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Thuringiensin: a toxin from *Bacillus thuringiensis*

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Abstract *Bacillus thuringiensis* (*Bt*) is an entomopathogenic bacteria widely used in practice for biological control of insect pests, nematodes and disease vectors. Your toxicity is related to the ability to produce many virulence factors, including thuringiensins (β-exotoxins). Most toxins produce by this microorganism are highly specific, therefore the use of it in biological control is considered environmentally safe and so this bacteria is extensively used in the production of biological insecticides and genetically modified plants. However, the thuringiensin is considered toxic to almost all life forms, including humans, due to its ability to inhibit the biosynthesis of RNA polymerase, an enzyme essential to the transfer of genetic information in almost all organisms. This way, the release of new strains of *Bt* with insecticidal properties for the biological control of pests must pass by verification of the absence of production of exotoxin, so that non-target organisms are not affected and the use of *Bt* in this field remains safe. Thus, this revision will discussed the knowledge about features, structure, genetic determinants, biosynthesis, mechanism of action, insecticide spectrum, security assessment and procedures for identification of thuringiensin in *Bt* strains.
Keywords Biological control; environmental security; β-exotoxins; thermostable toxin

Background

Bacillus thuringiensis Berliner (1911) (*Bt*) (Eubacteriales: Bacillaceae) are a ubiquitous soil bacteria and was first isolated in 1901, in Japan, by Shigetane Ishiwata, in sick larvae of *Bombyx mori* (Lepidoptera: Bombycidae) (silkworm), being initially named sotto disease. Seven years later, Iwabuchi (1908) called it as *Bacillus sotto*. In 1911, the same bacteria was found in the province of Thuringia, Germany, by bacteriologist Ernst Berliner from infected larvae of *Ephestia kuehniella* (Lepidoptera: Pyralidae) (moth flour-of-Mediterranean) (Bravo et al., 2012; Ramirez-Lepe and Montserrat 2012). To analyze it, Berliner reported the presence of parasporal protein crystals (Cry), but did not know how to describe the role of these proteins in this microorganism, which remained unknown until 1950 (Federici et al., 2010). Currently, it is known that *Bt* can establish lethal infections in susceptible organisms to break the midgut cells and replicate itself inside the hemolymph of living host (Raymond et al., 2010).

Because of their entomopathogenic properties, *Bt* is widely used in biological control of insect pests practices, nematodes and vectors of human diseases (Bravo et al., 2012). The first tests in field using this bacterium were carried out in 1929, by Husz, in corn fields, for the control of *Ostrinia nubilalis* larvae (Lepidoptera: Crambidae) (European corn worm). The positive results of the experiments caught the attention of agribusiness industries in this entomopathogenic organism (Kaya and Vega, 2012). The first commercial formulation of *Bt*, the insecticide spray Sporeine, was developed in France in 1938, but had low selectivity and action limited to few species. The large-scale production was started only in the 50s, reaching countries such as Russia, Czechoslovakia, France, Germany and the United States. However, few strains of *Bt* were used for the production of spray insecticides to date, representing about 2% of the marketable insecticides (Pardo-López et al., 2013).

On the other hand, with the development of molecular techniques and biotechnological advances, it has become possible the genetic manipulation of plants, through the insertion of small sequences of genes from *B. thuringiensis* expressing toxic proteins of the

bacterium directly into the genotype of the plant (Pinto-Zevallos e Zarbin, 2013). In the early 80s, Schnepf and Whiteley (1981) conducted the first cloning and sequencing of the first genes encoding these proteins, obtained through an isolated from *Bt* subsp. *kurstaki* strain and expressed it initially in tobacco and tomato plants, which were the first transgenic-*Bt* plants to be marketed (Ali et al., 2010; Rodr ́guez et al., 2012). Thus, it was possible the introduction of *Bt* genes in other plants to introduce resistance to insect pests, such as corn and cotton cultivars which are the two major *Bt* crops grown worldwide (Ali et al., 2010).

