXINS AGAINST LEPIDOPTERAN LARVAE.

Environmental Target	Target Insect	<i>Bt</i> variety	Transformed Host Organisms	Advantage	Author
vascular system of corn plants	larvae of <i>Ostrinia nubilalis</i> (European corn borer)	<i>kurstaki</i>	vascular system colonizing bacterium <i>Clavibacter xyli</i> subsp. <i>cynodontis</i>	delivery to internally feeding insects	Beach, 1990
soil (corn roots)	soil caterpillars	<i>kurstaki</i>	root colonizing bacterium, <i>Pseudomonas fluorescens</i>	persistence, delivery to soil	Obukowicz et al., 1987
foliage	caterpillars (e.g., <i>Manduca sexta</i> , <i>Keiferia lycopersicella</i> , <i>Helicoverpa virescens</i>)	<i>kurstaki</i>	tobacco, tomato, potato, cotton plants	foliar persistence, delivery to internally feeding insects	Fischhoff et al., 1987; Gasser and Fraley, 1989; Honee et al., 1989; Vaeck et al., 1987
foliage	caterpillars (e.g., <i>Plutella xylostella</i>)	<i>kurstaki</i>	<i>Pseudomonas fluorescens</i> (killed) CellCap encapsulation system	foliar persistence	Barnes and Cummings, 1987
foliage	beetles (e.g., <i>Leptinotarsa decemlineata</i>)	<i>san diego</i>			
foliage	foliar feeding caterpillars (e.g., <i>Trichoplusia ni</i>)	<i>kurstaki</i>	<i>Autographa californica</i> nuclear polyhedrosis virus	inhibit insect feeding; reduce LT ₅₀ and increase potency; target larger larvae	Merryweather et al., 1990
foliage	foliar feeding caterpillars (e.g., <i>Pieris brassicae</i>)	<i>aizawai</i>	<i>Autographa californica</i> nuclear polyhedrosis virus	inhibit insect feeding; reduce LT ₅₀ and increase potency; target larger larvae	Martens et al., 1990
foliage	caterpillars (e.g., <i>Pieris</i>), beetles (e.g., <i>Phaedon</i>), mosquitoes (e.g., <i>Aedes</i>)	<i>aizawai</i>	<i>Bt</i> variety <i>tenebrionis</i>	expanded host range; synergy of delta endotoxin mixture	Crickmore, et al., 1990
foliage, water	caterpillars (e.g., <i>Pieris</i>), beetles (e.g., <i>Phaedon</i>), mosquitoes (e.g., <i>Aedes</i>)	<i>tenebrionis</i>	<i>Bt</i> variety <i>israelensis</i>	expanded host range; synergy of delta endotoxin mixture	Crickmore, et al., 1990

tion to insect developmental stage than conventional synthetic chemical products. Several of the technologies described above, including development of *Bt* expressing plants and plant colonizing bacteria, address the issue of improved persistence as well as enhanced delivery of delta endotoxins. A somewhat different approach, the CellCap™ encapsulation system developed by Mycogen Corporation, utilizes dead bacterial cell walls to protect delta endotoxins from environmental degradation. In this system, *Bt* delta endotoxin genes are transferred from *Bt* to a non pathogenic isolate of *Pseudomonas fluorescens*. When the recombinant cells are grown in fermentors, the endotoxin is produced and forms a crystal within the cell. Unlike *Bt*, the recombinant *Pseudomonas* cells do not break apart at the completion of the growth cycle. Instead, while still in the fermentation tank, the intact cells are subjected to a chemical treatment which kills and fixes the *Pseudomonas* cell wall, causing it to become thicker and more rigid through cross linking of cell wall components. The rigid wall of the dead *Pseudomonas* cell then serves as a protective biological microcapsule for the enclosed toxin (Barnes & Cummings 1987). Replicated small plot and large scale grower trials conducted over the past three years have demonstrated that the CellCap encapsulation system results in a two fold improvement in residual activity of the *Bt* delta endotoxins, compared to that for the nonencapsulated endotoxins present in conventional *Bt* formulations. Mycogen has developed several products based on the CellCap encapsulation system including MVP® Bioinsecticide, based on an encapsulated delta endotoxin from *Bt* variety *kurstaki* and targeted against caterpillar larvae, and M-Trak™ Bioinsecticide which controls beetle pests such as the Colorado potato beetle and is based on an encapsulated delta endotoxin from *Bt* variety *san diego*. Because these engineered products contain only dead microorganisms, concerns regarding the release and spread of living recombinants are not considered pertinent by regulatory agencies. In 1991, MVP and M-Trak became the first genetically engineered biopesticides to be registered for use by the United States Environmental Protection Agency.

