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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 proteinic 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 worldwide 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 worldwide (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; Drobniewski 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 *Ephesia kuehniella* (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).
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 on ingestion, and feeding activity depends on environmental conditions

2.4 Classification of strains of Bacillus thuringiensis

Bt is a crystalliferous spore-forming bacterium close genetic relationship with B. cereus, B. anthracis and B. mycoides (Höfte & 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. Mycoides 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. mycoides 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 classification 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 classification, although still in use as a basic method to classify 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 classification 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 classified 42 Bt crystal protein genes into 14 distinct genes grouped into 4 major classes. The classes were CryI (Lepidoptera-specific), CryII (Lepidoptera-and Diptera-specific), CryIII (Coleoptera-specific), and CryIV (Diptera-specific).
<table>
<thead>
<tr>
<th>Serotype</th>
<th>Serovar</th>
<th>Serotype</th>
<th>Serovar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>thuringiensis</td>
<td>28a, 28c</td>
<td>jeghatensan</td>
</tr>
<tr>
<td>2</td>
<td>finitimmus</td>
<td>29</td>
<td>amagiensis</td>
</tr>
<tr>
<td>3a, 3b, 3c</td>
<td>kurstaki</td>
<td>31</td>
<td>toguichini</td>
</tr>
<tr>
<td>3a, 3c</td>
<td>aesti</td>
<td>32</td>
<td>cameroun</td>
</tr>
<tr>
<td>3a, 3d</td>
<td>sumiyoshiensis</td>
<td>33</td>
<td>leesis</td>
</tr>
<tr>
<td>3a, 3d, 3e</td>
<td>fukoakaensis</td>
<td>34</td>
<td>konkukian</td>
</tr>
<tr>
<td>4a, 4b</td>
<td>soto/dendrimums</td>
<td>35</td>
<td>seoulensis</td>
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<td>kenya</td>
<td>36</td>
<td>malayensis</td>
</tr>
<tr>
<td>5a, 5b</td>
<td>galleriae</td>
<td>37</td>
<td>andralousiens</td>
</tr>
<tr>
<td>5a, 5c</td>
<td>canadensis</td>
<td>38</td>
<td>owaldocruizi</td>
</tr>
<tr>
<td>6</td>
<td>entomocidus/subtoxicus</td>
<td>39</td>
<td>brasiliensis</td>
</tr>
<tr>
<td>7</td>
<td>aizawai/pacificus</td>
<td>40</td>
<td>huazhongensis</td>
</tr>
<tr>
<td>8a, 8b</td>
<td>morrisoni</td>
<td>41</td>
<td>sooncheon</td>
</tr>
<tr>
<td>8a, 8c</td>
<td>ostriniae</td>
<td>42</td>
<td>jinghongiensis</td>
</tr>
<tr>
<td>8b, 8d</td>
<td>nigeriensis</td>
<td>43</td>
<td>guiyangiensis</td>
</tr>
<tr>
<td>9</td>
<td>tolworthi</td>
<td>44</td>
<td>higo</td>
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<td>10a, 10b</td>
<td>darmstadiensis</td>
<td>45</td>
<td>roskildensis</td>
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<tr>
<td>10a, 10c</td>
<td>londrina</td>
<td>46</td>
<td>chanpaisis</td>
</tr>
<tr>
<td>11a, 11b</td>
<td>toumanoffi</td>
<td>47</td>
<td>wratislaviensis</td>
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<tr>
<td>11a, 11c</td>
<td>kyushuensis</td>
<td>48</td>
<td>balearica</td>
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<td>12</td>
<td>thompsoni</td>
<td>49</td>
<td>muju</td>
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<td>13</td>
<td>pakistani</td>
<td>50</td>
<td>navarrensis</td>
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<tr>
<td>14</td>
<td>israelensis</td>
<td>51</td>
<td>xiaaguangiensis</td>
</tr>
<tr>
<td>15</td>
<td>dakota</td>
<td>52</td>
<td>kim</td>
</tr>
<tr>
<td>16</td>
<td>indiana</td>
<td>53</td>
<td>asturiensis</td>
</tr>
<tr>
<td>17</td>
<td>tohokuensis</td>
<td>54</td>
<td>poloniensis</td>
</tr>
<tr>
<td>18a, 18b</td>
<td>kumamotoensis</td>
<td>55</td>
<td>palmanyolensis</td>
</tr>
<tr>
<td>18a, 18c</td>
<td>yosoo</td>
<td>56</td>
<td>rongseni</td>
</tr>
<tr>
<td>19</td>
<td>tochigiensis</td>
<td>57</td>
<td>pirenaica</td>
</tr>
<tr>
<td>20a, 20b</td>
<td>yunnanensis</td>
<td>58</td>
<td>argentenensis</td>
</tr>
<tr>
<td>20a, 20c</td>
<td>pondicheriens</td>
<td>59</td>
<td>iberica</td>
</tr>
<tr>
<td>21</td>
<td>colmeri</td>
<td>60</td>
<td>pingluonsi</td>
</tr>
<tr>
<td>22</td>
<td>shanongiensis</td>
<td>61</td>
<td>sylvestriensis</td>
</tr>
<tr>
<td>23</td>
<td>japonensis</td>
<td>62</td>
<td>zhaodongensis</td>
</tr>
<tr>
<td>24a, 24b</td>
<td>neolonensis</td>
<td>64</td>
<td>azorensi</td>
</tr>
<tr>
<td>24a, 24c</td>
<td>novosibirsk</td>
<td>65</td>
<td>pulsiensis</td>
</tr>
<tr>
<td>25</td>
<td>coreanensis</td>
<td>66</td>
<td>graciosensis</td>
</tr>
<tr>
<td>26</td>
<td>silo</td>
<td>67</td>
<td>vazensis</td>
</tr>
<tr>
<td>27</td>
<td>mexicanensis</td>
<td>none</td>
<td>wuhanensis</td>
</tr>
<tr>
<td>28a, 28b</td>
<td>monterrey</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

