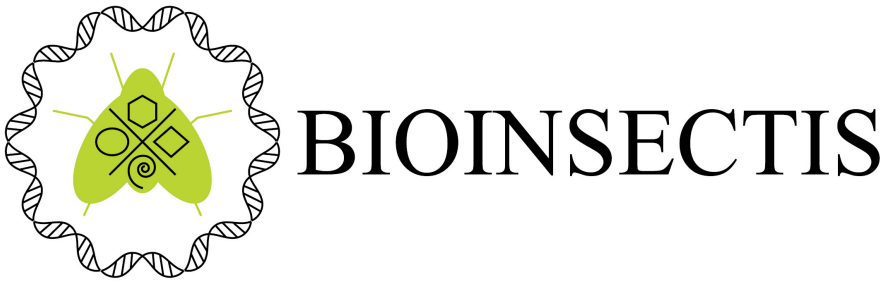
BIOINSECTIS

TESIS DOCTORAL

Identification and quantification of crystal components of *Bacillus thuringiensis* strains and their contribution to insecticidal activity

JAVIER CABALLERO SÁNCHEZ

Pamplona 2019

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Memoria presentada por

JAVIER CABALLERO SÁNCHEZ

para optar al grado de Doctor por la Universidad Pública de Navarra

Identification and quantification of crystal components of *Bacillus thuringiensis* strains and their contribution to insecticidal activity

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INFORMAN

que la presente memoria de Tesis Doctoral titulada "Identification and quantification of crystal components of *Bacillus thuringiensis* strains and their contribution to insecticidal activity" elaborada por Don **JAVIER CABALLERO SÁNCHEZ** ha sido realizada bajo nuestra dirección y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor.

Y para que así conste, firman la presente en Pamplona a 23 julio de 2019

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AGRADECIMIENTOS

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RESUMEN

Bacillus thuringiensis (Berliner, 1915) (Bt) es una bacteria Gram positiva que forma una espora de resistencia durante la fase estacionaria de crecimiento y un cristal parasporal compuesto por δ-endotoxinas (proteínas Cry y Cyt) con actividad insecticida por ingestión. Bt es el agente entomopatógeno más empleado para el control de plagas en campo y su uso es compatible con otros métodos de control que tienen cabida en programas de Gestión Integrada de Plagas (GIP). La diversidad de las toxinas Bt, y su amplio espectro de huéspedes, hacen que esta bacteria sea utilizada: i) como recurso genético para la obtención de factores tóxicos utilizados en la construcción de plantas resistentes contra las plagas, y ii) para el desarrollo de insecticidas basados en Bt.

Desde los años 70, la potencia de los insecticidas basados en Bt se expresa en Unidades Internacionales (UI). Este valor, se determina para cada lote de producto técnico comparandolo con la potencia de una cepa estándar de Bt a la cual se le ha atribuido un valor arbitrario frente a larvas de un insecto de referencia. Sin embargo, este dato no aporta ninguna información relevante sobre la actividad insecticida del producto cuando el objeto del tratamiento son otras especies plaga distintas al insecto de referencia. El principal objetivo de esta tesis ha sido desarrollar un método proteómico que permite analizar cualitativa y cuantitativamente las proteínas que componen el cristal producido por distintas cepas silvestres de Bt. El método emplea un sistema de cromatografía líquida acoplada a un espectro de masas (LC-MS/MS) en combinación con una monitorización de múltiples reacciones (MRM). Para llevar a cabo el análisis, es necesario conocer la secuencia del genoma de la cepa Bt para determinar los potenciales genes insecticidas que podrían formar parte del cristal parasporal. El uso de herramientas bioinformáticas permite la selección de péptidos proteotípicos que detectan de forma específica la presencia de cada una de las proteínas de la mezcla. Estos péptidos proteotípicos, marcados isotópicamente, permiten determinar la proporción relativa de cada proteína en el cristal. El método fue validado utilizando dos mezclas artificiales de tres proteínas recombinantes (Cry1Aa, Cry2Aa y Cry6Aa), donde la proporción relativa de cada proteína era conocida. La aplicación del método permitió detectar las tres proteínas de forma independiente y cuantificar la proporción relativa de cada una de ellas con gran