The main toxins produced by *Bt* presenting entomopathogenic activity are the δ-endotoxins (Cry and Cyt) and parasporins, which are intracellular proteins; besides the α-exotoxins; thuringiensins (β-exotoxins); toxins VIPs (vegetative insecticidal proteins); S-layer proteins (SLP) and exoenzymes (lipases, proteases, chitinases and phospholipase C), which are extracellular macromolecules enabling virulence (Arora et al. 2013; Bravo et al. 2011; Vu et al. 2012). As biolarvicides, *Bt* proved to be innocuous in tests that evaluated the potential toxicity of the most of their toxins in mammalian cells, and organisms non-target (Thomas and Ellar, 1983). All of these toxins have been studied for its potential use in biological control practices. However, not all can be used in pest control, such as the thuringiensin, which has a broad spectrum of biological toxicity to a variety of non-target species, including mammals. Therefore, these present review discuss current knowledge about characteristics, types, genetic determinants, biosynthesis, mode of action, insecticide spectrum, safety assessment and procedures to identification of thuringiensins in *Bt* strains.

1 Features of Thuringiensins

Thuringiensins are secondary metabolites, nonproteic and soluble in water. It is also a heat-stable exotoxin that maintains their bioactivity to 121℃for 15 min (Farkas et al., 1969). It is known that are more stable at pH 7.0, but their stability decreases with increasing temperature (Zhou et al., 2013). As in other extracellular insecticides toxins, their production occurs during the vegetative growth phase of some strains of *Bt*, when are secreted in the culture medium where the bacteria is inoculated (Liu et al., 2010; Obeidat et al., 2012). However, according to (Arg ̂lo-Filho et al., 2014), the secretion of this secondary metabolite varies temporally, thus the knowledge of the temporal pattern of secretion or activity in the culture medium is necessary to avoid the lack of identification of this undesirable exotoxin. The different toxicity scale of thuringiensin, highly dependents of the time of cultivation of the producing strain, may also be explained by different volumes of culture, and the variable conditions of the culture medium (aeration level; pH value) (Arg ̂lo-Filho et al., 2014). (Jing-Wen et al., 2007) reported that the pH and the concentration of glucose had an important effect on the synthesis and efficiency of thuringiensin. Physiological differences between strains can also result in a variation of the secretion of toxins, even when it is inoculated the same quantities of cells, generating toxicity profiles temporally distinct for each strain, and, consequently, a potential wrong classification of producer isolates (Arg ̂lo-Filho et al., 2014).

The exotoxin thuringiensin was discovered by McConnell and Richards (1959), which describe it as a substance thermostable toxic to insects. Heimpel (1967) proposed the name β-exotoxin to designate it, but over time, this term was considered inappropriate, due to the structure of this toxin. Instead, several authors suggested the thuringiensin synonym that is currently used (Kim and Huang, 1970; Pais and De Barjac, 1974; Farkas et al., 1977).

The chemical formula of the thuringiensin is $C_{22}H_{32}N_5O_{19}P$ (De Rijk et al., 2013). Initially, (Farkas et al., 1969) reported that the structural formula of thuringiensin consisted of adenosine, glucose, a phosphoric acid and a gluconic diacid. Šebesta and Horska (1970) suggested that exotoxin is composed of adenine, aleric acid phosphorylated and a sugar moiety formed by D-ribose and D-glucose linked by an ether unusual. Currently, it is known that the thuringiensin has a unique structure and, as a polymer of monosaccharides, has asymmetric carbon atoms (Liu et al., 2010) (Figure 1).

Many *Bt* strains belonging to different serotypes excrete the toxin (Jing-Wen et al., 2007), as well as some strains of *B. subtilis*, and *B. megaterium* (Pinto et al., 2010). Some studies reported that strains of *B. cereus* also are able of producing thuringiensins (Carlberg 1986; Krieg and Lysenko 1979; McConnell and Richards 1959; Ohba et al., 1981). Perchat et al.,

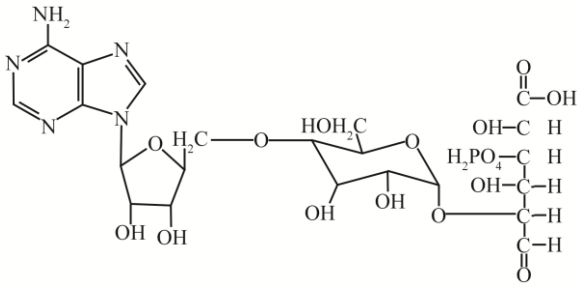

Figure 1 Structure of thuringiensin I (Belder e Elderson. 2013)

(2005), for example, performed a screening of 575 strains of *B. cereus* and found 270 strains producers of thuringiensins of type II.

