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Chapter

Toxic Potential of *Bacillus thuringiensis*: An Overview

David Fernández-Chapa, Jesica Ramírez-Villalobos and Luis Galán-Wong

Abstract

The toxins of *Bacillus thuringiensis* (Bt) have shown great potential in the control of harmful insects affecting human health and agriculture, used as the main biological agent for the formulation of bioinsecticides due to its specificity to target different insects’ orders. This has led Bt-based products to become the best-selling biological insecticides in the world since the genes encoding insecticidal proteins have been successfully used in novel insecticidal formulation, genetically engineered (GE) crops, and development of transgenic rice that produce insecticidal toxins derived from *Bacillus thuringiensis*. It has been proven that insecticidal activity of Bt protein crystals can prolong their toxicity in shelf life or field under specific conditions, and this can improve the use of special strains and formulations to control insect vectors of diseases. Bt toxins have shown well-documented toxicity against lepidopterans, coleopterans, hemipterans, dipterans, nematodes, Rhabditida and human cancer cells of various origins. These crystal toxins may be responsible for other novel biological properties suggesting a pluripotential nature with different specificities.

Keywords: *Bacillus thuringiensis*, Cry toxins, bioinsecticide, resistance, Dulmage

1. Introduction

In the modern era, *Bt* was isolated for the first time in Japan by the bacteriologist Ishiwata Shigetane in 1901, and it was considered the microorganism responsible for the disease of the silkworm *Bombyx mori*. The author named it *Bacillus sotto*, which means soft and flaccid, in reference to the appearance of the infected larvae. He noted that young bacterial cultures were not pathogenic to larval insects; in contrast old cultures that suffered sporulation were highly toxic. However, the first valid description was until 1911, when the German scientist, Ernst Berliner, isolated it from diseased larvae of the flower moth *Anagasta kuehniella*. He named it *Bacillus thuringiensis*, which derives from Thuringia, the German town where moths were found [1].

*Bacillus thuringiensis* is a ubiquitous gram-positive, rod-shaped soil bacterium, that has been isolated worldwide from a great diversity of ecosystems including soil, water, dead insects, dust from silos, leaves from deciduous trees, diverse conifers, and insectivorous mammals [2–4], known by its ability to produce crystalline inclusions during sporulation (Cry toxins) which contain insecticidal proteins called
δ-endotoxin. Crystalline inclusions from Bt are showing well-documented toxicity to a wide variety of insect pests, such as Lepidoptera, Coleoptera, and Diptera [5], hemipterans, as other biological activities such as molluscicidal, nematicide (human and animal parasites, and free living; Rhabditida), acaricide and even against human cancer cells [2, 6–10].

Bt toxins have been applied to the environment since 1933 and began to be used commercially in France in 1938, and by 1958 their use had spread to the United States. From the 1980s Bt becomes a pesticide of global interest [11]. Bt crystal and secreted soluble toxins are highly specific for their hosts and have gained worldwide importance as an alternative to chemical insecticides. Bt toxins have been considered as the most successful bioinsecticide during the last century. Currently, it consists of more than 98 (424 million USD) of formulated sprayable bacterial pesticides [12] and is the most common environmental-friendly insecticide used and is the basis of over 90% of the pesticides available in the market today [13].

2. Bioinsecticide activity of Bacillus thuringiensis proteins

The main difference between Bacillus thuringiensis and other closely related bacillus is the formation, during the sporulation process, of one or more crystalline bodies of a protein nature adjacent to the spore. Some of these parasporal crystals known as δ-endotoxins (Cry and Cyt) confer the pathogenic capacity against larvae of different orders of insects, mostly Lepidoptera, Diptera, Coleoptera and in some cases against species of other phyla [14]. By synthesizing parasporal crystalline inclusion during sporulation, the bacterium can ensure its survival, since a dead insect can provide sufficient nutrients that allow the spores to germinate [15].

Bt strains synthesize crystal (Cry) and cytolytic (Cyt) toxins (also known as δ-endotoxins), at the onset of sporulation and during the stationary growth phase as parasporal crystalline inclusions. Additionally, Bt isolates can also synthesize other insecticidal proteins during the vegetative growth phase; these are subsequently secreted into the culture medium, the vegetative insecticidal proteins (Vip) [5, 16], and the secreted insecticidal proteins (Sip) [17].

This part refers to the nomenclature first used for Cry genes, on the next part of the page it explains the nomenclature currently used for Bacillus thuringiensis genes [18] (Table 1).

