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The potential of *Bacillus thuringiensis* as a biological control agent in the order Diptera

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Abstract

Bacillus thuringiensis (Bt) is a spore-forming gram-positive bacterium that produces a variety of insecticidal proteins effective against various insect orders, especially Diptera. This review highlights the diversity, mode of action, and target specificity of Bt toxins-mainly Cry and Cyt proteins-against dipteran pests, including mosquitoes (Aedes, Anopheles, Culex), houseflies (*Musca domestica*), and fruit flies (*Bactrocera* spp). Some Bt serovars, particularly Bt israelensis, produce synergistic toxic combinations with higher larvicidal activity. The review also examines bioassay findings that demonstrate the effectiveness of purified delta-endotoxins and recombinant strains for integrated vector control. In particular, Bt kyushuensis (strain Btk176) showed promising activity against *M. domestica*, suggesting a new bioinsecticide candidate. The ecological specificity and low environmental toxicity of Bt make it a sustainable alternative to chemical insecticides for pest control of dipteran.

Keywords: *Bacillus thuringiensis* (Bt); Biological Control Agent; Insect; Pesticide; Diptera; Toxic

1. Introduction

Bacillus thuringiensis (Bt) has been isolated from the most diverse habitats on our planet. Its discovery was in 1901, and its correct scientific explanation was in 1915. It has led to the characterization of many Bt strains taken together, revealing this bacterium's enormous genetic diversity. This genetic diversity corresponds to a wide range of functions these bacteria play in natural and altered ecosystems (agriculture and forestry). Some of the most relevant functions attributed to Bt from an applied point of view are plant growth-promoting activity, bioremediation of different heavy metals and other pollutants, biosynthesis of metal nanoparticles, production of polyhydroxy alkanoate biopolymers, and anticancer activity. In agriculture, these bacteria are undoubtedly the most widely used due to their usefulness as biological pest control agents and as the most significant source of insecticidal genes for developing resistant transgenic crops (also known as Bt crops) for several crops. One of the agricultural pests, it is also an efficient biological control agent for insect vectors (especially mosquitoes) of significant diseases in human and animal health [1].

Bt is a gram-positive rod-shaped bacterium that can form resistant spores, belonging to the Bacillaceae family. Sporulating Bt cells typically form parasporal crystals composed of proteins that are active against several insect species from different orders, such as Lepidoptera, Diptera, Coleoptera, Hymenoptera, Hemiptera, Orthoptera, as well as other organisms such as mites and nematodes. When the Bt crystal reaches the insect's gut, it's dissolved to release one or more protoxins. These protoxins are then proteolyzed and activated by midgut proteases along with the toxins that can bind and disrupt the cell membrane. Binding and insertion of the toxins in the membrane trigger the formation of pores and, as a result, is an extinct of the insect.

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Crystals or inclusion bodies, formed by combinations of delta endotoxins (historically referred to as Cry and/or Cyt proteins, with Cry toxins now divided into structural groups in the new nomenclature), can exhibit different shapes (bipyramidal, spherical, etc.) and sizes (smaller, equal to, or larger than the spore size), which are usually characteristic for each wild Bt strain [2]. The genes encoding these proteins are mostly located on the original megaplasmid (>100 kb). The size and number of plasmids harboring these genes vary greatly for each strain, with some of them (conjugative plasmids) being transferred from one Bt strain to another. Crystal synthesis requires a huge metabolic investment from the cell. In addition, the high levels of protein expression that occur in the stationary growth phase are controlled at the transcriptional, post-transcriptional, and post-translational levels.

Based on their molecular structure and homology, the largest group of crystallin proteins is formed by the 3-domain Cry proteins. Domain I consists of a bundle of seven antiparallel ÿ helices and is the pore-forming domain. Domain II consists of three antiparallel ÿ sheets (ÿ-prism structure) and is involved in toxin-receptor interactions. Domain III consists of two antiparallel ÿ-sheets twisted into an ÿsandwich and has a role in receptor binding and pore formation. The three-domain Cry toxins are divided into two main types, those with a large protoxin form of ~120–140 kDa and those with a smaller 65–70 kDa protoxin that lacks the C-terminal region seen in the larger form. Larval midgut proteases convert these protoxins into active fragments through protein processing [3]. Although these groups of Cry proteins have a very similar and conserved three-domain structure, they differ significantly in their amino acid sequences. There are many other proteins previously designated as Cry proteins that do not have a three-domain structure and these include Etx_Mtx2 proteins, Toxin_10 proteins, and alpha-helical toxins. In contrast to Cry proteins, Cyt proteins exhibit general cytolytic (hemolytic) activity in vitro and dipteran specificity in vivo. Their three-dimensional structures suggest that Cyt proteins are composed of a single domain with an ÿ-sheet surrounded by two ÿ-helical layers.

2. Methods

In the steps of compiling this review, the technique used is the library study technique by searching for sources or literature in the form of primary data in the form of national journals and international journals for the last 10 years (2014-2024). In addition, in making this review, data searches in addition carried out using online media, such as Google and journal sites (NCBI, PuMed, etc).

