

Biological Control of Mosquito Larvae by *Bacillus thuringiensis* subsp. *israelensis*

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1. Introduction

Chemical insecticides provide many benefits to food production and human health and has proven very effective at increasing agriculture and forestry productivities. However, they also pose some hazards as contamination of water and food sources, poisoning of non-target fauna and flora, concentration in the food chain and selection of insect pest populations resistant to the chemical insecticides (Wojciech & Korsten 2002). It is well documented that chemical pesticides reduced natural-enemy populations and chemical applications can disrupt biological control and may cause outbreaks of secondary pests previously suppressed by natural enemies (Bartlett, 1964) and pest species develop pesticide resistance but natural enemies not (Johnson & Tabashnick, 1999).

The use of synthetic organic pesticides has had serious economic, social and environmental ramifications. Economically, the rapidly increasing cost for development and production of petrochemically derived insecticides, together with the declining effectiveness due to widespread insect resistance. As a result the chemical pesticide industry continues to develop new more expensive compounds and increasing pesticide prices. Socially and ecologically they have caused death and disease in human and damaged the environment. It is estimated that only a minute fraction of the insecticides applied is required for suppression of the target pest. The remainder, more than 99.9%, enters the environment through soil, water and food cycles (Metcalf, 1986).

Alternative methods of insect management offer adequate levels of pest control and pose fewer hazards. One such alternative is the use of microbial insecticides, that contain microorganisms or their by-products. Microbial insecticides are especially valuable because their toxicity to non-target animals and humans is extremely low. Compared to other commonly used insecticides, they are safe for both, the pesticide user and consumers of treated crops. Microbial insecticides also are known as biological pathogens, and biological control agents. Chemical insecticides are far more commonly used in the world than microbial control, however some microbial control agents, at least in part, can be used to replace some hazardous chemical pest control agents. A number of biological control agents formulated with bacteria, fungi, virus, pheromones, and plant extracts have been in use mainly for the control of insects responsible for the destruction of forests and agriculture crops (McDonald & Linde, 2002).

The microbial insecticides most widely used in the world are preparations of *Bacillus thuringiensis* (*Bt*). The insecticidal activity of *Bt* is due to the proteic parasporal inclusions that are produced during sporulation. Insecticides based on the proteinaceous δ -endotoxin of *Bt* constitute part of a more ecologically rational pest control strategy. *Bt* strains have been isolated world wide from many habitats, including soil, insects, stored-product dust, and deciduous and coniferous leaves, all of which have a limited host range, however together span a wide range of insects orders which include: Lepidoptera, Diptera, Coleoptera, Hymenoptera, Homoptera, Phthiraptera, Orthoptera, Acari, and Mallophaga and other organisms such as nematodes, mites, and protozoa (Federici, 1999). *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) or serotype H-14, exhibit acute toxicity towards dipteran insects such as larval mosquitoes and black flies (de Barjac 1978) and is currently used in mosquito control programs world wide (Priest, 1992). The World Health Organization's (WHO) Onchocerciasis Control Program (OCP) in West Africa using *Bti* toxins has been one of the success stories of international co-operation in the control of infectious diseases program (Webb 1992; Drobniowski 1993). Due to the importance of *Bti* to control several tropical diseases such malaria and dengue, our purpose in this chapter is to provide an overview for non-*Bt* specialists of the basic knowledge of *Bti*.

2. *Bacillus thuringiensis* strains

2.1 Origin of some strains of *B. thuringiensis*

A large number of strain of *Bt* have been isolated from which to date. *Bt* as currently recognized is actually a complex of subspecies. They have grouped in 79 serotypes (Zeigler, 1999). The first *Bt* strain was isolated from diseased larvae of the silkworm, *Bombi mori*, in Japan by Ishiwata (1901). Iwabushi (1908) describe the bacillus as *Bacillus sotto*. Aoki & Chigasaki (1915) and Mitani & Wataral (1916) purified a highly toxic substance from sporulated *Bt* cultures. It was not officially described, however, until it was reisolated by Berliner in 1915 from diseased larvae of the Mediterranean flour moth, *Anagasta kuehniella*, in Thuringia, Germany, hence the derivation of species name *thuringiensis* (Federici, 1999). A *Bt* strain was isolated again by Mattes (1927) and described briefly the inclusion rhomboidal body. The activity of the *Bt* strains against lepidopteran larvae was described by Metalnikov (1930) and by Husz (1931). The association of the inclusion bodies of *B. thuringiensis* with toxicity against insects was established by Steinhaus. In 1951 published a paper which described the morphology of *Bt* and its possible use in the biological control against the alfalfa caterpillar. In France, a product named "Sporeine" was developed and used against *Ephestia kuhniella* (Lepidoptera) (Jacobs, 1950). Hannay, (1953) described a parasporal body in bipyramidal shape produced by the bacterium during sporulation and suggested that the crystal was involved in the toxic activity. The protein nature of the crystals was determined by Hanna and Fitz-James (1955).

2.2 Features of *B. thuringiensis* strains

Bt is a facultative anaerobic, gram-positive bacterium that forms characteristic protein inclusions adjacent to the endospore (Fig.1). The crystalline inclusion are composed of proteins known as ICPs crystal proteins. Cry proteins, or δ -endotoxins is the basis for commercial insecticidal formulations of *Bt*. Insecticides containing *Bt* in pest control programs is now considered as a viable strategy, which has proven to be both safe and reliable over the last 45 years (Chungjatupornchai et al. 1988).

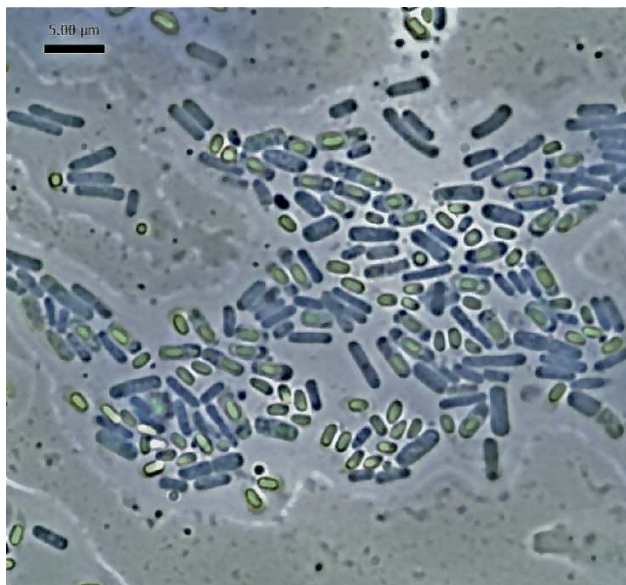


Fig. 1. *Bacillus thuringiensis* subsp. *israelensis*.

2.3 Advantages and disadvantages of *Bt*

According to Federici (1999) the main reasons for the success of *Bt* include (i) the high efficacy of its insecticidal proteins (ii) the existence of a diversity of proteins that are effective against a range of important pests (iii) its relatively safety to nontarget insect predators and parasites (iv) its easy to mass production at a relatively low cost, and (v) its adaptability to conventional formulations and application technology.