However, this nomenclature was not ideal, since the new toxins had to be tested against an increasing number of insects so that the toxin and the gene could be

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Table 1. Classification of Cry toxins according to their insect host specificities proposed by Crickmore et al. [18].
named; that was when the *Bacillus thuringiensis* Toxin Nomenclature Committee was created in 1993 and proposed a new classification system [18], which consists of giving the new toxin a four-rank name depending on its degree of pairwise amino acid identity to previously named toxins, using Arabic numbers for the first and fourth rank and uppercase and lowercase letters for the second and third ranks, respectively, for example, Vip1 and Vip2 if they share less than 45% pairwise identity, Vip3A and Vip3C if they share less than 78% pairwise identity, Vip3Aa and Vip3Ab if they share less than 95% pairwise identity, and Vip3Aa1 and Vip3Aa2 if they share more than 95% pairwise identity [19].

Based on the amino acid sequences, there are 75 families of Cry proteins, with 800 different *Cry* genes [20], while the Cyt proteins consist of three families with 38 genes [21].

Cry proteins have been reported to be toxic to Lepidoptera, Coleoptera, Hymenoptera, Hemiptera, Diptera, Orthoptera, and Mallophaga and also against nematodes, mites, and Protozoa (Figure 1) [22]. Some toxins have an expanded

![Figure 1](image_url)

*Insecticidal activity of Cry and Cyt δ-endotoxins against the orders Diptera, Coleoptera, Lepidoptera, Hemiptera, and Hymenoptera [15, 21, 23]*.
spectrum of action to two or more order or phylum [10]. For example, Cry1B is one of those that present a remarkable activity against larvae of Lepidoptera, Diptera, and Coleoptera. So, the combination of toxins present in a strain will define its spectrum of action [4].

In contrast, Cyt toxins have predominant activity against dipterous; however, they have toxic activity against some lepidopteran and coleopteran [24]; in addition, some Cyt toxins are able to establish synergy for insecticidal activity with other Bt proteins such as Cry or Vip3 and to reduce the resistance levels of Cry proteins toward some insect species of the Coleoptera and Diptera orders (Figure 1). The Cyt1Aa toxin from Bacillus thuringiensis var. israelensis is active against Chrysomela scripta and Culex quinquefasciatus and can prevent the development of resistance to the proteins Cry3Aa, Cry4, and Cry11Aa [14].

2.1 Bti toxins

Bacillus thuringiensis subsp. israelensis (Bti) was first isolated from a water pond in the Negev desert [25] and was the very first strain described for having insecticidal activity outside Lepidoptera. Bti serovariety, H-14, is a subspecies of the diversified Bacillus thuringiensis species. The serovariety H-14, Bti, produces four main toxins (Cry4Aa, Cry4Ba, Cry11Aa, and Cyt1Aa) specific to dipterans (mosquitoes, blackflies, etc.) which represent a serious threat to public health because of their hematophagous nature and vector capacity responsible for high morbidity and mortality in billions of people spread over almost half of the planet.

Bti toxin Cry4Ba is active primarily against Anopheles and Aedes and shows no toxicity to Culex species, in contrast to Cry4Aa toxin that is toxic to Culex larvae. Cry11 is the most toxic to Aedes, and Cyt1Aa shows low (Aedes, Culex) to non-toxicity at all (Anopheles). Cyt1Aa has a strong synergistic effect on the toxicity of Cry toxins in all mosquitoes. In addition to its own mosquitocidal and cytotoxic activity, Cyt1A was shown to act synergistically with the other Bti toxins [26, 27].

All Bti insecticidal proteins are produced as protoxins, and all must be activated in vivo by insect midgut proteases prior insecticidal activity.

2.2 Mechanism of Cry toxin action

Although the mechanism of action of Cry toxins against various insects has been widely investigated, there are still many controversies. Therefore, there are currently different models in the literature that seek to explain it [28].

The sequential union model is known as the classical mechanism. It has been detailed in studies with the Cry1Ab protein in Manduca sexta. It postulates that the toxic properties come from crystalline inclusions produced during the sporulation of Bt. The crystals and their subunits are inert protoxins and are not biologically active, and their mode of action can be plotted as follows: the δ-endotoxins are ingested, the crystals are solubilized by the alkaline pH of the intestine, the inactive protoxins are digested by proteases of the midgut which produces an active toxin of about 60–70 kDa resistant to proteases, and then the Cry toxins come into contact with the N-aminopeptidase receptors and cadherin on the surface of the membrane. The affinity between toxins and certain types of receptors results in proteolysis of the Cry protein that causes structural changes in the chains and forms oligomers that function as “pre-pores.” The N-aminopeptidase receptor anchors the pre-pore in the lipid bilayer, pore formation affects integrity of the membrane,
and electrophysiological evidence and biochemistry suggest that the pores cause an osmotic imbalance that causes cell death and lysis; the intestine is paralyzed, the insect stops feeding, and there is diarrhea, total paralysis, and finally death (Figure 2) [1, 29].