2 Types of Thuringiensins

Levinson et al., (1990) described two types of thuringiensins from assays of high-performance liquid chromatography (HPLC). Thuringiensins of the type I have low molecular weight, approximately 701Da, and are composed of adenosine, glucose, a phosphate group and gluconic diacid (Liu et al. 2010; Mac Innes and Bouwer 2009). For many years it was believed that thuringiensin I was a phosphorylated molecule analogous to the adenine nucleotide with great structural similarity to this nucleotide (Šebesta and Horska 1970; Šebesta and Sternbach 1970; Šebesta et al., 1981). However, recently, Liu et al., (2010) proposed that, in fact, it is an oligosaccharide from adenine nucleoside.

Because of the similarity to adenine, the toxicity of thuringiensin I was explained by the inhibition of RNA polymerase biosynthesis, one of the key enzymes in the transfer of genetic information. That's because this exotoxin acts essentially in the step of the polymerization of the polymerase reaction, competing for binding sites with ATP (Devidas and Rehberger 1992; Perchat et al., 2005). More specifically, thuringiensins I bind reversibly (without being incorporated into the polymer) to a portion of adenosine in the specific site of ATP (Šebesta and Sternbach 1970). The depression of RNA polymerase biosynthesis was seen in assays with rats (Šebesta and Horska 1969), and as it is a fundamental process for all types of life, thuringiensins I are toxic to almost all living organisms (Belder and Elderson 2013).

Levinson et al., (1990), analyzing strains of *Bt* subsp. *thuringiensis*, *Bt* subsp. *tolworthi* and *Bt* subsp. *darmstadiensis*, reported that the gene that encodes the thuringiensin I production is supported by plasmids. Ozawa and Iwahana (1986) provided evidences that the production of exotoxin is associated with a plasmid of 62 Mdal in a strain of *Bt* subsp. *darmstadiensis*, but that plasmids producers thuringiensins I are not ubiquitous in *Bt* strains. Initially, it was believed that an ABC transporter could be related to the secretion and production of this exotoxin (Espinasse et al., 2002a). The researches about thuringiensins are focused on tests to measure their insecticidal activity and in strategies for its detection and purification, because studies on genetic determinants involved in its biosynthesis are still scarce (Liu et al., 2014). Recently, it was discovered that the *Thu3* gene, which is homologous to ABC transporter is involved in the secretion thuringiensin I. According to Liu et al., (2010) synthesizers gene of this exotoxin are encoded by circular endogenous plasmids of 110 kb that harboring *thu* cluster besides synthesizers genes of the Cry1Ba proteins (Iatsenko et al., 2014). More specifically, in the thuringiensin biosynthesis, genes *thuA*, *thuC* and *thuD* encode proteins responsible by the synthesis of the key precursor of the toxin, a gluconic diacid (precursor A) from glucose-6-phosphate. The *thuF* and *thu1* genes encode proteins involved in the assemblage of thuringiensin I. The *thuE* gene encodes the enzyme responsible for the synthesis and phospho-rrylation of the toxin and the *thu3* gene encodes a protein that acts in the release of thuringiensin I mature, which may be secreted by the type IV-like secretion system (T4SS) towards the cell (Liu et al., 2010) (Liu et al., 2010).

Some studies report that the thuringiensin I production is related to the presence of plasmids that also harbor *cry* and *vip1*/*vip2* genes (Espinasse et al. 2002b; Cstagnola and Stock 2014; Iatsenko et al., 2014; Levinson et al., 1990). (Espinasse et al., 2002a) and (Perani et al., 1998), for example, report that the production of high levels of thuringiensin I is linked to the presence of plasmids carrying the gene encoding the crystal protein Cry1b. However, *Bt* strains that do not produce crystals may also produce thuringiensins I, such as mutant strain of *B. thuringiensis* 407-1 (Cry^-), which still synthesizes a pigment of soluble melanin also secreted in the culture supernatant. Therefore, the production of thuringiensin I can occur even in a lineage that lost the plasmids containing the genes cry (Espinasse et al., 2002b). It is believed that these

endogenous plasmids encoders thuringiensins I were acquired by horizontal gene transfer, in a co-evolution with insects through a host-parasite relationship. Representing a unique genetic resource and as part of adaptive genetic pool can play an important role in the biology and evolution of *Bt* where cells are hosted (Schnepf et al., 1998).