Advantages and disadvantages of *Bt* with chemical insecticides were summarized by Rowe and Margaritis (1987).

Advantages of *Bt*:

- High specificity, i.e. no mammalian or nontarget effects; use permitted up to date of harvest.
- No broad insect resistance observed or expected to develop.
- Adaptable to many types of formulations; potential to incorporate feeding stimulants or baits to increase the attractiveness of the formulations to the insects and thereby increase their efficacy.
- Probability of producing more potent formulations and reducing production costs through improved-fermentation technology.
- High probability that strain selection and/or genetic engineering will lead to better control of pest insects by newly found or created strains of *Bt* having novel host spectra or increased activity

Disadvantages of *Bt*:

- Narrow host spectrum
- Lack of patent protection on new strains
- Proper timing of application require due to slower effect than chemical insecticides

- Activity dependent of ingestion, and feeding activity depends on environmental conditions
- Relatively higher cost compared to chemical insecticides

2.4 Clasification of strains of *Bacillus thuringiensis*

Bt is a crystalliferous spore-forming bacterium close genetic relationship with *B. cereus*, *B. anthracis* and *B. mycooides* (Höffe & Whiteley, 1989). The classification of *Bt* is difficult because DNA sequencing studies of conserved gene regions of these species have suggested that they belong to a single specie. *Bt* strains are distinguished from *B. cereus*, *B. anthracis* and *B. Mycooides* by the ability to produce parasporal crystalline inclusions during sporulation (de Barjac, 1978). Crystal formation is the criterion for distinguishing between *B. cereus* and *Bt*, otherwise they could be considered as the same specie. Research based on a comparative study of 16s rRNA sequences, *Bt* and *B. cereus* var. *mycooides* differed from each other and from *B. anthracis* and *cereus* by less than nine nucleotides (Ash and Collins 1992). Chen & Tsen (2002) amplified 16S rDNA and *gyrB* gene by PCR and they found that the discrimination between *B. cereus* and *Bt* strains, when a large number of *Bacillus* strains were tested was difficult. They proposed, to distinguish *Bt* from *B. cereus*, a single feature, such as the presence of a parasporal crystal protein or cry gene is reliable.

Several attempts were made to classify *Bt* strains. de Barjac and Frachon (1990) didn't find correlation between biochemical reactions and 27 H serotypes using 1,600 *B. thuringiensis* isolates. They demonstrated that the current biochemical tests have no value as the sole criteria for differentiating *Bt* strains. Another approach of these authors for classification was the use of susceptibility to certain bacterial viruses called phages. There are 14 bacteriophages that have been used for *Bt* but phage typing is inconsistent with serotyping and does not permit classification. They found frequent cross-reactions.

One widely clasification system for *Bt* strains is based in the determination of the H-flagellar antigen technique described by Barjac & Bonnefoi (1962). This technique needs very motile bacterial cultures to prepare flagellar suspensions. These suspensions are titrated against antisera directed against *B. thuringiensis* strains of each serotype. Presently *B. thuringiensis* strains are classified within 79 serotypes. Table 1 shows the classification of *Bt* strains by serotypes.

Bt strains that had discovered previously to 1977 were pathogens towards lepidopteran larvae. However, in 1975 was discovered *B. thuringiensis* serovar *israelensis* toxic to mosquito larvae (Goldberg & Margarit 1977) and in 1983 a strain from *Bt* serovar *morrisoni* was found to be pathogenic to Coleoptera larvae (Krieg et al. 1983). According to these findings, serological clasification, although still in use as a basic method to clasify *Bt* strains could not be related with pathogenicity. Subsequently studies showed that, within a serotype, different activity, spectra can be found. For example, some strains of *B. thuringiensis* serotype *morrisoni* in their parasporal inclusion bodies contain different proteins and have activity against Diptera, Coleoptera or Lepidoptera.

With the knowledge of sequence of the genes that encode the proteins cry it was proposed a classification based on the cry toxin genes. Höfte and Whiteley (1989) proposed a nomenclature clasification scheme for *Bt* crystal proteins based in their structural aminoacid sequence, deduced from the DNA and host range. They named *cry* (crystal protein) genes and their related proteins, "Cry proteins". They clasified 42 *Bt* crystal protein genes into 14 distinct genes grouped into 4 major clases. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera-and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Dipter-specific).

Serotype	Serovar	Serotype	Serovar
1	thuringiensis	28a, 28c	jeghatensan
2	finitimus	29	amagiensis
3a, 3b, 3c	kurstaki	31	toguchini
3a, 3c	alesti	32	cameroun
3a, 3d	sumiyoshiensis	33	leesis
3a, 3d, 3e	fukuokaensis	34	konkukian
4a, 4b	soto/dendrolimus	35	seoulensis
4a, 4c	kenyae	36	malayensis
5a, 5b	galleriae	37	andralousiensis
5a,5c	canadensis	38	owaldocruzi
6	entomocidus/subtoxicus	39	brasilensis
7	aizawai/pacificus	40	huazhongensis
8a,8b	morrisoni	41	sooncheon
8a, 8c	ostrinae	42	jinghongiensis
8b, 8d	nigeriensis	43	guiyangiensis
9	tolworthi	44	higo
10a, 10b	darmstadiensis	45	roskildensis
10a, 10c	londrina	46	chanpaisis
11a, 11b	toumanoffi	47	wratislaviensis
11a, 11c	kyushuensis	48	balearica
12	thompsoni	49	muju
13	pakistani	50	navarrens
14	israelensis	51	xiaguangiensis
15	dakota	52	kim
16	indiana	53	asturiensis
17	tohokuensis	54	poloniensis
18a, 18b	kumamotoensis	55	palmanyolensis
18a, 18c	yosoo	56	rongseni
19	tochigiensis	57	pirenaica
20a, 20b	yunnanensis	58	argentiniensis
20a, 20c	pondicheriensis	59	iberica
21	colmeri	60	pingluonsis
22	shanongiensis	61	sylvestriensis
23	japonensis	62	zhaodongensis
24a, 24b	neolonensis	64	azorensis
24a, 24c	novosibirsk	65	pulsiensis
25	coreanensis	66	graciosensis
26	silo	67	vazensis
27	mexicanensis	none	wuhanensis
28a, 28b	monterrey		

Table 1. Classification of *B.thuringiensis* strains by serotype (modified after Zeigler, 1999).

The *cryI* genes can be distinguished from the other *cry* genes simply by sequence homology (>50% aminoacid identity) and encode 130 to 140 kDa proteins which accumulate in

bipyramidal crystalline inclusions during the sporulation of *Bt. cryII* genes encode 65-kDa proteins which form cuboidal inclusions in strains of several species. *cryIII* genes produces rhomboidal crystals containing one major protein, a 72kDa protein. *cryIV* class genes (*cryIVA*, *cryIVB*, *cryIVC*, and *cryIVD*) as well as *cytA* were all isolated from the same 72-Mda plasmid present in *Bt* subsp. *israelensis*.