The *cry* 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
bypiramidal crystalline inclusions during the sporulation of Bt. cryII genes encode 65-kDa proteins which form cuboidal inclusions in strains of several species. cryIII genes produce romboidal 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 fagellar 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 realltively small (0.1 to 0.5μm) 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.

<table>
<thead>
<tr>
<th>Vector</th>
<th>Disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anopheles</td>
<td>Malaria, lymphatic filariasis</td>
</tr>
<tr>
<td>Culex</td>
<td>Lymphatic filariasis, Japanese encephalitis, other viral diseases</td>
</tr>
<tr>
<td>Aedes</td>
<td>Yellow fever, dengue, dengue hemorrhagic fever, other viral diseases, lymphatic filariasis</td>
</tr>
<tr>
<td>Mansonia</td>
<td>Lymphatic filariasis</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Mosquito genus</th>
<th>Species</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aedes</em></td>
<td>40</td>
</tr>
<tr>
<td><em>Anopheles</em></td>
<td>27</td>
</tr>
<tr>
<td><em>Culex</em></td>
<td>19</td>
</tr>
<tr>
<td><em>Culiseta</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Mansonia</em></td>
<td>5</td>
</tr>
<tr>
<td><em>Psorophora</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Armigeres</em></td>
<td>3</td>
</tr>
<tr>
<td><em>Toxorhynchites</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Limatus</em></td>
<td>2</td>
</tr>
<tr>
<td><em>Trichophospon</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Uranotaenia</em></td>
<td>1</td>
</tr>
<tr>
<td><em>Tripteroides</em></td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>109</strong></td>
</tr>
</tbody>
</table>

Table 3. Larvicidal activity of *Bti* on mosquito species (Clare & 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.
μL/Larvae/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, where as 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.

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

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 quinquefascitus 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. israeensis 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 recomended 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 \( k_{l,a} \), 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, *Bti* 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 *Bti* have been sequenced by various researchers (Table 5).

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

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

In *Bti* 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 *Bti* 125 kb plasmid.

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

### 5.2 Bti 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 *Bti* 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 $\sigma^{35}$ and $\sigma^{28}$, specific sigma subunit of the RNA polymerase genes related with control of sporulation of *B.*
Insecticides – Pest Engineering

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

<table>
<thead>
<tr>
<th>Direct</th>
<th>Sequences (5′→3′)</th>
<th>Reverse Sequences (5′→3′)</th>
<th>Amplifies/Prod uct (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EE-4A(d)</td>
<td>GGGTATGGCACTC AACCCCACCT*</td>
<td>Un4(r) GCGTGACATACC ATTTCCAGGTCC*</td>
<td>cry4A/1529</td>
</tr>
<tr>
<td>Dip2A(d)</td>
<td>GGTGCTTCCTATTC TTTGGC**</td>
<td>Dip2B TGACCCAGTCCCT TGATTAC**</td>
<td>cry4A/1290</td>
</tr>
<tr>
<td>EE-4B(d)</td>
<td>GAGAACACACTA ATCAACCAACT*</td>
<td>Un4(r) GCGTGACATACC ATTTCCAGGTCC*</td>
<td>cry4B/1951</td>
</tr>
<tr>
<td>EE-11A(d)</td>
<td>CCGAACCTACTAT TGGCCA*</td>
<td>EE-11A(r) CTCCCTGCTAGGA TTCCGTC*</td>
<td>cry11A/445</td>
</tr>
<tr>
<td>gral cyt1(d)</td>
<td>AACCCTCAATCA ACAGCAAGG***</td>
<td>gral cyt(r) GGTACACATACAA TAAACCCACCC***</td>
<td>cyt1/522-525</td>
</tr>
</tbody>
</table>

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)***

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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 (Thanabal 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 (LC50 = 4.10 μg protein/mL) and purified GST-cry11A fusion protein (LC50 = 4.10 μg 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 LC50 for the cry11A protein of 1.175 μg/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 LC50 value reported against third-instar A. aegypti larvae was 0.023 μg/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 (LC50 of 0.267 and 0.008 μg/mL, respectively). These authors obtained 2 GST fusion proteins of 36 and 32 kDa from cry11A. The LC50 against C. pipiens larvae obtained using both proteins were 0.818 μg/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 diperans insects and proteolytic 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 membrane of epithelial midgut cell walls (v) After 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 a 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 required 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:

$$\text{Potency (A)} = \frac{\text{Potency (std) LC}_{50} \text{(std)}}{\text{LC}_{50} \text{(A)}}$$

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 homogeneously 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
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 protects 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). Briquets 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 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. Briquetts 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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11. References


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


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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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