(Levinson et al., 1990) also identified another type of thuringiensin, the type II, in a strain of *Bt* subsp. *morrisoni*. The thuringiensin of type II is analogous to uracil (UTP) and presents a greater toxicity than type I, mainly to Coleoptera (Levinson et al., 1990; Palma et al., 2014; Perchat et al., 2005). According Saukas et al., (2014), strains producing thuringiensins type II do not harbor the gene *thuE* in their plasmids and, therefore, these exotoxins are encoded by different genes. However, due to the low number of studies about this type of toxin, there structure remains unknown (Rodr ǵuez et al., 2003; Obeidat et al., 2012).

3 Production of tThuringiensin

(Espinasse et al., 2002a) reported that the production of thuringiensins may occur in response to particular environmental conditions. Other authors believe that this secondary metabolite can act as a molecular signal inside of the microorganism responsible for transcriptional control of the genes of sporulation (Johnson et al., 1975). As previously mentioned, the production of thuringiensins is linked with large plasmids (Levinson et al., 1990). According (Obeidat et al., 2012), the plasmidial DNA profiles of *Bt* strains that produce these exotoxins share three great bands (> 10 kpb), i.e. the regulatory genes of the synthesis of thuringiensin, or part of its structure, are encoded by common bands. (Espinasse et al., 2002b) suggested that the genetic determinants responsible for producing these toxins usually found in plasmids Cry-dependent, are regulatory elements, indicating that any strain of *Bt*, of any serotype, is able to acquire a plasmid that encodes thuringiensins by conjugation processes (Obeidat et al., 2012). Thus, several authors claim that thuringiensin production is a specific property of strains, rather than being a specific property limited to certain serovars of *Bt* (Rodr ǵuez et al. 2001; Hern ández et al. 2003; Lee et al. 2001; Ohba et al., 1981; Tanada and Kaya 1992).

Despite the serotyping of *Bt* strains be insufficient to determine the production of thuringiensins, there is a strong correlation between production or lack of production of this exotoxin with serotype (Barfod, 2010). In other words, strains belonging to *darmstadiensis*, *thuringiensis* or *tolworthi* serotypes, which are considered common producers, will have higher chances of producing this metabolite than those belonging to serovars *alesti*, *sotto*, *aizawai*, and *morrisoni*, classified as rare producers (Rodr ǵuez et al., 2003). In the literature, it has been reported the production of thuringiensins in at least 13 *Bt* serotypes (Table 1).

4 Toxicity of Thuringiensins

Due to its broad spectrum of nonspecific toxicity to invertebrates of the orders Lepidoptera, Coleoptera, Diptera and species of nematodes, which are representatives of agricultural pests and vectors of the world's most important diseases (Table 2), thuringiensins have promising applications in biological control practices. In addition to these taxa, these exotoxins also have harmful effects against insects of the orders Hymenoptera, Isoptera, Orthoptera, Hemiptera and Neuroptera, plus species of aphids and mites (He et al., 2011; Liu and Tzeng 1998; Liu et al., 2010; Pinto et al., 2010; Royalty et al., 1991; Tanigoshi et al., 1990; Zhou et al., 2013). However, this toxin also has cytolytic and hemolytic activity against non-target organisms and vertebrates, acting including in human erythrocytes (Belder e Elderson 2013; Iatsenko et al., 2014; Liu et al., 2010; Obeidat et al., 2012; Palma et al., 2014; Veloorvalappil et al., 2013).

When administered orally, thuringiensins are highly toxic to a wide range of insect species (Perchat et al., 2005). In sublethal doses, the effects can be teratogenic or are involved in critical stages of metamorphosis, as in the formation of ecdysis, in the transformation into pupae and in the emergence of adults (Burgerjon et al., 1969; Espinasse et al., 2002a; Glare and O'Callaghan 2000; Tsuchiya et al., 2002). In some cases, the duration of the larval stage may be prolonged, or the lifespan and fertility of the insects can be affected (Carlberg, 1973). Thuringiensins also prevents the regeneration of midgut cells of intoxicated insects larvae (Federici et al., 2010). Among the insects more susceptible to thuringiensins, are those who belong to the order Diptera (Carlberg, 1986) and have already been reported including mutagenic effects in fruit fly (*Drosophila melanogaster*) (Carlberg 1973).