The second proposed mechanism called signaling pathway model has similarities with the previous model; however, in this other causes for cell death are assigned. According to this theory, Cry proteins affect the cell in two ways: first by the formation of pores in the membrane, as mentioned in the sequential binding model and, second, by the production of successive reactions that alter the cellular metabolism. According to this hypothesis, Cry toxins bind to cadherin receptors, which stimulate heterotrimeric G protein and adenylyl cyclase with an increase in cAMP production. The cAMP activates the protein kinase A, which stimulates apoptosis with an activation of the Mg$^{2+}$ channels in the plasma membrane. The
opening of these channels causes an abnormal movement of the ions in the cytosol, stimulating the process of apoptosis (Figure 3) [1, 3, 30].

The germination of the spores also contributes to the death of insect, since the vegetative cells can replicate within the host’s hemolymph and cause septicemia; however, the δ-endotoxins alone are sufficient to kill some insect species if they are produced in high doses. This feature has been exploited by expressing the delta endotoxin genes in bacteria that better adapt to a particular environment, as well as its expression in genetically modified plants [31, 32].

3. Howard T. Dulmage’s methods and contributions on Bt

Howard T. Dulmage was a microbiologist who established his line research in the study of pathogenic bacteria insects [33] and is considered one of the most important pioneers in the development of technologies for the implementation of Bt as a control agent of biological pests [34].

Working at the US Department of Agriculture, at the Agricultural Research Service (USDA-ARS), in Brownsville, Texas, Howard T. Dulmage from the pink worm, Pectinophora gossypiellia, a strain of Bacillus thuringiensis variety kurstaki, in 1969, which is 200 times more active in laboratory tests against the pink bollworm, Pectinophora gossypiella; the tobacco budworm, Heliothis virescens; and the cabbage looper, Trichoplusia ni [21], higher than that of the known strains, which is marketed as “Dipel” by the company United States of America [35–37].

Strain HD-1 is one of the best-studied strains, since it is characterized by the carrying of a variety of Cry anti-Lepidoptera genes, Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, Cry2Ab, and Cry1Ia, and since its discovery, the outlook for Bt-based products has expanded and is still the most commercial success of microbial control of pest [4, 38].

H. Dulmage sets up the basis for the fermentation and formulation procedures of Bt culture extracts for their commercialization [39] and were among the most important pioneers in the development of technologies for the implementation of Bt as a biological pest control agent. He established diverse methodologies for mass production product formulation and power standardization [40].

At the beginning of the 1970s, two great advances were obtained by Dulmage, the first was based on the recovery of the spore-crystal complex by means of precipitation with lactose-acetone to produce powders and wettables, which was rapidly developed and adapted in the industry. The second was the adoption of a standardized system to calibrate the potency of the different preparations of Bt and the establishment of international toxicity units (ITU)/mg, which allowed the comparison of the different products developed [41, 42]. The equation proposed by Dulmage [43] is the following:

$$\text{Test extract potency (ITU/mg)} = \frac{\text{standard LC}_{50}[\text{standard potency (ITU/mg)]}}{\text{test extract LC}_{50}}$$  (1)

Dulmage established better bioassay methods to assess the effectiveness of powders [37, 44].

In 1984, Dulmage participated in the establishment of a bioassay protocol for toxicity assessment of Bacillus thuringiensis var. israelensis powders. This protocol differs from the one previously suggested by WHO in the Guidelines for Bti Production regarding the follow aspects:
• Specifies a standard cup for larval exposure to Bti extracts.

• Establishes a number of 20 larvae per cup and three replications for the concentrations assayed.

• If a minimum of six extract concentrations is tested, a repetition of the assay is required.

• A computational probit analysis is required for evaluating the toxicity as LC50.

• A mortality or pupation higher than 5% in the control invalidates the bioassay.

Additionally, the study suggested a variability coefficient of less than 20% for each repetition. Dulmage, together with a team of colleagues, tested the validity of this protocol and suggested some considerations for the management of the reference standard strains and for the establishment of new ones.

3.1 Howard T. Dulmage’s fermentation extracts

From 1970 to 1988, Dulmage established the largest Bt collection in the Americas, and he collected more than 800 isolates that were named using his HD code, belonging to 21 serovarieties. From these 800 isolates, 17 belonged to the H-14 serovariety, corresponding to Bti. He conducted a series of fermentation experiments with Bt in order to optimize the production and to assess the effectiveness of powder; hundreds of fermentation extracts were generated, and some of them were donated by the US Department of Agriculture in 1989 to the International Collection of Entomopathogenic Bacillus of the Faculty of Biological Sciences of the University of Nuevo León, Mexico, which has approximately 4000 stored fermentation extracts of which 3000 of them correspond to HD strains, and currently extracts are found in the form of dry powder, with different times of storage [38].

3.2 Bti strain collection

In the 1970s, Dulmage continued to the control of disease-transmitting mosquito larvae using lepidopteran-active isolates having some reported dipteran activity. When Dulmage became aware of the discovery of a new Bt subspecies capable of attacking dipteran larvae, especially simuliiids (Bacillus thuringiensis subsp. israelensis) (Bti), he quickly perceived the great value of this discovery, because of the possibility to control dangerous human disease vectors, and began to be involved in studies on Bti as dipteran biocontrol agent.