3. Bt Active Toxins Against Dipterans

Within Bt, there are several serovars (including israelensis, jegathesan, darmstadiensis, kyushensis, medellin, fukuokaensis, and higo) either of which contains a large number of individual Bt strains containing proteins with known insecticidal activity against an increasing number of dipteran species. The current list of Bt genes encoding proteins with anti-dipteran activity includes cry1, cry2, cry4, cry10, cry11, cry19, cry20, cry24, cry27, cry30, cry39, cry44, cry47, cry50, cry54, cry56, mpp60, tpp80, cyll and cyt2 [18].

4. Cry Poison from Bt ser. *Israelensis*

Bt ser. *israelensis* (Bt) was the first Bt serotype found to be toxic to dipteran larvae. Bt is a highly potent and environmentally friendly alternative biological component in integrated disease vector control programs. Bt is much more effective against many mosquito species and black fly larvae than previously known biocontrol agents. Bt crystals potentially consist of up to six crystal proteins (Cry4Aa, Cry4Ba, Cry10Aa, Cry11Aa, and Mpp60A/Mpp60B (formerly Cry60A/Cry60B)), while the strains may lack the mpp60 gene and three Cyt proteins (Cyt1Aa, Cyt2Ba, and Cyt1Ca). Most of the genes encoding these toxins remain contained in the 128 kb plasmid pBtoxis, despite the fact that some Bt strains contain another plasmid called pBtc100, which carries two additional pesticide protein genes, encoding the toxins Mpp60Aa and Mpp60Ba [4]. Bt has been the most studied serovar for many years with most marketed Bt products being based on strains within this serovar. The pesticide proteins of Bt (Cry4A, Cry4B, Cry11A, and Cyt1A) have also been studied extensively; however, other proteins, which may have lower expression, have been studied less (Cry10Aa, Cyt2Ba, Mpp60Aa, and Mpp60Ba). Their activities, as well as possible interactions between them, open up new possibilities for study in the search for alternative dipteran control.

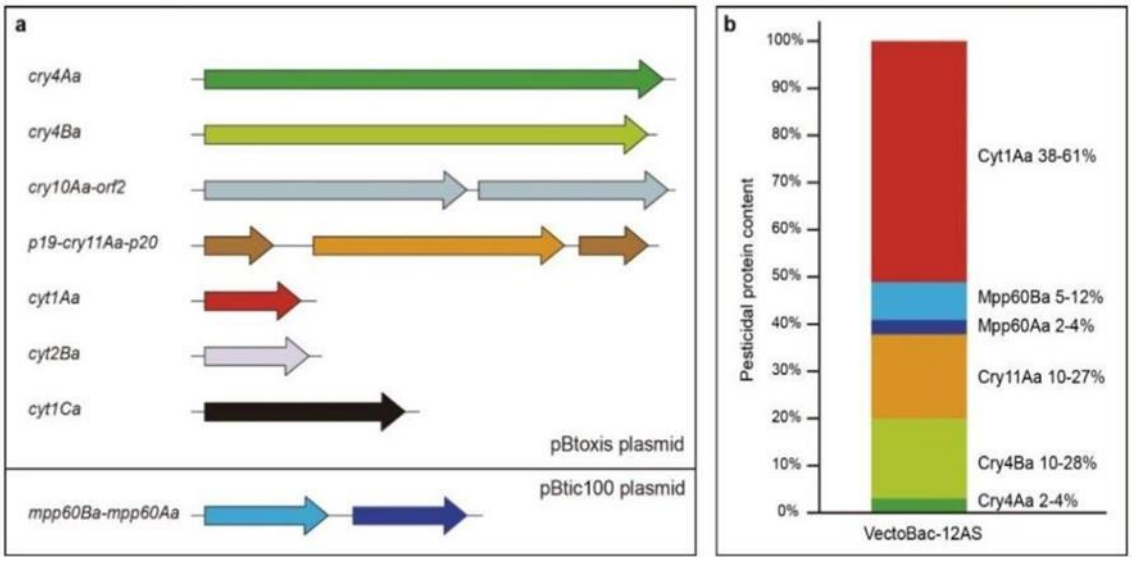

Figure 1 Pesticidal protein composition of Bti strains [6]

Cry4 Protein, Bt strains can produce two members of the large Cry 3-domain protoxin family: Cry4Aa (135 kDa) and Cry4Ba (128 kDa). These 3-domain protoxins form crystals spontaneously through inter- and intra-molecular disulfide bonds by the conserved C-terminal portion. The target range of Cry4Aa includes the following mosquito species: *Aedes aegypti, Anopheles stephens, Anopheles gambiae, Culex pipiens*, and *Culex quinquefasciatus*. Several studies have provided evidence that Culex species are the most susceptible to this protein, while species from the genera Anopheles and Aedes are less susceptible. Cry4Ba is one of the major crystal proteins produced by Bt [5]. This protein shows high toxic activity against *A. aegypti* and *An. stephensi*, but is completely inactive against larvae in the genus Culex. Toxicity of Cry4Ba toxin against *A. aegypti* and *An. stephensi* larvae are higher than Cry4A. Putative loops 1 and 2 of domain II of the protein are responsible for its activity, and mutations in putative loop 3 result in increased toxicity to Culex. Cry4Ba also has toxicity to *Chironomus tepperi* (Diptera, Chironomidae) and is the most effective Bt toxin against *Simulium* spp. (Lower activity was seen for Cry4Aa).