The mode of action of thuringiensins is quite different from the mode of action of δ-endotoxin (Vachon et al.,

Table 1 Serovars of *Bacillus thuringiensis* producers of thuringiensins (type I and II) and the reference

Serovars of *Bt*	Type of thuringiensin	Reference
darmstadiensis	I	Ohba et al., (1981); Mohd-Salleh and Lewis (1982); Levinson et al., (1990); Rodr ǵuez et al., (2001); Tsuchiya et al., (2002); Rodr ǵuez et al., 2003; Belder and Elderson (2013)
galleriae	I	Belder and Elderson (2013);
israelensis	I	Obeidat, Horani and Al-Momani (2012);
kenyae	I	Rodr ǵuez et al., (2001); Tsuchiya et al., (2002); Obeidat et al., (2012);
kunamotoensis	I	Ohba et al., (1981);
kurstaki	I	Levinson et al., (1990); Tsuchiya et al., (2002); Obeidat et al., (2012);
morrisoni	I e II	Ohba et al., (1981); Levinson et al., (1990); Rodr ǵuez et al., (2001);
pakistani	I	Obeidat et al., (2012);
tenebrionis	I	Belder and Elderson (2013);
thuringiensis	I	Cantwell et al. (1964); Burgerjon and Galichet (1965); De Barjac and Burgerjon (1972); Mohd-Salleh et al., (1980); Mohd-Salleh and Lewis (1981); Ohba et al., (1981); Mohd-Salleh and Lewis (1982); Levinson et al., (1990); Tsuchiya et al., (2002); Rodr ǵuez et al., 2003; Belder and Elderson (2013);
tohokuensis	I	Obeidat, Horani and Al-Momani (2012);
tolworthi	I e II	Mohd-Salleh et al., (1980); Mohd-Salleh and Lewis (1982); Ohba et al., (1981); Levinson et al., (1990); Rodr ǵuez et al., (2001); Rodr ǵuez et al., (2003)
toumanoffi	I	Ohba et al., (1981);

Table 2 Toxic effects of thuringiensins in major taxa of agricultural pests, with their references

Taxa	Lethal effects or others	References
Coleoptera	Larvae Mutagenic Teratogenic	Burgerjon et al. (1969); Burgerjon (1974); Espinasse et al. (2002a); Tsuchiya et al. (2002); Pe ña et al. (2006)
Diptera	Adult Larvae Mutagenic Teratogenic	Bond et al. (1969); Ignoffo and Gard (1970); Bond et al. (1971); Gingrich and Eschle (1971); Carlberg (1973); Wasti et al. (1973); Ohba et al. (1981); Šebesta et al. (1981) Haufler and Kunz (1985); Carlberg (1986); Gingrich (1987); Mohd-Salleh et al. (1980); Mullens et al. (1988); Mullens and Rodriguez (1988); Marec et al. (1989); Yamvrias and Anagnou (1989); Levinson et al. (1990); Carlberg et al. (1991); Karamanlidou et al. (1991); Robacker et al. (1996); Johnson et al. (1998); Lee et al. (2001); Arango et al. (2002); Mac Innes and Bouwer (2009); Mwamburi et al. (2009); Hayes et al. (2011); Elleuch et al. (2015)
Lepidoptera	Larvae Teratogenic	Dubois, (1986); Gardner et al. (1986); Ignoffo and Gregory (1972); Vankov á et al. (1974); Ohba et al. (1981); Mohd-Salleh and Lewis (1982); Moar et al. (1986); Barreto et al. (1999); Robacker et al. (2000); Perchat et al. (2005)
Nematodes	Adult Larvae Inhibits the hatching of eggs Decreases of the reproductive rates	Prasad et al. (1972); Ignoffo and Dropkin (1977); Bone et al. (1985); Devidas and Rehberger (1992); Iatsenko et al. (2014)

2012; Pardo-L ópez et al., 2013; Sober ón et al., 2010). With effect only on young larvae of insects, this exotoxin has no immediate action on the digestive tract and its effect is dependent on high doses. Somerville and Swain (1975) claim that 2 mM of exotoxin are required to inhibit protein synthesis of insect cells. Moreover, in certain nematode species, this exotoxin can act lethally, regardless of the stage of development, reaching therefore, all phases of the lifecycle (Devidas and Rehberger, 1992).

5 Toxicity to Non-target organisms

(Cantwell et al., 1966), analyzing the effect of thuringiensin in *Apis mellifera*, reported that the

highest level of exotoxin (2.5 mg) killed 100% of individuals in 7 days. Sharma and Sahu (1977) performed tests with root meristem cells of *Allium cepa* (onion) and report that thuringiensins are able to inhibit spindle and cytokinesis and induces micronuclei, and minor in spindle activity. According to the authors, the binucleated cells also undergo mitosis giving biprophases and bimetaphases. Finally, they claim that microtubule systems and chromosomes are implicated as primary targets of this exotoxin.