fiabilidad y precisión. Una vez verificada su validez, el método fue aplicado para determinar la composición del cristal de cuatro cepas Bt silvestres que component el ingrediente activo de los productos comerciales más vendidos a nivel mundial para el control de distintos órdenes de insecto: DiPel® y XenTari® para lepidópteros, VectoBac® para dípteros, y Novodor® para coleópteros. El cristal parasporal de la cepa ser. *kurstaki* ABTS-351 (DiPel®) resultó estar compuesto por cuatro proteínas: Cry1Aa (13-22%), Cry1Ab (16-29%), Cry1Ac (6-12%) y Cry2Aa (40-64%); al igual que la cepa ser. *aizawai* ABTS-1857 (XenTari®) Cry1Aa (26-33%), Cry1Ab (57-60%), Cry1Ca (7-11%) y Cry1Da (3-4%). La cepa AM65-52 (VectoBac®) sintetizó un cristal formado por Cry4Aa (2-4%), Cry4Ba (10-28%), Cry11Aa (10-27%), Cry60Aa (2-4%), Cry60Ba (5-12%) y Cyt1Aa (38-61%) y el ingrediente activo de Novodor®, la cepa ser. *tenebrionis* NB-176, contenía Cry3Aa (70-75%), Cry23Aa (14-16%) y Cry37Aa (10-14%). Adicionalmente, se determinó la actividad de la cepa ABTS-1857 en larvas de tres especies del género *Spodoptera*: *S. exigua, S. littoralis* y *S. frugiperda. S. exigua* fue la especie más susceptible (CL_{50}= 7.8 ng/µl), seguida de *S. littoralis* (CL_{50}= 28 ng/µl). *S. frugiperda* se mostró como la especie más tolerante (CL_{50}= 120.2 ng/µl). Para determinar la contribución de cada proteína individual a la toxicidad general de la cepa ABTS-1857 contra cada una de las tres especies de insectos, se construyeron cepas Bt recombinantes que producían individualmente las proteínas Cry1Aa, Cry1Ab, Cry1Ca y Cry1Da. Los resultados de los bioensayos, utilizando el "droplet feeding method", revelarón una elevada toxicidad de Cry1Ca para las larvas de *S. exigua* y *S. littoralis* y de Cry1Da frente a *S. littoralis* y *S. frugiperda.* Las mezclas artificiales de dos o tres proteínas produjeron mortalidades atribuibles a la cantidad de Cry1Ca, en el caso de *S. exigua*, de Cry1Ca y Cry1Da, en el caso de *S. littoralis*, y de Cry1Da en el caso de *S. frugiperda*. La mezcla artificial de cuatro proteínas, que reflejaba la composición natural del cristal, dio valores de actividad concordantes con los producidos por el cristal natural de la cepa ABTS-1857. Aumentos de la proteína Cry1Da, en detrimento de las proteías Cry1Aa y Cry1Ab, produjo incrementos en la actividad insecticida para larvas de *S. littoralis* y *S. frugiperda.* Estos resultados indicaron que la metodología empleada para el análisis de los cristales es válida para su empleo en la caracterización y estandarización de los productos comerciales basados en Bt, aportando la información necesaria para expresar su potencia insecticida.

SUMMARY

SUMMARY

Bacillus thuringiensis (Berliner, 1915) (Bt) is a Gram-positive bacterium that forms a resistance spore during the stationary phase of growth and a parasporal crystal which is comprised by δ-endotoxins (Cry and Cyt proteins) that show insecticidal activity when ingested. Bt is the most commonly used entomopathogenic agent for pest control in the field and its use is compatible with other control methods in Integrated Pest Management (IPM) programs. The diversity of Bt toxins, and their broad spectrum of hosts, allow the use of this bacterium: 1) as a genetic resource for obtaining toxic factors used to engineer plants resistant to pests, and 2) for the development of Bt-based insecticides.

Since the 1970s, the potency of Bt-based insecticides is expressed in International Units (IU). This value is determined for each batch of technical product by comparison with the potency of a standard Bt strain, which has been assigned an arbitrary value against larvae of a reference insect. However, this data does not provide any relevant information concerning the insecticidal activity of the product when the targets of the treatment are other pest species than the reference insect. The main aim of this thesis has been the development of a proteomic method that allows a qualitative and quantitative analysis of the proteins that make up the crystal produced by different wildtype Bt strains. The method uses a liquid chromatography system coupled to a mass spectrum (LC-MS/MS) in combination with a multiple reaction monitoring (MRM). To carry out the analysis, it is necessary to know the genome sequence of the Bt strain to determine the potential insecticidal genes that could be part of the parasporal crystal. The use of bioinformatic tools, allow the selection of proteotypic peptides that specifically detect the presence of each of the proteins in the mixture. These proteotypic peptides, isotopically labelled, allow to determine the relative proportion of each protein within the crystal. The method was validated using two artificial mixtures containing three recombinant proteins (Cry1Aa, Cry2Aa and Cry6Aa), in a known relative proportion. The application of the method allowed the detection of the three proteins independently and the quantification of the relative proportion of each of them with great reliability and precision. Once the method was validated, it was applied to determine the crystal composition of the Bt strains used as active ingredients of four of the best sold commercial products worldwide for the control of different insect orders: DiPel® and