The Cry10Aa protein is a minor component of the crystals produced by Bt ser. Israelensis strains and, unlike Cry4Aa and Cry4Ba, is a short 3-domain protoxin protein. However, the 2025 bp cry10Aa gene (orf1) is followed (after a gap of 66 nt) by a second gene (orf2) that encodes a sequence similar to the carboxyl terminus of the Cry4Aa and Cry4Ba proteins. Orf2 codes for a 56 kDa with two proteins of 68 (orf1) and 56 (orf2) kDa and is expressed consequently when the complete operon has been cloned. The parasporal bodies formed by the complete Cry10Aa (Orf1- Orf2) are as active against *A. aegypti* as the Cry4 toxin. Cry11 also belongs to a large group of ÿ-endotoxins consisting of three structural domains and consisting of proteins and is active against dipteran targets ([6]. Cry11Aa of Bt is a 72 kDa protoxin located in an operon where the primary gene (1941 bp) was flanked by two other small genes known as p19 and p20. Among the individual toxins of Bt, Cry11Aa, together with Cry4Ba, is the second most abundant toxin produced, after Cyt1Aa. The Cry11Aa protein has high toxicity against the genera Aedes and Culex because its insecticidal activity is lower against the larvae of Anopheles species. This protein is activated in the insect midgut by proteolytic cleavage that produces two fragments of 38 and 30 kDa in the manner of the capacity to bind to midgut microvilli.

In the case of *A. aegypti*, Cry11Aa can interact with different midgut brush border membrane receptors, a GPI-anchored alkaline phosphatase (GPI-ALP), an aminopeptidase N, and a cadherin. The protein also binds to Cyt1Aa as a membrane-bound receptor, enhancing its activity. In *Anopheles albimanus*, alpha-amylase is already been described as a putative binding receptor for Cry11Aa. Abide other midgut proteins, such as ATP-binding protein, that enhance the toxicity of this protein to third-instar larvae of C. quinquefasciatus [7]. Cry11Aa is also toxic to other dipterans such as *Chironomus tepperi* (Diptera, Chironomidae), *Tipula oleracea*, and *Simulium species*. Two other Cry11 proteins, Cry11Bb (94 kDa) produced by Bt ser. Medellin and Cry11Ba (81 kDa) produced by Bt ser. jegathesan, has the same insect specificity and higher activity than Cry11Aa. Three different *A. aegypti* midgut proteins, cadherin, AaeALP1, and AaeAPN1, are involved in the binding of Cry11Ba to the *A. aegypti* midgut brush border membrane.

In Bt ser. jegathesan, the mpp60A (960 bp) and mpp60B (912 bp) genes form an operon. These two ORFs have also remained detected in Bt ser. malayensis 4AV1 and Bt ser. israelensis ATCC 35646. Interestingly, the operon containing the mpp60A and mpp60B genes has the same structure in these three Bt strains, it is typify classified into three different

serovars. Both proteins belong to the Etx/Mtx2 protein family. Individual or coexpression of the mpp60A and mpp60B genes in Bt strains (acrystalliferous) resulted in crystalline components (33 and 35 kDa, respectively) that showed moderate insecticidal activity against fourth instar larvae of *C. quinquefasciatus*. Despite being part of the same operon, Mpp60Aa and Mpp60Ba should not be considered a binary toxin because neither of them depends on the other to be expressed or succeed in exerting its insecticidal activity on target insects [8].

5. Other Poisons Specific to Diptera

In addition to the *Bt ser. israelensis* toxin, there are several other 3-domain Cry proteins from different Bt serovars with toxicity against several Diptera species. For example, Cry19Aa, identified in the *Bt ser. jegathesan* strain, together with ORF2 (encoded in an operon structure similar to the cry10- orf2 format of Bt), shows toxic activity against *C. pipiens* and *An. stephensi* Cry19Ba, a close member of this family derived from Bt ser. Higo shows activity against *Culex molestus* larvae, but not against *An. stephensi*. Cry20Aa is another mosquito-killing protein slightly toxic to *A. aegypti* and *C. quinquefasciatus* larvae and seems to be produced by the *Bt ser. Fukuokaensis* strains. However, its toxicity is not high, probably due to rapid protein degradation. The Cry24Ca protein also shows larvicidal activity against *A. aegypti* [9].