One of the greatest contributions of Dulmage to Bti research was the compilation of a protocol guide for Bt H-14 serovariety local production. This guide was an extension of the procedures developed by him for the production, formulation, and standardization of lepidopteran-specific serovarieties. These guidelines were presented and discussed in the informal consultation on local H-14 Bt production, in Geneva, Switzerland, in October 1982. The 128-page booklet was prepared by Dr. Dulmage, at the request of the Scientific Working Group on biological control of vectors of the Special Program for Research and Training in Tropical Diseases of the World Health Organization, and was published in 1983 [45].

In 1985, Dulmage and a research group proved the tested strain was Bti HD-968-S-1983, which resulted to be 4.74 times more potent than the standard use
(IPS-78); the potency assigned to it was 4740 ± 398 ITU/mg. They recommended the use of this strain as the potency reference standard for comparison with any Bti formulation.

Twenty samples of the strain HD-500 and HD-567 of Bti fermentation extracts from the collection of Dulmage et al. [44] recovered by lactose-acetone coprecipitation during the period from 1978 to 1983 maintained their residual toxic activity against the mosquito *Aedes aegypti*. All extracts evaluated presented toxicity at the highest tested doses (1000 ppm), and two of the stored extracts (3260 and 3501) showed LD50 of 0.12 and 1.16 ppm, respectively [40].

Bti protein crystals from fermentation extracts showed persistence of toxic activity of fermentation extracts after more than three decades. This opens the possibility of improving the use of special strains and improved formulations to control insect vectors of diseases.

4. New Cry toxins

Despite the success of the application of Bt crystal proteins for the biological control of pests, at present it is still necessary to identify new Cry toxins with greater toxicity; this approach is considered one of the best ways to counter the potential resistance evolved by insects as well as in developing products against a wider spectrum of insect pests. Traditionally, Bt isolates were screened for their insecticidal spectrum by the time-consuming and laborious insect bioassays [22, 46]. Since only a limited number of cry genes have been used for insect control either in sprays or transgenic crops so far, novel insecticidal genes are required [31].

The most common technique used to predict toxicity is the polymerase chain reaction (PCR), through the identification of new cry genes [47], but high-throughput sequencing technology has also been used in the discovery of toxins [20]. Seventy-two antigenic groups (serovariety) have been distinguished for *Bacillus thuringiensis* [48]. Crickmore et al. [19] have designed an especial database for Bt toxins with links to information on host insects, based on the last update (www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/). About 952 toxin genes, encoding different entomopathogenic proteinaceous toxins, have been identified and characterized in the Bt strains isolated all around the world; however, only a small proportion of these proteins are highly toxic and therefore used in the production of bioinsecticides. This can be accomplished by either finding new wild-type strains or engineering Cry proteins with enhanced activity or altered insecticidal spectrum by swapping domains and site-directed mutagenesis; nevertheless a thorough knowledge of Cry protein structure and binding interactions with target receptors is a must [49].

Additionally, the construction of Bt DNA libraries in *Escherichia coli*, followed by screening by Western blotting or a hybridization-based method, or the development of DNA libraries in an acrystaliferous mutant of Bt followed by microscopic observation and/or SDS-polyacrylamide gel (SDS-PAGE) detection of expressed genes has also been used to detect novel Cry protein genes [44].

Moreover, a combination of genomics, transcriptomics, proteomics, and metabolomics could be used to study Bt toxin proteins with different characteristics and activities [21]. However, due to the interaction between different toxins produced by a strain in insect midgut, bioassays provide complementary and necessary characterization information. Due to the money, time, and material costs associated with insect rearing and time-consuming characteristics of insect bioassays, cell-based assays have been employed for toxicity characterization of Bt strains or toxins [50].
Furthermore, recent studies have confirmed more new potentials of different Bt strains. These new features are including plant growth promotion [51], bioremediation of heavy metals and other chemicals [1, 52], anticancer activities [53], polymer production [54], and antagonistic effects against plant and animal pathogenic microorganisms [55].

5. Bacillus thuringiensis development on rice crops

Genetically engineered or transgenic crops producing Cry proteins from Bacillus thuringiensis are key management tools against several important insect pests. GE plants expressing Bt insecticidal proteins selectively target insect pests while having little impact on beneficial insects. Bt toxins have been widely adopted worldwide; it was calculated that over 100 million hectares of crops contained Bt genes by 2017 [56].