XenTari® for Lepidoptera, VectoBac® for Diptera, and Novodor® for Coleoptera. The parasporal crystal of the Bt subsp. *kurstaki* strain ABTS-351 (DiPel®) was comprised by four proteins, including: Cry1Aa (13-22%), Cry1Ab (16-29%), Cry1Ac (6-12%) and Cry2Aa (40-64%); in the Bt subsp. *aizawai* strain ABTS-1857 (XenTari®) four proteins were also detected: Cry1Aa (26-33%), Cry1Ab (57-60%), Cry1Ca (7-11%) and Cry1Da (3-4%). The Bt subsp. *israelensis* strain AM65-52 (VectoBac®) synthesized a crystal formed by Cry4Aa (2-4%), Cry4Ba (10-28%), Cry11Aa (10-27%), Cry60Aa (2-4%), Cry60Ba (5-12%) and Cyt1Aa (38-61%). Finally, the active ingredient of Novodor®, the Bt subsp. *tenebrionis* strain NB-176, contained Cry3Aa (70-75%), Cry23Aa (14-16%) and Cry37Aa (10-14%). Additionally, the activity of the strain ABTS-1857 was determined for three species of the genus *Spodoptera*, including: *S. exigua, S. littoralis and S. frugiperda*. *S. exigua* was the most susceptible species (LC_{50} = 7.8 ng/µl), followed by *S. littoralis* (LC_{50} = 28 ng/µl). *S. frugiperda* was the most tolerant insect (LC_{50} = 120.2 ng/µl). To determine the contribution of each individual protein to the overall toxicity of the strain ABTS-1857 against each of the three insect species, recombinant Bt strains producing Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da proteins, were engineered.

The results of the bioassays, using the droplet feeding method, revealed a high toxicity of Cry1Ca for *S. exigua* and *S. littoralis* larvae and Cry1Da against *S. littoralis* and *S. frugiperda*. Artificial mixtures containing two or three proteins produced mortalities attributed to the amounts of Cry1Ca, in the case of *S. exigua*, of Cry1Ca and Cry1Da, in the case of *S. littoralis*, and of Cry1Da in the case of *S. frugiperda*. The obtained activity values for the artificial mixture containing the four proteins, which reflected the natural composition of the crystal, were consistent with those obtained with the natural crystal of the strain ABTS-1857. Increasing amounts of Cry1Da protein, in combination with decreasing quantities of either Cry1Aa or Cry1Ab proteins, produced an enhanced insecticidal toxicity for *S. littoralis* and *S. frugiperda* larvae. These results indicated that the method used for the analysis of the Bt crystals is valid for its use in the characterization and standardization of Bt based commercial products, providing the necessary information to express its insecticidal potency.

CHAPTER I

CHAPTER I

Introduction

1. GENERAL INTRODUCTION

In 1939, the Swiss Paul Hermann Müller discovered the insecticidal properties of DichloroDiphenylTrichloroethane (DDT) (Metcalf, 1973). The use of this synthetic chemical became significantly extensive during the second half of World War II to prevent Allied soldiers from contracting insect-transmitted diseases, such as malaria, dengue fever and schistosomiasis (Fishel, 2016). By the end of the war, its use was confined to solving problems related to crop protection. Since then, synthetic pesticides such as organochlorines (e.g. DDT), organophosphates (e.g. glyphosate, malathion), carbamates (e.g. aldicarb) and synthetic pyrethroids (e.g. fenvalerate) (Nicolopoulou-Stamati et al., 2016), were massively sprayed in agricultural practices. This lead to the rise of the chemical industry, initiating a new era in pest control (Fishel, 2016).

Chemical insecticides in general have contributed to significantly increase agricultural production by providing season-long protections against a wide variety of insect pests at a relatively low cost. DDT and other synthetic organic pesticides work through similar mechanisms either by disrupting the ion balance of nerve axons, thus producing tremors, convulsions and eventually death; or by inhibiting cholinesterase, leading to the accumulation of acetylcholine at the neuron synapsis and causing paralysis (Das, 2013). Due to their unspecific mode of action, non-target organisms such as beneficial insects or pollinators may be negatively affected. As a result, reductions in the yield and quality of crops or outbreaks of secondary pests may occur (Devine and Furlong, 2007; Sánchez-Bayo et al., 2016). One of the most notorious disadvantages of chemical pesticide usage is the appearance of resistant insect biotypes in the pest populations due to a high selection pressure (Tabashnik, 2008). A few years after the introduction of DDT as the ultimate solution for crop protection, populations of resistant insects arose and in some cases even developed cross resistance to all classes of chemical insecticides (Das, 2013). High stability (low degradation) and long persistence of chemical residues played an important role in their effectiveness, but at the same time they were also key factors in producing negative impacts on the environment. In 1962, Rachel Carson published *The Silent Spring* (Carson et al., 2002), where she expressed her concerns for generic pesticides and their negative impact as they

bioaccumulated in the adipose tissue of animals and lead to their biomagnification higher up in the food chain (Jarman and Ballschmiter, 2012). A decade later, the U.S Environmental Protection Agency (EPA) banned the use of DDT in the United States (Fishel, 2016).