Within the Cry27 family, resides reported that the Cry27Aa protein produced by *Bt ser. Higo* strain showed activity against *An. stephensi* but was not toxic to species classified in the genera Culex or Aedes. The Cry39Aa protein has been found to be highly toxic to *An. stephensi* larvae [10]. In contrast, Cry44Aa from *Bt ser. entomocidus*, showed high toxic activity against *Culex pipiens* and *A. aegypti*, although its activity against *An. stephensi* was lower. The aerolysin-like Mpp46Ab (previously known as Cry46Ab and also called parasporin-2Ab) and Cry50Ba formed were described to be highly active against *C. pipiens* and *C. quinquefasciatus* larvae. Tpp80Aa (previously known as Cry80Aa) also showed toxicity against *C. pipiens*. Lastly, Cry47Aa appeared to be active against dipteran species, such as the sheep fly *Lucilia cuprina* (Diptera, Calliphoridae).

6. Anti-Diptera Cyt Toxin

Proteins of the Cyt1 family bear no resemblance to any of the currently described Cry family (including those recently renamed to other structural classes). Of all the proteins advised in the Cyt1 family, the Cyt1Aa protein is undoubtedly the most studied. Cyt1Aa is the main component of Bt crystals and adopts a typical cytolysin fold containing an ÿ-sheet held together by two surrounding alpha-helical layers [11]. The insecticidal activity of Cyt1Aa for larvae of various dipteran species has been reported by several authors. Efficient expression of the Cyt1Aa protein (molecular mass 27 kDa) requires the presence of a 20 kDa “helper” polypeptide. Proteolytic digestion of the Cyt1Aa protein yields a 22-25 kDa fragment that is more effective than the native protoxin *in vitro* [12].

This toxin exhibits *in vitro* hemolytic and cytolytic activity on vertebrate and invertebrate cells due to interactions between its hydrophobic segments and membrane phospholipids of midgut epithelial cells. However, different activities against insects and red blood cells were considered. Cyt1Aa was tested as a fully soluble protein, mixed with food, against several species of the suborder Brachycera, toxic to first instar *Lucilia sericata* (Diptera, Calliphoridae), *Lucilia cuprina* (Diptera, Calliphoridae), and *Calliphora stygia* (Diptera, Calliphoridae). In these experiments, trypsin treatment of the soluble toxin increased the activity 4-6-fold, regardless of whether pure insoluble Cyt1Aa crystals were not toxic. Cyt1Aa also showed toxic activity against larvae of *Tipula paludosa* [13].

Radically, no highly toxic Bt toxin remained against *Ceratitis capitata* (Diptera, Tephritidae) in the field. However, several authors have shown that, under controlled laboratory conditions, the soluble Cyt1A protoxin exhibits moderate toxicity to *C. capitata* larvae. *Bacillus thuringiensis* crystals do not appear intended to solubilize easily below pH 9, and the pH of C. *capitata* third instar larvae and the midgut of adults was calculated to be 8 and 7.5, respectively. The development of more accurate and reproducible quantitative methods may aid in the determination of toxic properties for insecticidal pathogens that act on adult Diptera by ingestion [14]. Cyt1Ab, a protein with 86% identity to Cyt1Aa, is also active, albeit to a lesser extent than Cyt1Aa, against Aedes, Anopheles, and Culex larvae. The ability of Cyt1Ba to induce death and reduce damage caused by the mining larvae of *Liriomyza trifolii* (Diptera, Agromyzidae) has been described. Cyt1Ca is approximately twice the size of other Cyt proteins and, in addition to the Cyt-like region, has an additional C-terminal lectin-like domain. No hemolytic activity or effects were observed for the protein encoded in the pBtoxis plasmid of Bt.

Proteins of the Cyt2 family have been identified and characterized in several Bt serovars: Cyt2Aa from Bt ser. Kyushensis and darmstadiensis, Cyt2Ba from Bt, Cyt2Bb from Bt ser. jegathesan and Cyt2Bc from Bt ser. Medellin. Cyt2Aa1 from Bt ser. kyushensis shows low identity (39%) with Cyt1Aa from Bt, but similarity is 70%. Furthermore,

both are processed in similar domains, probably because they share a high potency of structural similarity. Cyt2Aa1 is a 29.2 kDa protein accompanying the crystal structure that has been solved. It consists of a single ÿ-ÿ domain containing two outer layers of ÿ-helix hairpins and an ÿ-sheet in between. The protein does not show hemolytic activity as a protoxin, and despite that, the N- and C-terminal segments are cleaved by primary proteolysis to dimer dissociation and toxin activation. Cyt2Aa1 showed LC50 values in the range of 0.5 and 4 µg/mL against Culex, Anopheles, and Aedes larvae. Cyt2Aa2 produced by Bt ser. Darmstadiensis showed moderate activity against Culex and Aedes larvae and hemolytic activity against sheep erythrocytes. Cyt2Aa3, from Bt strain MC28, showed toxic activity against *Ch. tepper* (Diptera, Chironomidae) and *C. quinquefasciatus* larvae [8].