3. *Bacillus thuringiensis* subsp. *israelensis* as an important part of mosquito control

3.1 Properties of *Bti*

In 1975-76 under a World Health Organization sponsored project, a new *Bt* strain was discovered in the Negev desert in Israel by Goldberg and Margalit (1977). The strain was isolated from *Culex* sp. dead larvae mosquito. Later was identified as *Bt israelensis*, serotype H14 according to its flagellar antigenicity by de Barjac (1978).

Bti has all the features taxonomic, morphological, growth, sporulation, of isolation, cultivation of other varieties of *B. thuringiensis* (Fig.1). The larvicidal activity of *Bt israelensis* on mosquito transmitted diseases was the most important feature of the strain. The insecticidal properties of this bacteria are due primarily to insecticidal proteins produced during sporulation. The key proteins are Cyt1A(27.3 kDa), Cry4A (128 kDa), Cry4B (134 kDa) and Cry11A (72 kDa) and in three different inclusion types assembled into a spherical parasporal body held together by lamellar envelope (Ibarra & Federici, 1986). The inclusions are relatively small (0.1 to 0.5 μ m) and there are usually two to four inclusions per cell which vary in shape from cuboidal to bipyramidal, ovoid or anamorph (Charles & de Barjac 1982; Mikola *et al.* 1982; Yamamoto *et al.* 1983)

3.2 Mosquitoes, important vectors of tropical diseases

Mosquitoes are important vectors of several tropical diseases that suck blood from human and animals. They are vectors of multiple of diseases of man through transmission of pathogenic viruses, bacteria, protozoa and nematodes (Priest, 1992). From the medical point of view, mosquitoes are among the most important insects due their capacity to transmit human diseases such as malaria and dengue.

Vector	Disease
<i>Anopheles</i>	Malaria, lymphatic filariasis
<i>Culex</i>	Lymphatic filariasis, Japanese encephalitis, other viral diseases
<i>Aedes</i>	Yellow fever, dengue, dengue hemorrhagic fever, other viral diseases, lymphatic filariasis
<i>Mansonia</i>	Lymphatic filariasis

Table 2. Some diseases transmitted by mosquito (Rawlins, 1989; Walsh 1986).

There are about 3000 species of mosquito, of which about 100 are vectors of human diseases. Some of the more important of these diseases are listed in Table 2. It is estimated two billion people worldwide living in areas where these are endemic (World Health Organization, 1999). Thus, there is an urgent need for new agents and strategies to control these diseases.

3.3 Susceptibility of mosquito species to *B. thuringiensis* serotype *israelensis*

Bti is highly pathogenic against *Culicidae* (mosquitoes) and *Simuliidae* (blackflies), and has some virulence against certain others Diptera, especially Chironomidae (midges). Mosquito have four distinct stages in their life cycle: egg, larva, pupa and adult. Depending on the specie a female lays between 30 and 300 eggs at a time on the surface of the water, singly (*Anopheles*), in floating rafts (*Culex*) or just above the water line or on wet mud (*Aedes*). Once hatched the larvae grow in four different stages (instars). The first instar measures 1.5 mm in length, the fourth instar about 8-10 mm. The fully grown larvae then changes into a comma shaped pupa. When mature, the pupal skin splits at one end and a fully developed adult emerges. The entire period from egg to adult takes about 7-13 days under good conditions (Wada, 1989). First instar is more susceptible to *Bti* than fourth instar (Mulla *et al.* 1990). Pupa does not feed and therefore is not affected by *Bti*. For almost all species tested, increasing age of the larvae resulted in reduced susceptibility in mosquito (Chen *et al.* 1984; Mulla *et al.* 1985). *Bti* was found to be specific toxic to larvae of 109 mosquito species (Table 3).

Mosquito genus	Species
<i>Aedes</i>	40
<i>Anopheles</i>	27
<i>Culex</i>	19
<i>Culiseta</i>	5
<i>Mansonia</i>	5
<i>Psorophora</i>	3
<i>Armigeres</i>	3
<i>Toxorhynchites</i>	2
<i>Limatus</i>	2
<i>Trichophospon</i>	1
<i>Uranotaenia</i>	1
<i>Tripteroides</i>	1
	Total 109

Table 3. Larvicidal activity of *Bti* on mosquito species (Glare & O'Callaghan 1998).

Among mosquitoes, different preparations of *Bti* have shown different levels of toxicity to host species. Others factors influencing the susceptibility of mosquito larva to *Bti*. For example, the effect of a given dosage of toxin could produce different results depending on weather the lethal dose is administered all at once or in same doses over a long period (Aly *et al.* 1988).

Bti has an LC50 in the range of 10-13 ng/ml against the fourth instar of many mosquito species (Federici *et al.* 2003). Generally, *Culex* and *Aedes* are highly susceptible while *Anopheles* are less susceptible, but can be killed with *Bti* (Balaraman *et al.* 1983). Much higher concentrations of *Bti* are required to induce mortality in anopheline larvae than in *Aedes aegypti* larvae (Goldberg & Margalit 1977; Nugud & White 1982)

The exception is *A. franciscanus* that is as susceptible as other genera (García *et al.* 1980, Sun *et al.* 1980). Furthermore, even within one genus, some species are more susceptible than others (Chui *et al.* 1993). Sun *et al.* (1980) suggested that a difference in feeding behavior might account for differences in susceptibilities. Filtering rates vary between genera and species. For example Aly (1988) showed that in the absence of *Bti*, larvae cleared the suspensions with constant relative filtration rates of 632 (*Ae. aegypti*), 515 (*Cx. quinquefasciatus*) or 83.9

$\mu\text{L}/\text{Larvae}/\text{h}$ (*An. albimanus*). Another factor to be considered for the susceptibility to *Bti* is the behavior of the larvae. *Anopheles* larvae filter-feed on food particles present at the surface of water or a few centimeters below it, whereas *Culex* and *Aedes* larvae not only feed faster but are capable of filter-feeding at much deeper water depths (Aly et al. 1988).

Mosquito species which are not filter feeders do not seem susceptible. For example, against *Culicoides occidentalis* (Colwell 1982) and *Coquillettidia perturbans*, *Bti* larvicides had no effect (Walker et al 1985). Solubilization of the protein reduced the toxicity dramatically (50- to -100 fold) this is attributed to reduced level of toxin ingestion by larvae owing to their filter feeding behaviour (Chungjatupornchai et al. 1988).

3.4 Susceptibility of mosquito species to purified *Bt* proteic crystals

Several efforts have been made to purify the proteins Cry of *Bti* and other *Bt* subps. with the purpose of studying the chemistry of Cry proteins, the synergism between them, and the effect of each crystal on larvae of different mosquito species. It is somewhat difficult to separate the *Bt* spores and crystals because they are of similar size and surface characteristics. For that reason several methods have been used to purify *Bt* crystals proteins. Using NaBr gradients (Chang et al. 1992), sucrose gradients (Debro et al. 1986), renografin gradients (Aronson et al. 1991), and in a separatory funnels (Delafield et al. 1968). Bioassays of purified Cry proteins have been allowed to know that not only *Bti* Cry proteins have activity on mosquito larva. Other purified Cry proteins of other *Bt* strains have also activity on mosquito larvae. Table 4 shows the mosquito toxicity of purified crystals of some *Bt* strains.