Concerning human health, skin contact, inhalation, ingestion or high occupational exposure to synthetic chemical insecticides may result in cancer, immune system disorders and dermatological, gastrointestinal, neurological, respiratory, reproductive and endocrine effects (Alewu B, 2018; Gunnell et al., 2007; Nicolopoulou-Stamati et al., 2016; WHO, 1990). Usually the limits of pesticide residues that can be found in fruit juices, water or cooked meals among others, do not exceed the safety levels indicated in the legislation. Nevertheless, the potential risk on human health when exposed to two or more of these substances simultaneously has been traditionally overlooked (Kortenkamp, 2007; WHO, 1990). Moreover, the chemical compounds found in these type of insecticides are often bioaccumulated in the fatty tissues of the human body, which may affect neonates through breast feeding, endangering their health (Pirsaheb et al., 2015).

The increasing awareness of chemical pesticide hazards has resulted in a critical need for the development of new safe control products from a health and environment viewpoint (Nicolopoulou-Stamati et al., 2016). Such urge for an alternative strategy to conventional pesticides has been made evident by the EU through Directive 2009/128/EC, which aims at the use of sustainable pesticides in order to reduce the risk and negative impact of chemical molecules in human health and the ecosystems. According to this, Integrated Pest Management (IPM) must be promoted and new techniques for crop protection that avoid the introduction of chemicals should be explored. Microbial insecticides based on bacteria, virus, fungi or protozoa constitute a real alternative and have been proven as a potential alternative for pest management (Chandler et al., 2011; Lacey et al., 2001). When compared to chemical pesticides, microbial control agents (MCA) present numerous advantages in terms of efficacy and cost. Similar to natural enemies, many entomopathogens are often specific to certain species or group of insects, which translates into high safety levels for humans and other nontarget organisms such as honey bees, parasitoids or predators (Hokkanen and Hajek, 2003). Analogously, reduction of pesticide residues on food and increased activity of natural enemies

and biodiversity in managed ecosystems are considered beneficial side effects of the use of MCAs in comparison with chemical pesticides. However, their brief persistence in the field, reduced speed of kill and elevated production costs are some of their still unresolved limitations. Despite their numerous advantages, MCAs should not be used as a unique solution for crop protection. Instead they may be considered as invaluable components of integrated pest management programs with natural enemies, resistant plants or even soft pesticides (Lacey, 2016). Although several researchers experimented with MCA in the late 19th century, it was not until the development of *Bacillus thuringiensis* (Bt) based biopesticides that the use of microbes for pest control became widespread. This bacterium produces highly specific toxins to target insects which are innocuous to humans, mammals and plants, and are completely biodegradable (Meher et al., 2002), making it a viable alternative for pest control and disease vectors of importance in human public health.

2. THE ENTOMOPATHOGENIC BACTERIUM *Bacillus thuringiensis*

2.1. Discovery and history

B. thuringiensis was first identified in 1902 by the Japanese biologist Shigetane Ishiwata, who found the microorganism infecting a silkworm larva (*Bombix mori*) and named it *Bacillus sotto* (Beegle and Yamamoto, 1992). About a decade later, in the region of Thuringia (Germany), Ernest Berliner isolated a Gram-positive bacterium causing an infection to the Mediterranean flour moth larvae (*Ephestia küehniella*) and named it *Bacillus thuringiensis*. Although it was the same bacterial species previously described by Ishiwata, the new name was quickly adopted by the scientific community and has persisted until our days (Melo et al., 2016). Berliner observed that a crystal inclusion body was formed when the microorganism reached the sporulation phase. Later, Angus demonstrated that such parasporal crystals that were formed during the sporulation were responsible for Bt insecticidal activity (Angus, 1956).

2.2. Interspecific classification

The genus *Bacillus* is a very diverse genera in the class Bacilli and includes Gram-positive, spore-forming, rod-shaped, aerobic and facultative