Cyt2Ba1 protein (30.1 kDa) from Bt shows 41% identity with Cyt1Aa1 and 67% with Cyt2Aa1. It is less active than Cyt1Aa against *A. aegypti, C. pipiens, C. quinquefasciatus* and *An. stephensi* larvae. In addition, solubilization or activation by trypsin is essential for its hemolytic activity. The crystal structure of the proteolytically cleaved form of Cyt2Ba has been elucidated and resembles the protoxin form of Cyt2Aa and the fungal volvatoxin A2. Cyt2Bb, from *Bt ser. jegathesan* (30.1 kDa) shows mosquitoicidal activity against *A. aegypti* larvae [12]. Its toxicity is lower than Cyt1Aa, in spite of both proteins having similar hemolytic activity. It's Cyt2Bc from *Bt ser. Medellin* (29.7 kDa) showed mosquitoicidal activity against *A. aegypti, An. stephensi, C. pipiens* and *C. quinquefasciatus*. However, its toxicity was lower than Cyt1Aa and Cyt2Ba, and trypsin treatment was required for its hemolytic activity.

7. Poison with Synergistic Activity against Diptera

For several insecticidal bacteria, including Bt, the high toxicity of the complete crystal compared to that expected, in terms of the additive effect of the toxicity of the individual constituent proteins, has been attributed to the synergistic activity of its components. The previously mentioned has been studied further in Bt than in other Bt serovars. The combinations Cry4Aa+Cry4Ba, Cry11Aa+Cry4Aa, and Cry11Aa+Cry4Aa+Cry4Ba shown to interact synergistically for a large number of species classified in the mosquito genera Aedes, Anopheles, and Culex. In addition, Cry4Ba has a synergistic effect with Cry10Aa against C. pipiens and with Cry11Aa against *A. aegypti* and *An. albimanus* larvae. Although Cyt1A is the most toxic, it is the strongest synergist among the ÿ-endotoxins against A. aegypti. Cyt1A interacts synergistically with Cry11A against *C. quinquefasciatus* and *An. albimanus*, and with Cry4Ba against *An. albimanus*. In addition to significantly reducing lethal concentrations, the synergistic effect of Cyt1A also plays a required role in slowing the emergence of resistance to Cry protein case concern of *C. quinquefasciatus* [16].

Furthermore, Cyt2Ba, which is present in the lowest amounts in Bt crystals, has some synergistic effects with Cry4A and shows one of the highest synergistic interactions described so far with Cry10Aa against *A. aegypti*. Another Cyt protein from Bt ser. darmstadiensis, Cyt2Aa2, has a highly synergistic activity with Cry4Ba from Bt against *A.aegypti* and *C. quinquefasciatus* larvae. The mechanism of Cyt synergy has advanced because the Cyt1A protein can function as a membrane-bound receptor for both Cry4Ba and Cry11A [10]. About the Cry11Aa protein, it has been suggested that Cyt1Aa inserts its ÿ-sheet into the membrane with two components (the ÿ6-ÿE loop and part of ÿ7) that bind with high affinity to Cry11Aa, which is integrated into the epithelial membrane of the larvae. Cyt1Aa appears to facilitate the formation of pre-pore oligomeric structures capable of forming pores in synthetic lipid membrane vesicles. However, oligomerization and membrane insertion of Cyt1A are not essential for its synergistic activity. Similarly, Cyt2Aa2 has been proposed to act as an alternative membrane receptor capable of binding specifically to Cry4Ba.

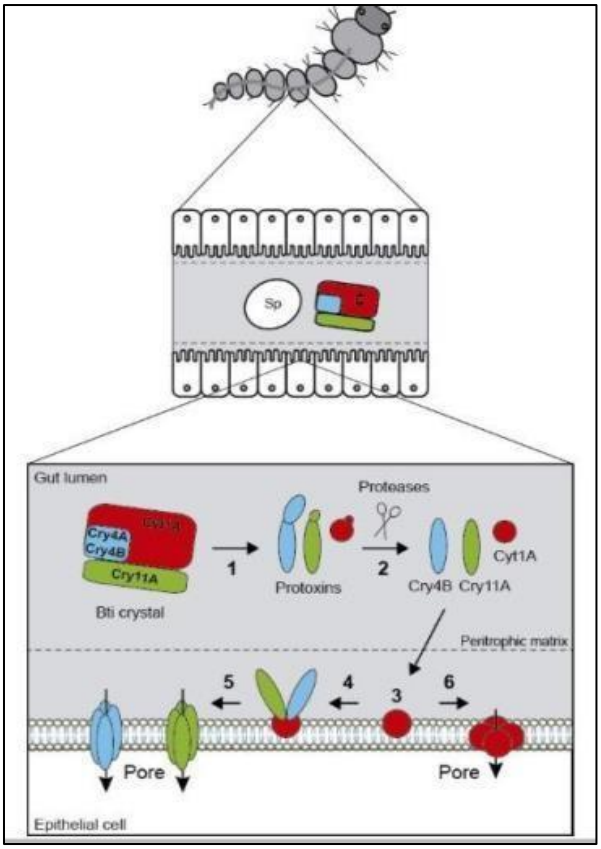

Figure 2 Synergy mechanism diagram after Bti ingestion by a mosquito larvae [6]

Bt Cry toxins can also synergize with proteins from other serovars. For example, Cry11Ba from *Bt ser. jegathesan* and Mpp46Ab (formerly Cry46Ab) from Bt TK-E6 exhibit synergistic activity in combination with Cry4Aa from Bt against *C. pipiens* larvae. The proteins Mtx1 and Mpp2 (formerly Mtx2) from L. sphaericus were additionally found to interact synergistically with various Cry-toxins from Bt against *C. quinquefasciatus*. Consequently, exploiting synergies between different types of Bt or *L. sphaericus* toxins is an excellent strategy to enhance the virulence of these microorganisms against relevant dipteran species [17].