6 Toxicity to Mammals

The proven effects of thuringiensins on vertebrates support the realization of safety tests in mammals, including toxicity and mutagenicity assays with intravenous, subcutaneous and intraperitoneal injections; cultures with human lymphocytes and allergenicity testing. Several tests using cultures of human blood and bone marrow cells are evaluated in the cytological effects after *in vitro* treatment with acute and chronic exposures of thuringiensins (Meretoja et al., 1977).

The toxicity to mammals was reported by several authors (Bishop and Robinson 2014; Robacker et al., 2000; Tsai et al., 2006). From these studies, it is possible to infer that there is a considerable difference in the toxicity of thuringiensins in insects and mammals. According (Vankova et al., 1974), the median lethal dose (LD50) in rats is 30 times higher than for *Galleria mellonella* larvae (Lepidoptera: Pyralidae). In high concentrations, thuringiensins are able to cross the cellular and nuclear membranes in mammalian systems, and have been reported neurotoxic, mutagenic and teratogenic effects in rats treated with these exotoxins (Meretoja et al., 1977).

Injections of lethal amounts of thuringiensins (10 ml / kg) can cause liver disorders, cellular degeneration and necrosis, hemorrhage and hepatic steatosis in mice (Meretoja et al., 1977). According to (Meretoja et al., 1977), human blood cultures treated with toxic concentrations of thuringiensins showed a significant increase in the incidence of chromosomal aberrations. Clastogenic effects have also been reported in bone marrow of rats that ingested thuringiensin for a month, or animals that received lethal doses of the toxin, much higher than the amounts normally used for control of insect pests. (Tsai et al., 2004) evaluated the effect of thuringiensins on the activity of the enzyme adenylate cyclase in the cerebral cortex of rats. The authors observed that the exotoxin is able to activate the enzyme and it may disturb cAMP-mediated signal transduction (adenosine 3' 5' - monophosphate), which is an important mediator of the action of various physiological functions in neuroendocrine systems.

The effect of thuringiensins by intratracheal instillation in rats induces pulmonary emphysema, fibrosis and death (Tsai et al., 1998). (Tsai et al., 2003) reported that intratracheal instillation thuringiensins results in highly inflammatory effect with a significant infiltration of polymorphonuclear neutrophils, which can lead to neurological disorders and damages to the cells of terminal bronchioles in the lung, in addition display fibrotic properties, due to accumulation of collagen. According to (Tsai et al., 2006), the intratracheal instillation of thuringiensin by rats resulted in lung damage, such as increase in weight; alkaline phosphatase decrease; lipid peroxidation increased; decrease in the activities of superoxide dismutase and glutathione; release of pro-inflammatory cytokines; excessive production of oxidants and may induce the lethality in some animals.

However, when dephosphorylated enzymatically or chemically, thuringiensins become biologically inactive (Bond et al., 1969). So when this toxin is ingested by mammals via gastrointestinal tract, can occur enzymatic dephosphorylation, which finishes with the toxicity of the molecule (Šebesta et al., 1981; Šebesta and Horska 1970). This could explain the lack of effects in the liver of rats treated with thuringiensins to long-term (Meretoja et al., 1977); the excretion of thuringiensin in the unchanged form in the urine of rats treated with the toxin via intraperitoneal; or the non-cumulation of the exotoxin in mice tissues (Šebesta and Horska, 1970). Corroborating this, (Šebesta et al., 1981), (Tsai et al., 2003) and (Vankova et al., 1974) stated that thuringiensin is more toxic when administered by intraperitoneal route than orally.

Although many studies have proven the harmfulness of thuringiensins when administered at high doses, there are also reports that expose his harmlessness in low doses. Carlberg (1973) demonstrated that feeding rats with thuringiensin for a long period, not presented pathological differences between the treated animals