Bt crops produce either a single toxin or more than one Bt toxin; these are called pyramided crops. Bt pyramided crops delay evolution of resistance to target pests, insects resistant to one toxin are killed by other toxins in the pyramid [57, 58]. Nevertheless, pyramided Bt crops are vulnerable to the development of cross-resistance. The use of Bt pyramids and the simultaneous planting of non-Bt crops are the main strategies applied to produce susceptible pest insects (known as the “refuge strategy”) [59].

Rice is a primary food source for more than half of the world’s population making it one of the most fundamental crops. Since 1989 multiple insect-resistant genetically engineered (IRGE) rice lines expressing Bacillus thuringiensis insecticidal proteins had been developed [60], controlling lepidopteran pests. There are four major lepidopteran pest rice such as the rice stem borers Chilo suppressalis (Walker) (Lepidoptera: Crambidae), Scirpophaga incertulas (Walker) (Lepidoptera: Crambidae), Cnaphalocrocis medinalis (family Crambidae), and Sesamia inferens (Walker) (Lepidoptera: Noctuidae) [61].

Bt rice lines resistant to rice lepidopteran pests mainly express Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1Ca1, Cry2A, and Cry9C proteins [61–63].

Since Cry1Ab was first introduced into a japonica rice variety, many Bt genes have been found, and only a few of them were selected for developing transgenic crops [60]. Because deploying two or more Bt genes in one rice variety can delay the emergence of pest resistance [64, 74], scientists started to develop Bt hybrid rice lines with Cry1Ab/Cry1Ac into various rice plants which have both high grain yield and good grain quality [65].

Some advantages of expressing fusion proteins like Cry1Ab/Cry1Ac and Cry1Ab/Vip3A are the equalization of the expression level of the two proteins, trait integration in different crops, and highly efficient expression strains [66]. Studies on Cry1Ab/Cry1Ac fusion protein have demonstrated great effectiveness significantly reducing the incidence of Chilo suppressalis [67, 72].

Other B. thuringiensis proteins that present high affinity are Cry9Aa and Vip3Aa. These two proteins bind specifically to brush border membrane vesicles of the Asiatic rice borer Chilo suppressalis, which do not share binding sites [68]. Cry9Aa and Vip3Aa toxins have shown potent toxic synergy based on a specific interaction between them against C. suppressalis larvae with a synergism factor (SF) value of 10.6-fold [68].

The rice water weevil (Lissorhoptrus oryzophilus Kuschel) is another of the most destructive insect pests of cultivated rice (Oryza sativa) in the United States [69, 70]. This pest causes low yields in rice by damaging the roots from larval feeding in the submerged root zone [71].

Some of the strategies to control this insect pest are the use of pyrethroids, which are toxic to aquatic organisms [72], synthetic insecticides, and weed control
around fields to reduce habitat for rice water weevil adults. *Bacillus thuringiensis* spp. *galleriae* (Btg) have proven to be an environmentally friendly alternative against rice water weevil larvae. Studies indicate that Btg granular formulation has biological activity against this target pest and performs as well as the pyrethroids insecticides [73], showing promising potential for rice water weevil control.

6. Resistance to *Bacillus thuringiensis*

*Bt* insecticides consist of several types of insecticidal crystal proteins; hence, the development of insecticidal pesticide resistance is difficult or slow [47]. However, resistance has already been observed in laboratory, and the first case was a population of Indian meal moths, *Plodia interpunctella*, in 1985, and since then different insect species have been reported to be resistant to one or more *Bt* toxins under laboratory conditions. However, the situation in the field remains very different. To date, the only natural populations that have really developed resistance following *Bt*-based treatments have been populations of diamondback moth, *Plutella xylostella* [27, 74].

The use of transgenic plants has greatly increased the selection pressure on target pest populations and is likely to become much more acute in natural conditions if *Bt* use in agriculture and for human health applications spreads or in cases of the nonrational use of large-scale transgenic crops expressing *cry* genes [32].

In agriculture worldwide, repeated applications of *Bt* sprays and widespread adoption of *Bt* crops (transgenic crops protected from insects by the expression of *Cry* and/or *Vip3* genes) have led to resistance [75, 76]. Field populations of *Diabrotica virgifera* have shown resistance to eCry3.1Ab maize and cross-resistance among Cry3Bb1, mCry3A, and eCry3.1Ab, which are the *Bt* toxins most commercialized for management of western corn rootworm [77].

Resistance to *Cry* toxins can be developed by mutations in the insect pests that affect any of the steps of the mode of action of *Cry* toxins [78]. “Field populations” refers to insects on the field, since the conditions are distinct in vitro, can be developed by different mechanisms, such as altered activation of *Cry* toxins by midgut proteases sequestering the toxin by glycolipid moieties or esterases, by inducing an elevated immune response, and by alteration resulting in reduced binding to insect gut membrane; among all these mechanisms of resistance, the most common mechanism of toxin resistance is the reduction in toxin binding to midgut cells, which in different resistant insect species include mutations in *Cry* toxin receptors such as cadherin (CAD)-like proteins, alkaline phosphatase (ALP), or aminopeptidase N (APN) or mutations in the ABC22 transporter [78].