Name	Source Strain	Mosquito toxicity
Cry4Aa1	<i>B.t. israelensis</i> 4Q2-72	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cry4Ba1	<i>B.t. israelensis</i> 4Q2-72	<i>Aedes aegypti</i> (Diptera: Cuclidae)
Cry10Aa1	<i>B.t. israelensis</i> ONR60A	<i>Aedes aegypti</i> , (Diptera: Cuclidae)
Cry11Aa1	<i>B.t. israelensis</i> HD-567	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cry11Ba1	<i>B.t. jethansan</i> 367	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cry11Bb1	<i>B.t. medellin</i>	<i>Aedes aegypti</i> , <i>Anopheles albimanus</i> , <i>Culex quinquefasciatus</i> (Diptera: Cuclidae)
Cry16Aa1	<i>Clostridium bifermentans malasya</i> CH18	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cry19Aa1	<i>B.t. jethesan</i>	<i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cry20Aa1	<i>B.t. fukuokaensis</i>	<i>Aedes aegypti</i> , (Diptera: Cuclidae)
Cry21Aa1	<i>B.t. higo</i>	<i>Culex pipiens molestus</i> (Diptera: Cuclidae)
Cyt1Aa1	<i>B.t. israelensis</i> IPS82	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cyt1Ab1	<i>B.t. medellin</i> 163-131	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)
Cyt2Aa1	<i>B.t. kyushuensis</i>	<i>Aedes aegypti</i> , <i>Anopheles stephensi</i> , <i>Culex pipiens</i> (Diptera: Cuclidae)

Table 4. Mosquitocidal activity of Cry and Cyt proteins (Modified after "Zeigler, 1999").

3.5 Resistance

One major problem with insects control via chemical insecticides is the evolution in insects of resistance to those insecticides. The use of *Bti* on biological control of mosquitoes has no resulted in the development of resistance in host populations. Laboratory attempts to induce resistance by continual exposure to *Bti* have generally failed to detect resistance (Lee & Chong 1985; Georghiou & Wirth 1997).

The lack of resistance development to *Bti* could be due to its complex mode of action, involving synergistic interactions between up to four proteins (Becker & Maragrit 1993). Use of a single protein from *Bti* for mosquito control resulted in resistance after only a few generations in the laboratory (Becker and Margalit 1993). Georghiou & Wirth (1997) also showed that resistance could be raised in only a few generations when single *Bti* toxin was used (i.e. Cry 4Aa, 4Ba, 10Aa or 11Aa), and was progressively more difficult to raise in mosquitoes with combinations of two, and three toxins. When all four *Bti* toxins were used, resistance incidence was remarkably low. On the other hand Wirth et al. (2005) have shown that the lack of resistance in *Bti* is due to the presence of the Cyt1Aa protein in the crystal. For example, *Culex quinquefasciatus* populations resistant to CryIVA, Cry4B and Cry11A have been obtained in the laboratory but not mosquito larvae resistant to Cry and Cyt1Aa proteins (Georghiou & Wirth 1997)

3.6 Synergism

Bti produces four crystal proteins Cry (4Aa, 4Ba, 10Aa, and 11Aa) and two Cyt (1Aa and 2Ba) (Guerchicoff *et al.* 1997). No single crystal component is as toxic as the intact crystal complex (Chan *et al.* 1993; Wu *et al.* 1994; Chilcott & Ellar, 1998). One possible explanation for this is that two or more proteins act synergistically, yielding a higher activity than would be expected on the basis of the specific toxicity of the individual protein (Finney, 1971). For example, the toxicity against mosquito larvae of Cyt1Aa is lower compared to each of the four Cry proteins (Crickmore *et al.* 1995). However, *cytA* can potentiate the activity of the toxins and synergistic interactions that seems to account for the high toxicity of the *Bti* strains (Delecluse *et al.* 1993).

Tabashnick (1992) proposed a method to measure synergistic effect. Using the proposed method discuss the data reported by two authors: in bioassays with *A. aegypti* larvae, Wu and Chang (1985) found that mixtures of the 27- and 65-kDa proteins from *B. thuringiensis* subsp. *israelensis* were more toxic than expected on the basis of their individual toxicities, however, Chilcott and Ellar (1988) concluded from their own data that no synergism between these two proteins occurred. With this new interpretation of Tabashnick method, both studies support the same conclusions: positive synergism between the 27-kDa protein (CytA) and either of the CryIV proteins (65 and 130 kDa) and no such synergism between CryIV proteins (65 and 130 kDa). Other studies have been carried out with the aim of increasing the synergistic activity of *Bti*. Ramirez-Suero *et al.* (2011) evaluated the synergistic effect of *S. griseus* and *Bt aizawai* chitinases with *Bt israelensis* spore-toxin complex against *Aedes aegypti* larvae. The synergistic factor values according to Tabashnik (1992) method were 2 and 1.4, respectively.

3.7 Effect of *Bti* on no-target organisms

Bti has no direct effect on aquatic organisms other than mosquitoes, blackflies and chironomids. Other aquatic organisms, such as shrimps, mites and oysters are generally unaffected (Glare & O'Callaghan, 1998). This large safety margin of preparations of *Bti* for

non-target organisms indicate their suitability for mosquito control programs in areas where protection of the natural ecosystem is important (Sinegre et al. 1980)

Several authors have reviewed the non target effects of *Bti* (Becker & Margalit 1993; Lacey & Mulla 1990). Field applications have often been monitored for effects on non-target organisms but no significant non-target effects have been reported (Ali, 1981; Jackson et al. 1994; Hershey et al. 1995)

4. Production of *Bti* by fermentation

4.1 Culture medium for *Bti* production

Commercial production of *Bti* is performed using culture media based on complex nutrients sources. The main purpose of the fermentation is to obtain high quantities of *Bti* crystals. The *Bti* parasporal crystal can account for up to 25% of the sporulated cell dry weight. To optimize the cry production it is necessary to have a suitable culture medium because the toxicity obtained at the end of the fermentation depend on the culture medium and operating conditions. The culture media that have been reported in the literature for high growth and sporulation can be used for any variety of *Bt*. Not always a high cell growth ensures an elevated Cry protein production or an increased insecticidal activity. Various culture mediums have been used for high growth and sporulation of *Bt* in the laboratory: 2XSG medium (Leighton & Doi, 1971), PA medium (Thorne, 1968), G-Tris medium (Aronson et al. 1971), CDGS medium (Nakata, 1964). Other media with inexpensive substrates have been reported by Pearson & Ward (1988), Smith (1982), Foda et al. (1985), Dulmage et al. (1970), Salama et al. (1983), Goldberg et al. (1980).