anaerobic bacteria with a G+C content ranging from 32-69% (Garbeva et al., 2003). *B. thuringiensis* has been included as a member of the *Bacillus cereus* group, also known as *B. cereus sensu lato* (Helgason et al., 2000), in the Bacillaceae family (de Barjac and Frachon, 1990). It comprises six closely related species: *B. cereus, B. anthracis, B. thuringiensis, B. mycoides, B. pseudomycoides and B. weihenstaphanensis.* Although these species share a similar genetic background they clearly differ in virulence, host range and environmental distribution (Vilas-Bôas et al., 2007). *B. anthracis* is responsible for anthrax, an acute disease that might be lethal for humans and animals (Mock and Fouet, 2001). Some strains of *B. cereus* are opportunistic human pathogens causing food poisoning leading to diarrhoeas, eye infections and periodontal disease (Drobniewski, 1993; Helgason et al., 2000), while other strains may be non-pathogens acting as animal probiotics and biostimulant promoting plant growth (Jadamus et al., 2001; Saharan et al., 2011). *B. thuringiensis* is mainly distinguished from other *Bacillus* species due to its ability to synthetize a crystalline inclusion of proteinaceous nature in the mother cell during the sporulation process, making it phenotypically different (Baumann et al., 1984). However, the bacteria may occasionally lose this ability and become indistinguishable from other *Bacillus* as the genes coding for the crystal proteins are generally conferred to plasmids which can be transferred by matting to different subspecies (González and Carlton, 1984; Yamamoto, 2001). Within Bt strains, these may be distinguished among others according to crystal characteristics at the structural and size level and their host range. The crystal inclusion body has shown to be specifically toxic to some insect pests and some of them have been developed as biological control agents against a range of insect species of different orders (Van Frankenhuyzen, 2009).

2.3. Intraspecific classification

Since the early 1960s several characteristics, such as biochemical and serological, along with toxicity records, have been used in order to classify the uprising number of Bt isolated worldwide ever since (Sanchis et al., 1996). The immunological reaction to the bacterial flagellar antigen (H serotyping) was stablished as a typing method of choice for the characterization within Bt isolates (de Barjac and Bonnefoi, 1962) and has been used until date (de Barjac and Frachon, 1990). In *B. thuringiensis,* the *hag* gene is responsible for the expression

of flagellin, the protein that gives the immunological reaction in H serotyping. Specific flagellin amino acid sequences correlates to specific Bt serotypes, and today, *B. thuringiensis* strains are classified into at least 69 H serotypes (Lecadet et al., 1999). Although generally serotypes do not contain H antigen variation, some of them have shown to contain few antigen distinct subfactors, which has led to the subdivision of the 69 H serotypes into 82 serological varieties, referred to as serovars (Xu and Côté, 2008). Although there is a good correlation between Bt subspecies and host range at the family level, a classification of isolates based on their pathotype turns into a hopeless task. Frequently, multiple toxin genes are present in a single isolate, and combinations of them can be endless, so an overlapping of the host profiles may occur breaking down the correlation at the genus and species level (Lecadet et al., 1999). Besides, phylogenetic analysis of the bacterium have been performed by using the *gyrB* gene (Yamamoto and Harayama, 1995) to complement the 16S rRNA gene and discrimination among Bt H serotypes, serovars belonging to the same serotype and strains from the same serovar has been accomplished (Soufiane and Côté, 2009). Today, the serotyping techniques seem to be in disuse due to the inability to certainly correlate H serotype with biological activity. Thus, different tools including determination of number and size of plasmids, characterization of toxic genes, analysis of protein fragments by polyacrylamide gel electrophoresis (SDS-PAGE) and toxicity bioassays may be applied in order to characterize a Bt isolate (Iriarte et al., 2000).

2.4. Genome

B. thuringiensis has a larger genome than *B. cereus* and *B. anthracis,* consisting of a circular chromosome ranging from 2.4 to 5.7 Mb (Carlson et al., 1994). Its organization near the replication origin is similar to the one that occurs in *B. cereus,* but greater variability can be adverted in the terminal half of it (Carlson et al., 1996). The pathogenicity exerted by Bt is due to virulent components that reside in large circular or linear plasmids (Carlson et al., 1994). Some strains, like Bt subsp. *kurstaki,* may harbor up to 17 virulence plasmids ranging from 2 to 250 kb (Baum and Gonzalez, 1992; Lereclus et al., 1982) and horizontal plasmid transfer by conjugation between strains may happen. It is well known that these plasmids are the main reservoir for *cry* and *cyt* genes (Berry et al., 2002; Li et al., 2015) which encode crystal proteins (Cry proteins) and cytolytic toxins (Cyt proteins),

respectively, although they may be also present in the chromosome (Kronstad et al., 1983).

2.5. Life cycle

The cycle life cycle of *B. thuringiensis* has two clearly differentiated growth phases (Bulla et al., 1980). The first phase, also known as the vegetative phase of growth, takes place when favourable conditions occur, in terms of nutrients and aeration, the spore germinates, rising a vegetative cell where the formation of a septum through the plasma membrane will divide the mother cell into two identical daughter cells, and so on (Figure 1) (Osman et al., 2015). Although the growth temperature may vary between 15-45 °C, the optimal range goes from 26 to 30 °C, as above 42 °C plasmids containing the insecticidal genes may be cured (Mikesell and Vodkin, 1985). In terms of pH, conditions may vary from 5.5 to 8.5, but normally pH around 7 is the most suitable for its proliferation (Bernhard and Utz, 1993).