The emergence of resistant insects is a problem that both *Bt* sprays and plant products are likely to face in the future [32]. Several strategies, such as the use of spatial or temporal refugia, high or ultrahigh doses, and gene pyramiding to express two toxins, or two insect control approaches, such that the possibility of evolution of resistance to two toxins/approaches, independent of each other, is greatly diminished, can be a promising approach to prolong the efficacy of products based on *Bt* [36, 46].

There are different methods to counteract the resistance of insects to *Bt* toxins, for example, assisted mutation with UV light; the combination of *Bt* toxins with other toxins, such as *Bacillus sphaericus* proteins; and formulations with plant extracts.

Nevertheless, a new method has been used to combat resistance to *Bt* toxins, the phage-assisted continuous evolution (PACE), which rapidly evolves *Bt* toxins to bind a new receptor with high affinity and specificity, expressed on the surface of insect midgut cells. The PACE system enhances the insecticidal activity against
both sensitive and Bt-resistant insect larvae up to 335-fold, through more than 500 generations of mutation, selection, and replication to bind a new receptor [23]. Collectively, these methods establish an approach to overcoming Bt toxin resistance.

7. Formulations based on Bacillus thuringiensis

The production of toxic proteins has given Bacillus thuringiensis enormous interest in its inclusion in phytosanitary formulations. The efficiency of products based on Bt depends on the type of formulation, as well as various environmental factors. Formulation depends on the persistence of toxicity and the choice of application method; other important factors are UV radiation, agitation, sedimentation, water quality, contaminants, pH, temperature, susceptibility of insects, and competition with other microorganisms [79].

The wide variety of formulations based on spores and crystals intended for being ingested by the white insect are the result of many years of research. The development of a large variety of spore-crystal complex matrices allows for improvements, such as increased toxic activity, increased palatability to insects, or longer storage times. These matrices use chemical, vegetable, or animal products, which are constituted in such a way that they favor contact between crystals and insects, without harming humans or the environment [80].

Proper formulation can help to overcome several of the factors that limit or reduce its larvicidal activity and improve control performance by enabling greater contact with target larvae, ensuring stability under storage and field conditions, providing a variety of application options, and increasing the ease of handling. There are several types of formulations, among the most used are:

**Powder (DP)**

- Formulated by sorption of an active ingredient on finely ground mineral powder (talc, clay, etc.).
- Particle size of 50–100 μm.
- Powders can be applied directly to the target, either mechanically or manually.
- The inert ingredients for this formulation are anticaking agents, ultraviolet protectors, and adhesive materials to improve adsorption.
- Concentration of the active ingredient (organism) in the powder is usually 10%.

**Granules (GR)**

- Granular particles are larger and heavier than powder formulations.
- Particle size coarse of 100–1000 μm for granules and 100–600 μm for microgranules.
- Made of mineral materials (kaolin, attapulgite, silica, starch, polymers, dry fertilizers, and residues of ground plants) [81].
- Concentration of the active ingredient (organisms) in granules ranges from 5 to 20%.
- Once applied, the granules slowly release their active ingredient.
Some granules require soil moisture to release their active ingredient [3, 82].

**Wettable powders (WP)**

- Finely ground dry formulations that will be applied after suspension in water.
- Produced by mixing an active ingredient with surfactants, wetting and dispersing agents, and inert fillers, followed by milling.
- Particle size approximately 5 μm.
- Long storage stability, good miscibility with water, and convenient application with conventional spray equipment [83].

**Water dispersible granules (WG)**

- Designed to be suspended in water.
- The granules break to form a uniform suspension similar to that formed by a wettable powder.
- Compared to powdered products, these WGs are relatively dust-free and with good storage stability.
- The products contain a wetting agent and dispersing agent similar to those used in wettable powders, but the dispersing agent is usually at a higher concentration.

**The emulsions**

- Consist of liquid droplets dispersed in another immiscible liquid.
- Size of the droplets in the dispersed phase varies from 0.1 to 10 μm.
- The emulsion can be oil in water (EW), which is a normal emulsion, or water in oil (EO), an inverted emulsion. Both products are designed to be mixed with water before use.

**The suspension concentrate (SC)**

- A mixture of a finely ground solid active ingredient dispersed in a liquid phase, usually water.
- The solid particles do not dissolve in the liquid phase, so that the mixture needs to be stirred before application to keep the particles evenly distributed.
- The composition of the suspension concentrate is complex and contains wetting-dispersing agents, thickening agents, antifoaming agents, etc., to ensure the required stability.
- They are produced by a wet milling process.
- Particle size distribution of 1–10 μm.
Oil dispersions (OD)

- Dispersions of solid active ingredients in a nonaqueous liquid intended for dilution before use.
- The nonaqueous liquid is more often an oil (vegetable oil).
- Oil dispersion provides several important characteristics, such as the ability to supply water-sensitive active ingredients and the ability to use an adjuvant fluid instead of water that can increase and extend pest control.