4.2 Factors affecting Cry production

There are several factors that influence the production of crystals: (1) Carbon source. Glucose is the most appropriate carbon source either for high *Bt* growth and sporulation (Smith, 1982). When glucose has been exhausted in the fermentation, the absence of this can trigger sporulation. The use of one or other carbon source affects the biological activity and the morphology of the crystals (Dulmage, 1970). (2) Nitrogen source. An appropriate source of aminoacids provides high growth rates and high sporulation of *Bt* strains. Its absence delays sporulation and low yield in Cry proteins (Goldberg et al. 1980) (3) Carbon:Nitrogen ratio. Higher C:N rates glucose do not deplete at the end of fermentation and biomass yield decrease. Several authors have recommended a carbon nitrogen ratio of 7.5:1. (Salama et al. 1983; Foda et al. 1985) (4) Oxygen. High aeration rates are important for high spore and toxin formation. As $k_{1,a}$, increase biomass and Cry protein formation are increased (Rowe & Margaritis, 1987)(5) pH. Optimum pH for *Bt* growth is 6.8-7.2. If pH rises to 9.0 Cry protein can be dissolved (6) Temperature. Optimum temperature of *Bt* is 28-32°C. Higher temperatures favours plasmid losses or *Bt* mutants (Rowe & Margaritis, 1987).

5. Molecular biology of *Bti*

5.1 *Bti* cry and *cytA* genes

All *Bt* strains contain extrachromosomal DNA. *Bt* strains are well known for its numerous plasmids ranging in size from 1.5 MDa to 130 MDa. Plasmids have been found in each variety examined and the plasmid profiles appear to be strain specific. However these plasmid profiles depend on the media type and growth rate of the strain and can be readily

gained or lost (Federici, 1999). The mere presence of plasmids in *Bt* does not prove that they are involved in crystal formation, many non-crystalliferous bacteria also contain plasmids. The *cry* genes are located on large plasmids although some *Cry* genes have been reported on the chromosome (Baume & Malvar, 1995). As mentioned earlier, *Bt* produces four different *Cry* proteins: *CryIVA*, *CryIVB*, *Cry11A*, and the cytolytic *CytA* protein (Hoffe and Whiteley, 1989). The *Cry* proteins are codified by *cryIVA*, *cryIVB*, *cry11A*, and *cytA* genes, respectively. These genes responsible for the toxicity of *Bt* have been sequenced by various researchers (Table 5).

Gene name	GenBank Accession No.	Coding Region	Reference
<i>cryIVAa1</i>	Y00423	1-3540	Ward & Ellar, 1987
<i>cry4Aa2</i>	D00248, E01676	393-3935	Sen <i>et al.</i> 1988
<i>cry4Ba1</i>	X07423, X05692	157-3564	Chungjatupornchai <i>et al.</i> 1988
<i>cry4Ba2</i>	X07082	151-3558	Tungpradubkul <i>et al.</i> 1988
<i>cry4Ba3</i>	M20242	526-3930	Yamamoto <i>et al.</i> 1988
<i>cry4Ba4</i>	D00247, E01905	461-3865	Sen <i>et al.</i> 1988
<i>cry11Aa1</i>	M31737	41-1969	Donovan <i>et al.</i> 1989
<i>cry11Aa2</i>	M22860	1-235	Adams <i>et al.</i> 1989
<i>cyt1Aa1</i>	X03182	140-886	Waalwijk <i>et al.</i> 1985
<i>cyt1Aa2</i>	X04338	509-1255	Ward & Ellar, 1986
<i>cyt1Aa3</i>	Y00135	36-782	Earp a& Ellar, 1987
<i>cyt1Aa4</i>	M35968	67-813	Galjart <i>et al.</i> 1987

Table 5. *cry* and *cytA* genes DNA sequences of *Bt*.

In *Bt* the elements responsible of the toxicity against mosquito larvae are located in a large plasmid of 72 MDa (125 kb) and contribute to the formation of a complex parasporal body (Aronson 1993). Figure 2 shows the partial map of the *Bt* 125 kb plasmid.

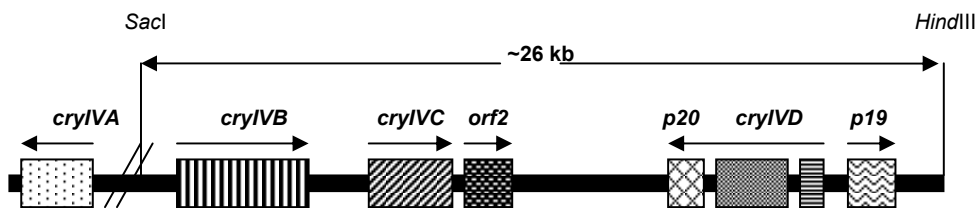


Fig. 2. Map of 26kb DNA fragment carrying the DNA genes responsible of the toxicity of *Bt* (Modified after Ben-Dov *et al.* 1996).

5.2 *Bt* operon of *cryIVD* gene

The DNA sequence indicated that *cryIVD* gene is the second gene of an operon which includes three genes. A gene that encodes a 19 kDa polypeptide, *cryIVD* gene and a gene that encodes a 20 kDa polypeptide (Dervyn *et al.* 1995). Transcription of *cryIVD* gene in *Bt* is induced 9 h after the beginning of the sporulation. DNA sequence analysis and potential promoters are recognized by the RNA polymerase associated with the σ^{35} and σ^{28} , specific sigma subunit of the RNA polymerase genes related with control of sporulation of *B.*

thuringiensis. *cryIVA* and *cryIVB* promoters are activated in the mid-sporulation phase (Ben-Dov et al. 1996), *cryIVA* is regulated by the σ^{35} RNA polymerase gene and *cryIVB* is under control of σ^{35} (Yoshisue et al. 1994). *cytA* gene is transcribed by two promoters, pBtI and pBtII, regulated by the RNA polymerase σ^{35} and σ^{28} , respectively (Brown & Whiteley, 1988, 1990). These results have demonstrated that *cryIVD* transcription is subjected to σ^{35} regulation.

5.3 Identification of *Bacillus thuringiensis* pesticidal crystal genes by PCR

The polymerase chain reaction (PCR) is a molecular tool widely used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The identification of *Bt* toxin genes by PCR can partially predict the insecticidal activity of a given strain. Several studies have reported that the type of *cry* and *cyt* genes present in a *Bt* strain correlates to some extent with its insecticidal activity (Porcar & Juarez-Perez). Thus, the identification of the gene content in a *Bt* strain can be used to predict its insecticidal potential. The PCR-based identification of *B. thuringiensis* *cry* genes was first developed by Carozzi et al. (1991), who introduced this technique as a tool to predict insecticidal activity. They found correspondence with the toxicity predicted on the basis of the amplification product profiles. Carozzi et al. proposed PCR as an accurate, fast methodology for the identification of novel strains and the prediction of insecticidal activity of new isolates, and they also forecast the possible use of PCR for the discovery of previously unknown *cry* genes. highly conserved regions and recognizing entire *cry* gene subfamilies are often used in a preliminary screening prior to performing a second PCR with specific primers. The primers used to amplify *cry4A*, *cry4B*, *cry11A*, and *cytA* genes have been designed by various researchers and are shown in Table 6.