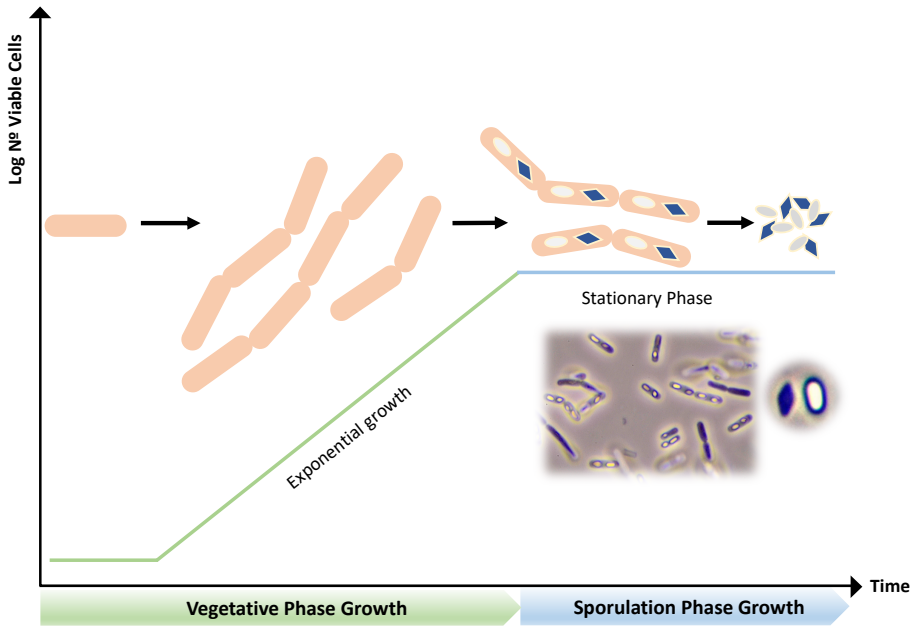

Figure 1. Schematic representation of the life cycle of *Bacillus thuringiensis.* During the vegetative phase of growth, the bacterium experiences an exponential growth forming collar chains. The lack of nutrients forces the bacterium to enter a stationary phase in which it sporulates and the proteins aggregate to form the parasporal crystal. Finally, the cell lysis releases the crystal and the spore, which will germinate again in favorable conditions, closing the cycle of this bacterium.

The cells form pairs or short chains, during the exponential proliferation of the bacterium, until the nutrients in the medium become scarce. At that moment the cell enters the stationary phase of its cycle, otherwise referred as the sporulation phase, in which the formation of a resistance spore takes place in the apical extreme of the cell, leaving room for the formation of a parasporal crystal of a proteinaceous nature at the other end. In the whole process the microorganisms undergoes up to 7 stages where the division turns asymmetric (Figure 1) (Bechtel and Bulla, 1982). The production of delta endotoxins is genetically regulated and give the microorganism a survival advantage, by exerting a lethal action against the insect host, providing sufficient nutrients for the germination of the dormant bacterial spores initiating the vegetative growth phase (de Maagd et al., 2001).

2.6. Ecology

B. thuringiensis is a Gram-positive, rod shaped, facultative anaerobe spore-forming bacterium (Claus and Fritze, 1989) which size varies between 1-1,2 µm in width and 2-5 µm in length. It has been isolated worldwide from many diverse environments, including soil samples (Vilas-Bôas and Lemos, 2004), the rhizosphere, the phylloplane (Smith and Couche, 1991), aquatic systems, grain dust, and dead insects (Iriarte et al., 1998; Raymond et al., 2010). Recently new isolates from marine sediments (Maeda et al., 2000) and Antarctic soil samples (Forsyth and Logan, 2000) have been reported. Despite its ubiquity, it has been always linked to the soil, presumable deposited by insects, where it may remain latent even in adverse conditions for its development (Glare and O'Callaghan, 2000) until the nutrients layout and growth conditions are propitious once again (Saleh et al., 2010). Unlike other Gram positive spore-forming bacteria, *B. thuringiensis* is able to live in the environment free and independent, without the need to interact with other microorganisms, even in unique environments such as the midgut and the hemocoel of insects (Nielsen-LeRoux et al., 2012; Raymond et al., 2010; Schnepf et al., 1998). Nonetheless, its role of the crystal in ecology is poorly understood and stills an enigma. Bt is an opportunistic pathogen, but it is not generally known for causing natural epizootics, although it occurred when it was first isolated by Ishiwata (Milner, 1994). It is part of the natural inhabitants that occur inside arthropods and may not provoke any injury to its host unless it is weakened by an infection caused by other pathogen (Eilenberg et al., 2000). Crystal toxins may provide a greater

chance for the proliferation of the bacterium in these situations by binding to the brush border, which seems to stimulate spore germination in the insect gut (Du and Nickerson, 1996) and subsequent vegetative cell proliferation (Hendriksen and Hansen, 2003) even in the dead corpse. When nutrients start to be limited sporulation occurs along with toxin production (Hansen and Salamitou, 2000) until suitable conditions arise. These crystal proteins may suppose up to 25-30% of the dry weight of the bacteria (Agaisse and Lereclus, 1995). In the case of mammals Bt appears as a transient microorganism in the intestine that have shown no harm for animals (Swiecicka et al., 2002) or humans after long term exposure (Jensen et al., 2002).