8. Mosquito Repellent Bacterial Insecticide

Currently, the main biological control alternatives for mosquito and black fly larvae involve bacterial toxins produced by Bt and *L. sphaericus*. Products based on Bt and L. sphaericus are marketed and used widely in the US and Europe, and several different formulations have been developed. The main commercial products are suspension concentrates, followed by wettable powders and, to a lesser extent, large-granular formulations. L. sphaericus, due to its better residual activity in polluted waters, has been used extensively against Culex species in the US, Central America, Brazil, India, Thailand, and China. To manage mosquito larvae, the formulated bacteria are sprayed or broadcast onto static or slow-moving water, attending sink at a rate determined by the formulation design [2].

An innovative formulation of Bt incorporated into ice pellets was used in a mosquito control program in the Rhine in Germany. The different feeding habits of larvae of particular species influence the effectiveness of the bacteria against mosquitoes. Culex larvae filter water up and down the water column and are often referred to as column feeders despite the fact that Aedes larvae tend to scavenge along the surface of the substrate, particularly at the bottom. Anopheles larvae feed on floating material trapped below the water surface. Under comparable conditions, the two Anopheles species filter water at 33 34 and 49 55 ullarvae/h, respectively, while C. quinquefasciatus filters 490-590 and A. aegypti 590-690 ullarvae/h [18]. The feeding habits of the larvae partly explain why Anopheles species consistently appear less susceptible to Bt suspensions than column- and bottom-feeding Culex and Aedes larvae in laboratory and field trials. Therefore, differently formulated products are needed for mosquito larvae of different feeding types. Floating products are required for anopheles, but some must remain suspended below the surface for column and bottom feeders. In habitual waters, rapid immersion should be obnoxious because a steady sediment of faces will soon cover the particles.

Black fly larvae live in fast-moving water currents and are controlled by pouring bacterial suspensions into the water at successive points, from where they go downstream.

The effectiveness of many bacterial formulations against mosquitoes and blackflies is short-lived in the field, often only 1–2 days. This is due to rapid settling, adsorption to plants and other substrates (which also filter particles out of the water), denaturation of the crystals by sunlight, and ingestion by filter-feeding fauna. The primary purpose of the formulation process is to extend the effective period. However, UV radiation in the water is not as important as particle settling, which is an essential factor in determining the effectiveness of a particular assumption, since water filters out most UV radiation. With Bt, only the effect of sunlight on the crystals reduces larval mortality because spores are not significant for mosquito and blackfly larvae. *L. sphaericus* is more susceptible to sunlight, becoming inactive in clear water a few centimeters deep in full sunlight, while strong sunlight reduces its effectiveness several-fold [19]. Sunscreen may benefit *L. sphaericus*, especially in formulations designed to float.

To increase the effectiveness of active Cry proteins against Diptera, they have been transferred to alternative hosts to increase their persistence in aquatic feeding areas. An improved biopesticide for mosquitoes was refined by inserting the Cry gene from Bt, which is highly toxic to mosquitoes, into the chromosome of *L. sphaericus*, which has a longer environmental persistence. The chromosomally integrated cry gene seemed to be maintained for several generations in the absence of selective pressure. Recombinant *L. sphaericus* produced high levels of the cry11A gene product of Bt, which is toxic to Aedes, Culex, and Anopheles larvae. Various other recombinants have also been produced and tested, as reviewed by Federici et al. [15].

9. The Effect of Bacillus thuringiensis as Bioinsecticide on Musca domestica

Musca domestica L. (Insecta: Diptera: Muscidae), is considered a prominent pest by medical, veterinary, and public health experts worldwide. The housefly has made it a potential pest in transmitting various diseases from human to human and/or animal to human. More than 100 animals remain experimentally associated with the housefly, including protozoan, bacterial, viral, and helminth infections. Therefore, some threats to humans and the poultry industry. *B. thuringiensis* is known to produce several toxins during its logarithmic growth phase, such as heat-labile α-exotoxins (lecithinase C) and β-exotoxins that are insoluble in water, heat-stable, and highly toxic to housefly larvae [20].