Capsule suspension (CS)

- Stable suspension of microencapsulated active ingredient in an aqueous continuous phase.
- Intended for dilution with water before use.
- The bioagent as an active ingredient is encapsulated in capsules (coating) made of gelatin, starch, cellulose, and other polymers.
- Protected from extreme environmental conditions (UV radiation, rain, temperature, etc.).
- Residual stability increases due to slow (controlled) release.
- The most frequently applied encapsulation method uses the principle of interfacial polymerization.

The extension of pesticide formulations containing Bt will depend essentially on our capacity to improve the performance of the products used [83]. Therefore, biotechnology companies have the task of providing not only formulations adapted to certain crops and insect pests, but also, they must look for and produce bioinsecticides based on the new high-potency strains originating from the agroecosystems where they are going to apply. It is expected that the new products that appear in the market will provide a spectrum of higher activity that will impact on a greater number of pests in other crops and can help develop sustainable agriculture [80].

8. Bioinsecticides based on Bt

Worldwide, the use of biopesticides increases 16% annually, which represents approximately 8% of the pesticide trade in the world [12]. The formulations derived from natural materials such as bacteria, animals, plants, or minerals offer a powerful tool to create a new generation of sustainable products [84]. About 90% of microbial biopesticides are derived from a single entomopathogenic species *Bacillus thuringiensis* [85].

*Bt*-based bioinsecticides are classified into first-line products up to the fourth generation: (1) They are made up of spores and crystals, have several drawbacks, since they present a narrow range of activity when more than one pest insect is present, have little persistence in the field to solar radiation, and do not reach insects that attack roots or internal parts of the plant. (2) They contain spores and toxins of strains as an active ingredient with the introduction of genes from other
strains, which is very useful to improve the action against the insect, generating a synergism, as well as diminishing the possibilities of resistance. (3) They contain recombinant bacteria, especially Pseudomonas fluorescens or Clavibacter xyli subsp. cynodontis, which are able to reach plant tissues and grow in the rhizosphere. (4) They constitute protein chimeras [86].

More than a century after its discovery, Bt has become an important tool for the management of insect pests, whether in the agricultural sector or in the fight against vectors of diseases. Since then the spectrum of its applications has been increasing and is no longer limited to its initial function. It has become evident that the potential of Bt would transcend the biological control of insects, and recent studies analyze new properties for this old acquaintance [88].

These new environmental features include the toxicity against nematodes, mites, and ticks, antagonistic effects against plant and animal pathogenic bacteria and fungi, plant growth-promoting rhizobacteria (PGPR) activities, bioremediation of different heavy metals and other pollutants, biosynthesis of metal nanoparticles, production of polyhydroxyalkanoate biopolymer, and anticancer activities (due to parasporins) [51–53].

Toxicity against nematodes with several classes of Cry toxin (Cry5, Cry6, Cry13, Cry14, Cry21, and Cry55) is well established. In addition to these Cry proteins, thuringiensin, chitinase, and a metallproteinase from Bt are also toxic to nematodes [89]. In contrast, the information about the effect of Bt on mites is rare, and a few in vitro and in vivo studies have reported the acaricidal activity of some Bt strains. In a study conducted by Dunstand et al. [90], the in vitro acaricidal activity was reported to be caused by the strain GP532 of Bt on the mite Psoroptes cuniculi. Histological alterations caused by Bt on this mite included the presence of dilated intercellular spaces in the basal membrane, membrane detachment of the peritrophic matrix, and morphological alterations in columnar cells of the intestine.

### Table 2

<table>
<thead>
<tr>
<th>Bt variety</th>
<th>Susceptible insects</th>
<th>δ-Endotoxin</th>
<th>Producer company</th>
</tr>
</thead>
<tbody>
<tr>
<td>kurstaki</td>
<td>Lepidoptera</td>
<td>Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa, and Cry2Ab</td>
<td>Abbott-Dupont and Certis</td>
</tr>
<tr>
<td>aizawai</td>
<td>Lepidoptera</td>
<td>Cry1Aa, Cry1Ab, Cry1Ba, Cry1Ca, and Cry1Da</td>
<td>Abbott-Dupont and Kenogard</td>
</tr>
<tr>
<td>san diego</td>
<td>Coleoptera</td>
<td>Cry3Aa</td>
<td>Thermo Trilogy, Columbia MD, Certis</td>
</tr>
<tr>
<td>tenebrionis</td>
<td>Coleoptera</td>
<td>Cry3Aa</td>
<td>Mycogen, and Novo Nordisk</td>
</tr>
<tr>
<td>israelensis</td>
<td>Diptera</td>
<td>Cry4A, Cry4B, Cry11A, and Cry11Aa</td>
<td>Abbott-Dupont, Novo Nordisk, and Certis</td>
</tr>
<tr>
<td>galleriae</td>
<td>Coleoptera</td>
<td>Cry1Da</td>
<td>Phyllo BioProducts</td>
</tr>
</tbody>
</table>