3. *B. thuringiensis* TOXIC FACTORS

Different toxins, including alpha(α)-exotoxin, beta(β)-exotoxin, gamma(γ)-exotoxin, delta(δ)-endotoxin, enterotoxin, louse and mouse factor exotoxins, water-soluble exotoxin and vegetative toxins (Heimpel, 1966; Osman et al., 2015), have been describe in *B. thuringiensis* strains. Thus toxins reporting insecticidal activity have been the most widely studied due to their commercial exploitation as active ingredients of biopesticides in the agricultural industry (Montesinos, 2003; Sanchis, 2011).

During the vegetative phase of growth Bt is capable of secreting soluble proteins to the medium, starting at mid-log phase and extending during sporulation, named vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip) (Palma et al., 2014). During the stationary phase of growth *cry* and *cyt* genes will encode the δ-endotoxins that accumulate in the cell forming the parasporal crystal, which may be composed by a single protein or a combination of Cry proteins and also, but less frequently, Cry and Cyt proteins (de Maagd et al., 2003).

3.1. Toxin nomenclature and classification

Since the earlies 1980s, when the first crystal protein gene was cloned in *E. coli* from the Bt strain HD1 (Schnepf and Whiteley, 1981), more than 700 *cry* genes, coding for Cry proteins have been reported during the last decades, and around two hundred of these have already been cloned (Crickmore, 2013). The increasing number of novel genes isolated from a wide range of Bt strains generated

the need for an organized nomenclature system (Crickmore et al., 1998).The first nomenclature system was based on the toxicity spectra of the crystal proteins and grouped them in four classes: CryI for lepidopteran-active toxins, which were 130-140 kDa in size; CryII for proteins yielding 65kDa that targeted both, lepidopterans and dipterans; CryIII consisted of proteins exhibiting toxicity against coleopterans; and CryIV was constituted of proteins exclusively toxic for dipterans (Höfte and Whiteley, 1989). A separate nomenclature was adopted for a toxin from a Bt subsp. *israelensis* that showed to have cytolytic activity, and so named CytA (Ward et al., 1986). But this system soon led to problems as toxins had to be tested against a wide range of insects in order to classify them, some did not show the expected activity and many others were named the same by their discoverers mixing up the classification. That how the *B. thuringiensis* Toxin Nomenclature Committee was created and a new nomenclature system, accepted until date, was proposed (Crickmore et al., 1998). This new system classified toxins exclusively on their amino acid sequence, not considering their structure, mode of action or biocidal activity. The nomenclature was based in a four-rank name according to the degree of pairwise amino acid identity to previously named toxins. For the first and fourth rank Arabic numbers were used and uppercase and lowercase letters for the second and third rank, respectively (Figure 2).

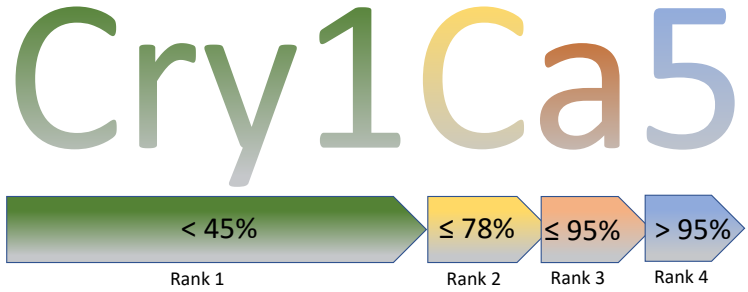

Figure 2. Nomenclature system adopted for insecticidal toxins produced by *Bacillus thuringiensis.* Four ranks are used to group up toxins according to their amino acid sequence identity. Primary, secondary and tertiary rank distinguish proteins with at least 45, between 45 and 78 and up to 95% sequence identities, respectively. Quaternary rank differentiates between alleles of the same gene found in different Bt isolates, although they may share the exact same sequence.