Direct	Sequences (5'→3')	Reverse Sequences (5'→3')	Amplifies/Product (bp)
EE-4A(d)	GGGTATGGCACTC AACCCCACTT*	Un4(r) GCGTGACATACCC ATTTCAGGTCC*	<i>cry4A</i> /1529
Dip2A(d)	GGTGCTTCCTATTC TTTGGC**	Dip2B TGACCAGGTCCCT TGATTAC**	<i>cry4A</i> /1290
EE-4B(d)	GAGAACACACCTA ATCAACCAACT*	Un4(r) GCGTGACATACCC ATTTCAGGTCC*	<i>cry4B</i> /1951
EE-11A(d)	CCGAACCTACTAT TGCGCCA*	EE-11A(r) CTCCCTGCTAGGA TTCGTC*	<i>cry11A</i> /445
gral <i>cyt1</i> (d)	AACCCCTCAATCA ACAGCAAGG***	gral <i>cyt</i> (r) GGTACACAATACA TAACGCCACC***	<i>cyt1</i> /522-525

Table 6. PCR primers pairs and the *cry* or *cyt* genes they amplify of *Bti*. Sources: Ben-Dov et al. (1997)*, Carozzi et al. (1991)**, Bravo et al. (1998)***

5.4 Expression of *Bti* genes in other strains

Expression of *Bti* genes either individually or in combination in crystal-negative *Bt* or other strains have been carried on by several researchers. The genes encoding these proteins have been expressed in *Caulobacter* (Thanabalu et al. 1992), cyanobacteria (Manasherob et al. 2002; Murphy & Stevens 1992), *Escherichia coli* (McLean & Whiteley 1987; Tanapongpipat et al. 2003), *Bacillus subtilis* (Ward et al. 1986), and *Bt* (Crickmore et al. 1990). However, *Bt* toxins have been expressed as active or inactive toxins, especially when expressed in *E. coli* (Ogunjimi et al. 2002).

Quintano et al. (2005) reported the expression of *cry11A* from *Bti* in *S. cerevisiae*. The *cry11A* gene was expressed as fusion proteins with glutathione *S*-transferase under the control of the *S. cerevisiae* *HXK1* promoter. The protein was purified by affinity chromatography using glutathione *S*-transferase-Sepharose beads. Insecticidal activity against third-instar *Aedes aegypti* larvae of the recombinant *S. cerevisiae* cell extracts ($LC_{50} = 4.10\mu\text{g protein/mL}$) and purified GST-*cry11A* fusion protein ($LC_{50} = 4.10\mu\text{g protein/mL}$) was detected in cells grown in ethanol.

Servant et al. (1999) constructed a recombinant *B. sphaericus* strain containing the *cry11A* gene from *Bti*. They found an LC_{50} for the *cry11A* protein of $1.175\mu\text{g/mL}$ against fourth-instar *A. aegypti* larvae. Poncet et al. (1997) constructed a recombinant *B. sphaericus* strain with *cry11A* and *p20* genes integrated into the chromosome. In this case, the LC_{50} value reported against third-instar *A. aegypti* larvae was $0.023\mu\text{g/mL}$. Xu et al. (2001) studied the expression of *cry11A* and *cry11A+p20* gene cluster in recombinant *E. coli* and *Pseudomonas putida*. They found that both recombinant bacteria contained higher levels of *Cry11A* protein when the adjacent *p20* gene was present on the same DNA fragment. Yamagiwa et al. (2004) reported that the solubilized *cry11A* protein, obtained from a nonrecombinant *Bti*, was less toxic against *Culex pipiens* larvae than the crystal itself (LC_{50} of 0.267 and $0.008\mu\text{g/mL}$, respectively). These authors obtained 2 GST fusion proteins of 36 and 32 kDa from *cry11A*. The LC_{50} against *C. pipiens* larvae obtained using both proteins were $0.818\mu\text{g/mL}$. In other study with the purpose to preserve the toxicity of sunlight-sensitive *Cry* proteins, Manasherob et al. (2002) constructed a transgenic cyanobacterium *Anabaena* PCC 7120 to express the genes *cry4Aa*, *cry11Aa* and an accessory protein (*p20*) under control of two tandem strong promoters. Cyanobacterium *Anabaena* can multiply in breeding sites of mosquito larvae and serve as their food source. Higher toxicity against *Aedes aegypti* larvae was obtained in this study.

6. Mode of action of *Bti* *Cry* proteins against mosquito larvae

6.1 Pore-forming-toxins

Bt *Cry* and *Cyt* toxins belong to a class of bacterial toxins known as pore-forming toxins (PFT) that are secreted as water-soluble proteins undergoing conformational changes in order to insert into, or to translocate across, cell membranes of their host (Bravo et al. 2007). In most cases, PFT are activated by host proteases after receptor binding inducing the formation of an oligomeric structure that is insertion competent. Finally membrane insertion is triggered, in most cases, by a decrease in pH that induces a molten globule state of the protein (Parker and Feil, 2005). *Cry* and *Cyt* proteins are PFT proteins. Both proteins are solubilized in the gut of susceptible dipteran insects and proteolytically activated by midgut proteases. For the *Cry 11Aa* protoxin, proteolytic activation involves amino-terminal processing and intramolecular cleavage leading to two fragments of 36 and 32 kDa that remain associated and retain insect toxicity.

6.2 Mechanism of action of *Bti* toxins

An accepted model for Cry toxin action against mosquito larvae is that it is a multistage process. (i) Ingestion of Cry protein by the larvae (ii) Solubilization of the crystals in the alkaline midgut (iii) Proteolytic activation of the insecticidal solubilised protein (iv) Toxin binds to receptors located on the apical microvillus membrana of epithelial midgut cell walls (v) Alter the toxin binds the receptor, it is thought that there is a change in the toxin conformation allowing toxin insertion into the membrane (vi) Electrophysiological and biochemical evidence suggest that the toxins generate pores in the cell membrane, thus disturbing the osmotic balance, consequently the cells swell and lyse (vii) The gut becomes paralyzed and the insect stops feeding. Most mosquito larvae die within few hours of ingestion, generally cease feeding within 1 hour, show reduced activity by two hours and die six hours after ingestion (Chicott *et al.* 1990; Marrone & Macintosh, 1993).

Several authors have studied the mechanisms of action of the δ -endotoxin of *Bti* on mosquito larvae. Thomas and Ellar (1983) found that δ -endotoxin active against mosquito larvae was inactivated by prior incubation with lipids extracted from epithelial midgut *Aedes albopictus* cells. They reported that toxin binds to membrane lipids (phosphatidyl choline, sphingomyelin and phosphatidyl ethanolamine). According to their results, they proposed a mechanism in which the interaction of toxin with membrane lipids causes a detergent-like rearrangement of the lipids and as a consequence cytolysis. Others authors have corroborated these results: Cyt protein, unlike Cry toxins, do not recognize specific binding sites and do not bind to protein receptors, directly interact with membrane lipids inserting into the membrane and forming pores (Knowles *et al.* 1989; Promdonkoy & Ellar, 2000) or destroying the membrane by a detergent-like interaction (Butko, 2003).