when compared to the control group. (Bishop et al., 1999) reported that rats that received oral doses (1 x 10^{12} spores/ml) for three weeks or a single subcutaneous dose (1 x 10^{6}) showed no effects on behavior, bacterial infection, or pathologies in their internal organs. All cultures of blood, heart, spleen and liver were negative for the presence of *Bt*. De Lecadet and Barjac (1975) demonstrated that rats excreted in the feces the thuringiensin previously administered orally; the presence of these toxins in the urine was negative and that there was no thuringiensin accumulation in tissues. (Meretoja et al., 1977) report that were not observed megakaryocytes in the bone marrow or polymorphs in blood cultures, which are typical defects of contamination by chemicals, radiation, or infection. The same authors did not observe the incidence of chromosomal aberrations in the bone marrow cells, even in animals that received a lethal dose of exotoxins. Despite all the adverse effects of thuringiensins observed in mice, there is only one report of human food poisoning attributable to *Bt* (Jackson et al., 1995). However, it is believed to be under-represented due to the lack of differentiation between strains of *Bt* and *B. cereus* which is an opportunistic human pathogen capable of causing gastroenteritis (Bishop et al., 1999). *Bt* strains have been found in skin and corneal ulcers in humans, but it is not proved they were the causative agent (Samples and Buettner 1983; Green et al., 1990). Fisher and Rosner (1959) exposed humans at doses of a commercial preparation of *Bt* subsp. *thuringiensis*, orally and via inhalation over five days, but there were no adverse clinical indications. Numerous other tests with *Bt* and vertebrates were performed without fatality reports after oral or subcutaneous administration (Burges 1981; Siegel and Shadduck 1990; Drobniewski 1994).

The positive responses in some studies with rodents have shown that they are good experimental models to evaluate the toxicity of thuringiensins to humans (Bishop et al., 1999). Assuming that the thuringiensins intake, is the most likely way of humans come in contact with this exotoxin, several studies have investigated the dose required for a possible poisoning. (Tan et al., 1997) suggested that the dose would be 10^{5} cells/g. Belder and Elderson (2013) indicated an LD_{50} acute oral of 170 mg/kg, or even more than 5000 mg/kg in rats. When injected intraperitoneally, thuringiensins are toxic to vertebrates at a LD_{50} for of 13-18 mg/kg body weight (Swadener, 1994). As many *Bt* products are wettable powders which can easily enter the airways of vertebrates, studies have also investigated the median lethal concentration (LC_{50}) for acute inhalation of thuringiensin in rats. Belder and Elderson (2013) affirm that it ranges from 0.024 to more than 0.3 mg/L, for approximately 4 h of exposure. (Tsai et al., 2003) indicate that the LD_{50} of the acute intratracheal instillation of thuringiensin in rats is 4.4 mg/kg body weight. From these results, it is possible to notice that the lethal doses of thuringiensins to humans are much higher than those that are required to cause lethality in target organisms.

7 Prohibition of Insecticides containing Thuringiensins

Biopesticides containing thuringiensins in its formulation, as Bitoxibacillin (Lipa, 1985), Muscabac (Carlberg, 1986), or Thuricide (Siegel, 2001), which are based on *Bt* subsp. *thuringiensis*, were widely used to control the larvae of *M. domestica* (Mullens et al., 1988; Mullens and Rodriguez 1988), besides flies that who did infest sheds, pigsties and slaughter houses (Carlberg et al., 1991; Meretoja et al., 1977; Mwamburi et al., 2009).

However, since Šebesta and Horska (1969) stated that thuringiensins ware lethal to rats, the commercial use of this toxin began to be criticized. Thus, its potential toxic to mammals restricts its wide application in biological control (Zhou et al., 2013) and, since 1999, the World Health Organization (who) has recommended not to use *Bt* thuringiensin-producing strains in the formulation of biopesticides (Belder and Elderson 2013; Bishop et al., 1999; Mac Innes and Bouwer 2009; Palma et al., 2014; Siegel 2001; Veloorvalappil et al., 2013; WHO 1999). For this reason, the production of thuringiensins is a limiting factor in the selection of new strains of *Bt* with potential use in biological control of pests (Arango et al., 2002).

Whenever a new entomopathogenic microorganism is presented as a candidate for use in biological control practices, its effect on non-target organisms must be investigated (Cantwell et al., 1966). Therefore, it is recommended that tests be performed in order to detect these undesirable exotoxins and, if the organism is considered able to produce them, should ensure that they are not present in doses of risk and that the product does not come into contact with media that allow its germination and/or growth at any time prior to use (Belder and Elderson, 2013).