Varieties of Bt used as bioinsecticides, susceptible insects, expressing δ-endotoxin, and companies that produce it.
Cry proteins synthesized by *Bt* do not show any antifungal activity. However, some *Bt* strains produce antifungal compounds, including cell wall-degrading enzymes, lipopeptide fengycin [21]. In a study conducted by Shrestha et al. [91], *Bt* strain C25 was antagonistic to *Sclerotinia minor* and *Sclerotinia sclerotiorum*, and it was found that the strain was capable of inhibited mycelial growth, suppressed sclerotia formation, and germination. On the other hand, strain C25 showed high activities of various cell wall-degrading enzymes such as proteases, β-1,3-glucanase, and chitins.

Some strains of *Bt* colonize plant roots and have plant growth-promoting characteristics. Many *Bt* strains produce some metabolites which enhance plant growth at abiotic stress conditions. These compounds include 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, indole-3-acetic acid (IAA), proline, phosphate solubilization enzymes, and siderophore production [92].

Different strains of *Bt* have been shown to produce many potential factors that could be of great interest in the biocontrol of phytopathogenic bacteria [55]. *Bt* produces bacteriocins, chitinases, acyl homoserine lactone lactonase, and zwitermicin, which collectively elicit detrimental effects on insect hosts and target bacteria; although the role of *Bt* bacteriocins in nature is enigmatic, it is possible that they assist in pathogenesis by attacking competing endosymbiotic or opportunistic bacteria, thereby facilitating propagation of this entomopathogen in the hemolymph of susceptible insects [93].

Parasporins are a heterogenous group of Cry proteins produced by noninsecticidal *Bt* strains that specifically act on human cancer cells without affecting normal ones, and it has been reported that Cry proteins, such as Cry31A, Cry41A, Cry45A, Cry46A, Cry63A, and Cry64A, present anticancer activity when digested with proteases [53].

### 10. Advantages and disadvantages

The biopesticide based on bacteria is probably the most used and is cheaper than the other methods of bioregulation of pests [94]. Almost 90% of the microbial biopesticides that are commercially available are *Bt* derivatives [95]. Among the advantages and disadvantages of using Bt as a bioinsecticide are the following [34] (Table 3):

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Performance</strong>: although each kilogram is more expensive, only a few grams per hectare are needed compared to 4 kg of chemical insecticides</td>
<td>Application with difficulty</td>
</tr>
<tr>
<td><strong>High toxicity</strong>: a small amount is needed to kill pests</td>
<td>It is not easy to produce it</td>
</tr>
<tr>
<td><strong>Specificity</strong>: it only kills the target organism</td>
<td>Little diffusion and acceptance by producers</td>
</tr>
<tr>
<td>It does not produce infectious: it is demonstrated that an infected larva does not harm other insects, animals, or even humans</td>
<td>Its quality could not be controlled. Sometimes it works, and sometimes it does not</td>
</tr>
<tr>
<td><strong>Limited time of permanence in the environment</strong>: after 3 or 4 weeks of application, traces of the bioinsecticide are no longer found</td>
<td>Variability in insect resistance</td>
</tr>
<tr>
<td><strong>Few cases of resistance</strong>: there are few cases reported, and only in extraordinary conditions there are certain degrees of resistance</td>
<td>Location. Its use may be limited to faunas of a certain region</td>
</tr>
</tbody>
</table>

Table 3. Advantages and disadvantages of bioinsecticides based on *Bt*.
11. Conclusion

*Bacillus thuringiensis* has undoubtedly been the most successful microbial agent for biological insect control of all time. However, different authors have warned of the generation of insect resistant to Bt-derived products, as well as genetically modified plants.

During the last two decades, new methods have been widely used on Bt to overcome resistance to insects, and it is expected that this advancing trend will be well continued in the future, including the search for new toxins and strains with increased toxic activity and the development of new biopesticides and technologies to maintain the success of this bioinsecticide which is a great challenge to overcome.

Nowadays there exist different lines of research that seek to use Bt in different applications, such as anticancer activity, promotion of plant growth; nematocide, antifungal and bactericidal activity among others. To achieve the implementation of these new features, it is necessary to know more about the biochemical and physiological pathways, as well as the mode of action of the new features. Such properties will undoubtedly lead to explore novel Bt strains with more potent insecticide activities or novel features which will enhance the implementation of these strains in other medical, agronomical, and industrial avenues. At the same time, technological development is necessary to allow new products to become a reality.

Conflict of interest

The authors declare that they have no conflicts of interest.

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