The high efficacy of *Bti* is because of the production of multiple toxins with different modes of action. Perez *et al.* (2005) reported that Cyt1Aa protein functions as a receptor for the Cry11Aa toxin and suggest that this interaction explains the synergism between the Cyt1A and Cry11A proteins. Further, the Cyt proteins in *Bti* synergize the toxic effect of Cry11A and Cry4 toxins and, even more, suppresses the resistance to these Cry toxins (Wirth *et al.* 1997).

7. Formulation of *Bti* toxins

7.1 Potency in *Bti* formulations

Formulation is a preparation of an insecticide for a particular application method. Formulation plays an important role in determining final virulence. The vast majority of the formulations of *Bt* have been developed to control agricultural and forest pests, mainly *Lepidoptera*. However, the feeding habits of *Lepidoptera* are different to feeding habits of *Diptera*. Mosquito larvae feed by filtering water and concentrate organic particles. Product formulations based on *Bti* should consider the mosquito larvae habits and the environmental conditions, promote that Cry proteins retain their toxic activity and promote that the larva have access to them. Products based on strains of *Bti* are given a potency based on bioassays on third or fourth instar mosquito larvae. Bioassays are conducted using 6 to 7 dilutions of the toxin by duplicate in 100 mL cups containing 20 third instar *Aedes aegypti* larvae. Duplicate cups with 20 mosquito larvae in 100 mL of deionized water without test material serves as a control (McLaughlin *et al.* 2004). Concentration-mortality data are obtained, transformed to a log-probit scale, and potency is obtained by comparing the estimated LC₅₀ of a test substance with that of a standard with a known potency (de Barjac

1985). The international standard recognized for *Bti* is IPS82. LC_{50} and LC_{90} are the dose require to kill 50 and 90 percent of the mosquito larvae of a tested population after 24h tested duration. Each sample is bioassayed at least 3 times on various days and the results are average values. LC_{50} and LC_{90} are measured in micrograms or milligrams of material per liter, or parts per million (ppm).

For potency calculations, it is used the international recognized standard for mosquito assay, IPS82 (15 000 ITU/mg) provided by Institute Pasteur, Paris, France. Standard vials are kept at -18°C.

Product potency is calculated by the Abbott (1925) formula:

$$\text{Potency (A)} = \text{Potency (std)} LC_{50} (\text{std})/LC_{50} (\text{A})$$

where (a) is the product and (std) is the standard.

The size of the particle could be a factor that influences the potency of the toxin. A product with small particles is more homogenously distributed in the water than a product with larger particles and small particles which sink slower. Changes in LC_{50} are not necessary regarded to reflect changes in amount of toxin, but could be a function of particle size/distribution (Skovmand et al. 1997). Change of particle size also change LC_{50} . Decreasing particle increased LC_{50} and thus decrease the calculated potency. The slope measured between LC_{50} and LC_{90} values should have high value. Higher value will require a smaller quantity to kill a greater number of larvae. The slope of the dosage-mortality curve is in function of the heterogeneity of the product effect. If product availability is in function of particle sizes, particles with broad ranges of particle size distribution will also have low slopes.

7.2 *Bti* formulations

A variety of *Bti* formulations have been studied for mosquito control under laboratory and field conditions. *Bti* fluid formulations are not stable in heat and high humidity and cannot be stored for months under tropical conditions (McGuirre et al. 1996). In many cases, and especially in areas exposed to the sun, the residual effect is very short (Leong et al. 1980) and the product has to be reapplied. Photoinactivation seems to be one of the major environment factors affecting the stability of *Bti* delta-endotoxin (Morris, 1983). Yu-Tien et al. (1993) reported that *Bti* completely lost its toxicity to mosquito larvae when exposed to irradiation at 253nm. Poszgay et al. (1987) showed that exposure of *B. thuringiensis* toxin to 40 h of ultraviolet light irradiation resulted in lost activity. Cry proteins inactivation by the solar radiation is the result of the destruction of the tryptophan (Pusztai et al. 1991). Research and development efforts are focusing on formulations to avoid the ultraviolet light effect. Ramirez-Lepe et al. (2003) encapsulated *Bti* spore-toxin within aluminum/carboxymethylcellulose using green malachite, congo red or ponceau red as photoprotective agents against ultraviolet light in lab conditions. The encapsulated form of the *Bti* spore-toxin complex with photoprotectors avoided the limitation in controlling mosquito larvae caused by ultraviolet light. Yu-Tien et al. (1993) achieved photoprotection of the spore-toxin complex by addition of melanin.

Other *Bti* formulations have been developed. For example, Ramirez-Suero et al. (2005) evaluated maltodextrin, nixtamalized corn flour and corn starch for entrapping active materials in *Bti* spore-toxin complexes dried by aspersion. Dried products had water activity values below 0.7 suggesting that the formulations are long shelf-life because keep

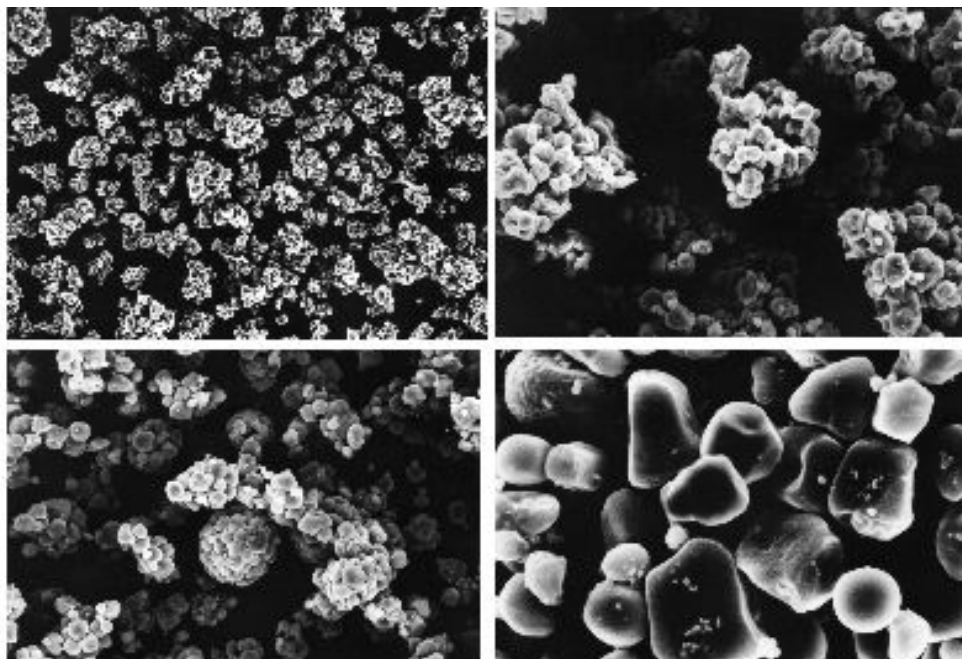


Fig. 3. Scanning electron micrograph of spray/dried formulation of *Bti* spore/toxin complex with Grits a) 379X and b)1500X and Nixtamalized corn flour c) 370X and d) 1500X.