A bioassay test was conducted by isolating *B. thuringiensis* that had undergone the sporulation phase, and bacteria had reached the autolysis phase (using the spore staining method), protein crystals, spores, and cellular waste were isolated from culture media by centrifugation. Protein crystals were then purified and separated by gel filtration chromatography. In the maintenance of *M. domestica* larvae, they are placed in another container (25-30°C, 80% humidity). At this stage, a mixture of wheat bran (0.2 mg/mL), flower straw (0.1 mg/mL), and grape juice (5% v/v) is foreowned as larval food. Furthermore, larval food was mixed with delta endotoxin poison and incubated for 10 days with larvae at concentrations of 0.43 mg/mL and 0.27 mg/mL. The bioassay results showed the cytotoxic effect of protein on fly larvae that 0.43 mg/mL protein can kill all larvae (100%) [20]. The protein crystal was further purified and separated by gel filtration chromatography, and four different products formed with molecular weights of 28, 68, 128, and 130 kDa from the right. The active part of the crystal is a 68 kDa protein or delta-endotoxin. Delta endotoxin is a glycoprotein with massive variations. Delta endotoxin was successfully separated into five main categories based on its activity against various groups of insects and worms. The gene of this crystal protein has several groups and subgroups. Several studies have cloned and sequenced 90 genes of the protein. Bioassay investigations have shown that the toxin has high cytotoxicity, while if varying proteins (raw proteins) became inherited in biological measurements, its cytotoxicity is much higher. Compared to chemical insecticides, the main advantage of B. thuringiensis is its highly defined activity against dipterans due to the occupancy of membrane receptors in the insect gut that constitute targets for bacterial toxins. Based on the common knowledge of toxins and spores that don't persist in the environment, with almost no residual effects, even in environments such as those submitted for seasonal applications. In this study, it was initiated that the protein crystals of Bacillus thuringiensis subsp. israelensis are a biologically effective weapon specifically valuable in the campaign against houseflies [20].

To determine the effect of *B. thuringiensis* on the life phase of *M. domestica*, Bt suspension processing was carried out at four concentrations (0.5, 1.0, 1.5, 2.0%). In *M. domestica* pupae, it was significantly affected by all concentrations applied. All pupa weights decreased at all concentrations used in the experiment, namely the pupa recorded the following average weights: (54.7 ± 12.20 mg), (55.1 ± 12.20 mg), (66.4 ± 10.01 mg) and (70.2 ± 12.80 mg) respectively, once compared to the experiment because it was recorded (73.5 ± 8.83 mg). The recorded results also showed that the age of the pupal phase was persuaded by the increase in the provision of larval feed in the bacterial treatment

environment with different concentrations, ranging between (3.67 ± 0.8.36) and (5.12 ± 0.119) days compared to the control group (3.04 ± 0.115) [21].

Bacillus bacteria produce different toxins during their life cycle, including external toxins (Alpha, Beta, Gamma) and one type of internal toxin known as internal toxin (Sigma). The ability of these toxins to cause death of dipteran insect larvae and scalp. Many scientists are interested in studying the toxicity, biochemistry, structure, and pathological and histological of these toxic protein molecules on insect larvae, especially dipterans. Studying the environmental activity of several Bt bacterial isolates against *Musca domestica* showed that all isolates have the mentioned activity against the tested larvae and agree with our results that Bt is a bio component against *Musca domestica* larvae [22].

B. thuringiensis var. kyushuensis exhibits mosquito-activating activity due to the presence of CytB toxin in the paraspore inclusions. Here, a preliminary evaluation of the potential of *B. thuringiensis* var. kyushuensis (strain Btk176) for controlling *M. domestica* larvae and adults conducted by histopathological effects on the intestinal epithelium of larvae examined after exposure. Neonatal fly larvae were conserved with three concentrations (100, 70, and 60%) of spore suspension and supernatant. Larvae from each treatment group and control at 6, 12, 24, and 48 h were collected to dissect their digestive tracts for transmission electron microscopy (TEM) examination. The posterior part of the midgut was identified based on the position of the Malpighian tubules. Examination of Btk176 sporulated cells by TEM revealed the presence of bipyramidal crystals. In contrast, mosquito-killing strains of this serovar showed irregular crystals. Btk176 strain showed significant pathogenicity to larvae. Here, a bioassay was performed that is commonly used to demonstrate the absence of Bt and provides a strong indication that the Btk176 strain is devoid of ÿ-exotoxin. Data generated from TEM analysis showed that the histopathological effects in the insect gut included a continuous and progressive disorganization of the cell structure that was absent in the control group. The histopathological damage recorded by TEM analysis in treated larvae has been reported to be a typical consequence of the toxin binding to its specific receptor, resulting in the formation of pores that increase cell permeability, consequential swelling, and rupture [23].

We speculate that the entomopathogenic activity of Btk176 may be directly related to Cry1Ba activity since statistically significant mortality, PCR amplification of the cry1B gene, positive cry1Ba gene, and crystal inclusions observed by electron microscopy. The initial analysis yielded promising data for strain Btk176. Further investigation of the sequencing of the cry1Ba PCR product confirming Cry1Ba expression was required through crystal isolation and proteomic analysis. The increased toxicity exhibited by strain Btk176, which can achieve at readily achievable spore concentrations without any exotoxin production, clearly suggests the use of a continuous screening program to identify new candidates for use as active ingredients in bioinsecticide products for the integrated control of M. domestica larvae [23].