Improved Insecticidal Activity:

Bt based products are most effective against newly hatched and small larvae. However, as insects mature, they grow increasingly more able to tolerate or overcome intoxication by *Bt* (Zehnder & Gelernter 1989, Bauer 1990). Insect specific baculoviruses, on the other hand, are capable of killing large lepidopteran larvae but their ingestion does not result in the same feeding inhibition response as *Bt*; this can result in insect feeding damage to the crop in the 2 to 10 days elapsing between virus ingestion and mortality. By developing recombinant baculoviruses that have been engineered to produce *Bt* delta endotoxin genes, it might be possible to achieve the best of both worlds—the efficacy of baculoviruses against large larvae, and the effects of *Bt* induced feeding inhibition and midgut disruption. Two research groups (Martens et al. 1990, Merryweather et al. 1990) have recently successfully engineered the *Autographa californica* nuclear polyhedrosis virus to produce *Bt* delta endotoxins from *Bt* varieties *aizawai* and *Bt kurstaki*. However, protein expression levels in the recombinant viruses are not optimized at this early stage in development, and it therefore still too early to accurately assess their efficacy.

Host Range

By transferring the delta endotoxin gene from the caterpillar active *Bt* variety *aizawai* into the beetle active *Bt* variety *tenebrionis*, Crickmore et al. (1990) have demonstrated improved activity on both caterpillar, mosquito and beetle pests with the transgenic bacterium, indicating that the delta endotoxin proteins may interact syner-

gistically. Similarly, Crickmore et al. (1990) have transferred the *Bt* variety *tenebrionis* endotoxin gene into mosquito active *Bt* variety *israelensis*, producing cells that have beetle and mosquito activity, as well as unexpected lepidopteran activity. Carlton et al. (1990) have taken a different approach towards improving the host range of *Bt* isolates where recombinant DNA technology is not utilized. In this case, the natural process of bacterial conjugation is utilized to transfer an entire *Bt* plasmid from one *Bt* to another. The resulting "genetically manipulated" or "transconjugant" organism now produces two different delta endotoxins—one from each "parent". This technique has allowed the biopesticide company Ecogen to successfully develop products with expanded host ranges. For example, the product Foil® is based upon a transconjugant *Bt* strain that produces two delta endotoxin crystals; one beetle active, and the other caterpillar active (Carlton et al. 1990).

ISSUES SURROUNDING THE USE OF BIOTECHNOLOGY FOR IMPROVING *Bt* BASED PRODUCTS

Recombinant DNA technology allows scientists to develop significantly improved biological insecticides. With this technology, the quality, quantity and diversity of efficacious biological insecticides available should dramatically increase over the next 20 years. Yet there are several issues which deserve continuing scrutiny and discussion as we progress towards this goal. These include:

Setting Realistic Expectations On Timing Of New Product Introductions

Although great progress has been made in the past five years towards development of improved *Bt* based insecticides, there are several technical and regulatory hurdles which will limit the number of genetically engineered products commercialized over the five to ten years. For example, many of the projects described above have not yet produced organisms with sufficiently high yields of delta endotoxin, and field efficacy has been demonstrated in only a few cases. Additionally, the United States and most European countries have not yet registered any products based on living recombinant microorganisms, and have only recently begun to develop guidelines for their testing and registration. Until these hurdles are overcome, the result will be a relatively slower paced, more gradual schedule of product introductions during the next several years.

Setting Realistic Expectations On Performance Of New Products

Too often, new technologies are positioned as a panacea. In the case of agricultural "biotech" products, it is important to establish that the performance of biological pesticides, whether they are engineered or not, will probably never quite equal the broad spectrum, fast acting toxicity of synthetic chemical insecticides. By their very nature, biopesticides will be more selective, and more sensitive to environmental conditions than their synthetic chemical counterparts. Thus, growers must still be trained in the proper use of these new products and must understand that increased management and scouting inputs are required for best results.

Avoiding Resistance

The tendency to over-use new, effective products has led to many incidences of insect resistance to formerly effective chemical products (Roush & Tabashnik 1990). This unfortunate trend was recently documented for certain conventional *Bt* products (Shelton & Wyman 1992, Tabashnik et al. 1991) which are used up to 25 times per year

for control of insecticide resistant diamondback moth larvae. Under this type of selection pressure, diamondback moth tolerance or resistance to *Bt* variety *kurstaki* has now been documented in at least two states within the U.S. (Florida and Hawaii). It is probable that *Bt* toxins will become more widely distributed and utilized in the near future. This, combined with the fact that persistence of toxins expressed in living recombinant plants or microorganisms is greatly enhanced, will increase the likelihood of the development of *Bt* resistance. Efforts to better understand the basis of *Bt* activity and resistance, and development of programs which incorporate other cultural and biological control measures into programs that currently rely heavily on *Bt* based products are essential if we are committed to preserving the use of *Bt* delta endotoxins for the future.

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BIOLOGICAL CONTROL OF INSECT PESTS OF CRUCIFERS IN SELECTED WEST INDIAN ISLANDS

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ABSTRACT

In the Caribbean, cole crops are attacked by many insects and other pests, most of which are controlled by insecticides. This paper provides data on the potentials for biological control of diamondback moth, *Plutella xylostella*, cabbage looper, *Trichoplusia ni*, Armyworms, *Spodoptera* spp., Cabbage white butterfly, *Ascia monuste monuste*, Cabbage budworm, *Hellula phidilealis*, and Cabbage aphid, *Brevicoryne brassicae*.