Another important factor in the assessment of environmental performance and safety, in addition to the physiological toxicity, is related to the stability of the thuringiensins (Zhou et al., 2013). Initially, several authors have reported that these exotoxins were quite persistent in the environment (Beebee and Korner 1972; Benz 1966; WHO 1999). Hitchings (1967) reported that thuringiensins are not degraded by exposure to UV radiation. However, (Zhou et al., 2013) recently reported that this exotoxin is unstable in aqueous solution and can be degraded in non-toxic compounds, being that this process can be reinforced by modification of biotic, physical or chemical factors, particularly with respect to pH and temperature. According to these authors, thuringiensins are highly unstable under simulated environmental conditions, have half-life around 2.72 to 16.19 days in the aqueous phase (significantly shorter than other common environmental pollutants) and can be 99% degraded without the presence of other microorganisms. Thus, under natural environmental conditions that contain different types of microorganisms, the degradation process can be accelerated. This data can decrease the concerns about the security issues of this toxin.

8 Procedures for the Detection of Thuringiensins

The first purification methods of thuringiensins involved centrifuging processes (Barjac et al., 1966); precipitation with $CaCl_2$ (Kim et al., 1970); micellar-enhanced ultrafiltration with surfactant cetylpyridinium chloride (CPC) (Tsun et al., 1999); fixation by calcium silicate and sodium phosphate dibasic for dissociation in the middle of fermentation, followed by high-performance liquid chromatography (HPLC) and electrodialysis to remove excess salts from solution (Tzeng et al., 2001); or precipitation for acetone and acetonitrile added to the supernatant (Gohar et al., 2001). Methods involving ultrafiltration have several drawbacks, including the high cost, low efficiency and extended duration. The method proposed by (Gohar et al., 2001) is able to detect very low levels of thuringiensins (0.3 μg/mL) and thus is the most widely used.

The most traditional method for detection of thuringiensins is based on toxicity bioassays (Gohar and Perchat 2001; Hernândez et al., 2001). As *Musca domestica* larvae are very susceptible to such exotoxins and do not develop into normal adults after exposure to the toxin, bioassays with these organisms may be used for the identification of these exotoxins (Bishop and Robinson 2014; Cantwell et al., 1964; Mac Innes and Bouwer 2009; Mechalas and Beyer 1963). Several authors propose high-performance liquid chromatography (HPLC) assays as a rapid method to detect and quantify thuringiensins. This method presents itself as an alternative to bioassays, which are time consuming (up to 9 days), enough variables, non-specific and can estimate inaccurate potencies due to impurities in the samples (Bubenschikova et al., 1983; Campbell et al., 1987; Rodr ́guez et al., 2003; Liu et al., 2010). The limit of detection of thuringiensins by HPLC ranges from 0.1 to 10 μg/mL (De Rijk et al., 2013). However, the HPLC method does not evaluate the direct toxicity, can provide false negatives and requires expensive equipment (Rodr ́guez et al., 2001; Mac Innes e Bouwer 2009). In turn the ELISA method is more sensitive than HPLC, can be used to quantify and quantitate the thuringiensins, with a detection limit of 0.1 ng/mL. (Liu et al., 2010) used the method of liquid chromatography mass spectrometry (LC-MS) for the qualitative confirmation of exotoxins, with the aim of detecting the presence of thuringiensins, even at very low concentrations. (Sauka et al., 2014) propose a method of Polymerase Chain Reaction (PCR) for the rapid prediction of the production of thuringiensins of type I by detection of the *Thue* gene, strongly associated with the synthesis of this exotoxin type. (Espinasse et al., 2002b), to report a strong correlation between production and the presence of Cry1B gene with the synthesis of thuringiensins, also suggest that a PCR reaction could allow indirect detection of the toxin. However, as the secretion of this exotoxin depends on the time of growth, the use of this technique does not seem to be reliable about the point of view of these authors (Arg ̂lo-Filho et al., 2014). In addition, other methods have been developed, including ion exchange chromatography, spectrophotometry and micellar electrokinetic capillary chromatography, quantification a limit of 0.028 mg/kg (Levinson et al., 1990; Campbell et al., 1987; De Rijk et al., 2013). The production of this toxin can still be evaluated in laboratory tests using rodent models (Burges 1981; Pinto et al., 2010).

Despite advances in research about thuringiensins of type I, detailed studies are needed on the structure, characteristics and mode of action of thuringiensins of type II. More specific tests with several non-target

organisms must also be made to provide more information about the lethal dose/concentration minimum and maximum needed to provide effects. The improvement of methods for the detection and quantification of thuringiensins should also be the focus of future research.

Authors' Contributions

SLFW developed and wrote the manuscript. HLP and LMF helped to draft the manuscript. All authors read and approved the final manuscript.

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