10. The Effect of Bacillus thuringiensis as a Bioinsecticide on Bactrocera sp.

Fruit flies (Diptera: Tephritidae) are a group of important agricultural pests worldwide that attack varying fruits and vegetables. The large number of fruit fly species poses a significant threat to fruit and vegetable production worldwide, causing both quantitative and qualitative losses. Based on the severity of the pest, host range, level of invasion, and frequency of infestation, *B. dorsalis* (oriental fruit fly), *B. cucurbitae*, and species of *Bactrocera* sp. were included in the Group A category, which contains widespread invasive polyphagous generalists or highly damaging specialists that have developed outside their native range. Decreased the attack caused by *Bactrocera* sp., a study is being conducted by applying Bacillus thuringiensis. B. thuringiensis produces various insecticidal proteins such as Crystal proteins (Cry), Cytotoxic proteins (Cyt), Vegetative Insecticidal Proteins (VIPs), and Secreted Insecticidal Proteins (Sips) during its growth phase [24].

The B*. thuringiensis* bioassay test was conducted by inoculating *B. thuringiensis* with a concentration of 12 ml on artificial media as feed for *B. dorsalis* larvae. The results showed that the percentage of larval mortality was 88%. In addition to the mortality rate, observations were also made on the physical condition of the larvae. The treated larvae showed physical damage. The toxins produced by the bacteria affected the weight of the larvae intensely. At the end of the observation, the treated larvae were significantly smaller in size (6.97 ± 0.97 mm) compared to the control larvae (10.08 ± 0.22 mm). In addition, the larvae also became lighter (0.01922 ± 0.001 g) compared to the control (0,02654 ± 0,0003 g).

Larvae that do not die when treated will develop into pupae to imago. However, the size of the pupae is significantly affected by the treatment. The pupae formed are smaller (4.0 ± 0.75 mm) compared to the control and are pale yellowish in color. Normal pupae (control) are average about 5.72 ± 0.28 mm in size and brown in color. Bt activity against B.

dorsalis larvae also causes a depressive effect on the rate of imago emergence. The rate of emergence for treated larvae was significantly lower (65.25%) compared to the control (93,01%) [25].

Isolate of *B. thuringiensis* isolated from Bangladesh city, encoded by JSc1, contains cry1, Cry2, and Cry 9 genes, which show significant toxicity to B. cucurbitae instar larvae. Isolate JSc1 has a protein molecular weight ranging from 23 kDa to more than 200 kDa. The results of the bioassay test conducted showed that the percentage of mortality in isolate JSc1 against B. cucurbitae larvae reached 93%. The synergistic effect of Cry proteins encoded by cry1, cry2, and cry9 causes the toxicity of isolate *B. thuringiensis* against *B. cucurbitae* [26].

Meanwhile, Bt isolates isolated from Amritsar encoded with B. thuringiensis VIID1 expressed a protein band of ~95 kDa when subjected to SDS-PAGE analysis. The alignment of partial cry sequences with the NCBI database indicated that the amplified gene sequence was related to the cry1 gene family. Isolate VIID1 contained spherical protein crystals. In this study, the bioassay results noted that the developmental period of larvae, pupae, and total development of B. cucurbitae were significantly overdue when given various concentrations of Cry protein isolated from B. thuringiensis VIID1 supplemented in artificial feed compared to the control. When 64-72 h-old larvae were fed with feed containing Cry protein, the mortality percentage reached 61.2% at the highest concentration (10 μg/ml) [27].

11. Conclusions

Control of dipteran pests is particularly relevant as some species in this order are a critical source of damage to extensive crops. Until the present, the use of bacteria in the field has been limited to the control of Diptera suborder Nematocera and the use of Bt strains. The use of these bacteria in control programs has often been highly successful. Significant resistance to Bt has not been reported in the field, and this is probably due to its various toxins, in particular, the role of the Cyt proteins.

Attempts were made to combine the activities of Bt and *L. sphaericus* toxins in a single strain despite the fact that no commercialized recombinant strain remains. Alternative strategies for the delivery of dipteran-active toxins against Nematocera seemed explored, including the incorporation of pesticide protein genes into a range of other organisms, including cyanobacteria, Caulobacter, Ancylobacter vacuolated gas, and the mosquito gut colonizing *B. cereus*, or encapsulation within Tetrahymena but, again, no products came to be produced which used of naturally occurring Bt and L. sphaericus strains remains the control method of choice.

B. thuringiensis var. kyushuensis bacteria from TEM analysis showed that the histopathological damage recorded by TEM analysis in treated M. domestica larvae was reported as a typical consequence of the toxin binding to its specific receptor, resulting in the formation of pores that increase cell permeability, leading to swelling and rupture. It is also known that the entomopathogenic activity of Btk176 may be directly related to the activity of Cry1Ba because the mortality was statistically significant and could be a new candidate for use as an active ingredient in bioinsecticide products for integrated control of *M. domestica* larvae.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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