the products without microorganisms for longer periods and increases the larval feeding and as a consequence have higher activity against mosquito larvae. Fig. 3 shows the scanning electron microscope of spray/dried formulations with grits and nixtamalized corn flour. It shows smooth spherical grits and corn particles entrapping the spore/toxin ingredient. Manasherob et al. (1996) encapsulated *Bti* in the protozoan *Tetrahymena pyriformis* and the activity against *A. stephensi* was enhanced 8 times when exposed to protozoan cells filled with *Bti* compared to exposed to the same concentration of *Bti* alone. Elcin (1995) encapsulated *Bti* in alginate microcapsules and increase its stability and its effect against *Culex* sp larvae. Another alternative to protect *Bti* crystals from ultraviolet light is obtaining mutants that protect *Bti* crystal. Hoti & Balaraman (1993) obtained a mutant of *Bti* that produced a brown pigment during sporulation, the pigment was identified as melanin. Other *Bti* formulations have been developed. Culigel superabsorbent polymer controlled-release system for the slow release of *Bti* to mosquito larvae (Levy et al. 1990). Combination of chemical and biological agents such as insect growth regulator s-methoprene + *Bti* (Bassi et al. 1989). Sprayed-dried *Bti* powder as a fizzy tablet (Skovmand & Eriksen 1993). Floating bait formulations designed to improve the effect of bacterial toxins, especially against *Anopheles* spp (Aly et al. 1987).

Highly concentrated liquid formulations are available for control of floodwater mosquitoes while formulations which float for as long possible have been developed for use in fast-flowing or turbulent waters. Formulations which settle and persist at the bottom are required for bottom feeders. Granules which float on the surface are the most effective against surface feeders such as *Anopheles* spp. (Mulla et al. 2004).

Briquettes for mosquitoes with continual successive generations like *Culex* spp (Becker & Margalit 1993; Kase & Branton 1986). Briquettes or pellets, in particular seem to be useful for overcoming lack of persistence, which is one of main limitations of *Bti*. Granules using plant, such corn (maiz) grits or clay carriers are particular useful in aerial application to breeding sites with dense foliage as salt marshes or rice fields. Sustained release formulations such as floating briquettes or semi submersible pellets are designed to provide long-lasting larvicidal activity in containers or small ponds. Ingestion of the toxin depends on the rate of feeding, the rate at which the toxin falls to the bottom of the pool and becomes inaccessible, and competition to ingestion from other suspended organic materials. In turbid and polluted waters the rate of application needs to be at least two-fold greater than in clear water (Mulla et al. 1985). The feeding habits of mosquito larvae influence formulation design. *Anopheles* larvae are surface feeders and ingest particulate material from water surface such as yeast or flour and filter feed poorly. This has led to the development of formulations that present the toxin at, or just below, the water surface and such preparations are particularly effective against certain *Anopheles* larvae (Aly et al. 1987)

8. Field application

One of the major drawbacks in the use of *Bti* is its rapid inactivation (24-48 h) in the environment (Mulla et al. 1993). Thus larvae populations of stagnant water mosquitoes recover within 5-7 days following treatment. Therefore the use of *Bti* is limited by the low efficacies of current preparations under field conditions (Tyanum & Mulla 1999). Since there is a little persistence of the toxin further applications are necessary to effect continuous control. Formulation and applications techniques can extend the persistence of activity for over one month in some situations, but activity remains sensitive to factors like UV degradation. In areas exposed to the sun, the residual effect is very short (Leong et al. 1980) and the product has to be reapplied. Other factors that affect the toxicity of *Bti* are particle sedimentation (Rushed & Mulla, 1989), protein adsorption onto silt particles, organic matter, elevated temperatures (Ohana et al. 1987), consumption by other organisms to which the toxin is not lethal (Blaustein and Margalit, 1991), dissolved tannins (Lord & Undeen, 1990) and inactivation by sunlight. Other factors that have been observed are that *Bti* does not recycle, under simulated field conditions, is unable to germinate and multiply in mud at the bottom of pools although it did remain viable for up to 22 days, and higher water depth where applied decrease its activity (Ohana et al. 1987)

Early reports showed that a primary powder formulations of *Bti* had virtually no residual effect against mosquito larvae beyond application (Aguilar-Meza et al. 2010), although the delta-endotoxin remained chemically stable in neutral and acid waters (Sinegre et al 1980). Extend persistence with *Bti* is possible through use of improved formulations. Gunasekaran et al. (2002) tested a floating sustained release formulation of *Bti* in polluted water habitats against *Culex quinquefasciatus* larvae. Briquettes may result in more prolonged control than liquid formulations as these products have greater persistence through slow release (Kase & Branton 1986).

On the other hand, Aguilar-Meza et al. (2010) tested the residual insecticidal activity after field exposure of an aluminum-carboxymethylcellulose microencapsulated formulation of *Bti* spore-toxin complex with malachite green as photoprotective agent. The formulation improved the activity against *Aedes aegypti* larvae for 30 days and was comparable to that of the chemical insecticide temephos.

9. Conclusions

Bti is a bacterium that has been applied with success in biological control programs against mosquitoes and flies larvae all over the world. The study in each of its facets addressed in this review will open new perspectives to improve their effectiveness in biological control.

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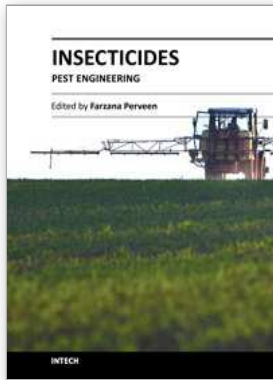
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This book is compiled of 24 Chapters divided into 4 Sections. Section A focuses on toxicity of organic and inorganic insecticides, organophosphorus insecticides, toxicity of fenitrothion and permethrin, and dichlorodiphenyltrichloroethane (DDT). Section B is dedicated to vector control using insecticides, biological control of mosquito larvae by *Bacillus thuringiensis*, metabolism of pyrethroids by mosquito cytochrome P40 susceptibility status of *Aedes aegypti*, etc. Section C describes bioactive natural products from sapindaceae, management of potato pests, flower thrips, mango mealy bug, pear psylla, grapes pests, small fruit production, boll weevil and tsetse fly using insecticides. Section D provides information on insecticide resistance in natural population of malaria vector, role of *Anopheles gambiae* P450 cytochrome, genetic toxicological profile of carbofuran and pirimicarp carbamic insecticides, etc. The subject matter in this book should attract the reader's concern to support rational decisions regarding the use of pesticides.

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