Proteins that shared at least 45% sequence identity were assigned a different primary rank (an Arabic number, e.g. Cry1 and Cry2); a different secondary rank was used to distinguish proteins sharing between 45% and 78% pairwise identity (an uppercase letter, e.g. Cry1A and Cry1B); a tertiary rank was reserved for differentiating those proteins between 79% and 95% identity (a lowercase letter, e.g. Cry1Aa and Cry1Ab); and finally different clones sharing more than 95% pairwise identity were assigned a different quaternary rank (an Arabic number, e.g. Cry1Aa1 and Cry1Aa2) (Crickmore et al., 2018). This nomenclature system is applied to δ-endotoxins (Cry and Cyt proteins), vegetative insecticidal proteins (Vip) and secreted insecticidal proteins (Sip).

3.2. Toxic factors produced during the vegetative phase of growth

3.2.1. Vip proteins

The vegetative insecticidal proteins (Vip) (Estruch et al., 1996) are synthetize and secreted to the culture medium during the vegetative phase of growth. The transcription of *vip* genes is detected at the start of the logarithmic phase of growth, reaching a high level of expression during the stationary phase (Bi et al., 2015; Shi et al., 2004). In contrast to crystal toxins, which can be effective in absence of a viable Bt cell, a vegetative growing cell is clearly required for secreted toxins to exert a pathogenic effect (Donovan et al., 2001; Zhu et al., 2006). The increasing cases of resistance to Cry proteins promoted the activation of screening programs focused on the identification of new insecticidal components, contained in the supernatant of Bt strains. These components were evaluated and found to be proteinaceous, rendering different band patterns when resolved in acrylamide gels (SDS-PAGE). Their toxicity was evaluated against different insect orders. To date, a total of four different classes of Vip proteins (Vip1, Vip2, Vip3 and Vip4) have been classified (Crickmore et al., 2018). The *vip1* and *vip2* genes encode proteins of 885 (80 kDa) and 462 (45 kDa) amino acidic residues, respectively. These proteins act as binary toxins, such as the cholera toxin, where two functional domains or subunits must assemble in order to compose a toxicity complex (Madshus and Stenmark, 1992). Although the molecular mechanism of their insecticidal activity is not well understood, it is believed that Vip1 inserts in the membrane and forms a pore

(Leaber et al., 2006) providing Vip2 a pathway to enter the cell via endocytosis in order to exert the lethal toxicity to the host (Barth et al., 2004). A total of 15 Vip1 and 20 Vip2 proteins have been reported, exhibiting insecticidal toxicity against species of the order Coleoptera (*e.g. Diabrotica spp*) (Shi et al., 2004) and Hemiptera (e.g., *Aphis spp*) (Sattar and Maiti, 2011). No activity has been reported when inoculated individually, supporting the fact that these toxins must act together to exhibit activity (Chakroun et al., 2016). The *vip3* genes encode proteins of 791 amino acids (88.5 kDa) which are processed in the insect midgut and consequently activated, binding to brush border membrane vesicles where ion channels are formed, provoking the disruption of the epithelial cells (Lee et al., 2006). Until date, 101 Vip3 proteins (http://www.btnomenclature.info/) with insecticidal activity against a broad spectrum of lepidopteran insect larvae of economic importance, that are less susceptible to δ-endotoxins (Estruch et al., 1996), have been described. So far, a new subfamily composed by the *vip4* gene has been classified, encoding a protein of 965 amino acids (108 kDa). No target insects have been found yet, although phylogenetically Vip4 seems to be closer to Vip1, sharing up to 34% amino acid identity, than to the rest of Vip proteins. An interesting feature of Vip proteins is that no homology with δ-endotoxins is shared, showing a different mode of action and binding to non-competitive sites in the insect midgut (Lee et al., 2006, 2003; Sena et al., 2009). This feature opens a new field for control strategies where Vip proteins could be included to overcome resistance problems that have arisen due to constant applications of Bt sprays (Ferré and Van Rie, 2002). Also, combination of secreted and crystal toxins may broaden the host range of Bt-based biopesticides (Bravo et al., 2011).

3.2.2. Sip proteins

The secreted insecticidal proteins (Sip) are also synthetized and secreted to the culture medium during the vegetative phase of growth. The first and only *sip* gene described in this new Bt insecticidal family, was identified in the strain EG2158 and encodes a protein of 367 amino acidic residues (41 kDa). Although the mode of action of this protein remains unknown, its limited homology with Mtx3 mosquitocidal toxins (Liu et al., 1996), suggests that pore formation would be responsible for its insecticidal activity against coleopteran larvae, including

Leptinotarsa decemlineata (Coleoptera: Chrysomelidae) or *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae) (Donovan et al., 2006). No homology in the amino acidic sequences has been found with Cry or Vip proteins (Donovan et al., 2001).

3.2.3. Other secreted toxic factors

Bt also produces several other toxins, such as α-exotoxin, hemolysin, enterotoxins, phospholipases and chitinases (Hansen and Salamitou, 2000). In some cases, certain Bt strains are capable of producing the well-studied ß-exotoxin, also referred to as thuringiensin (Šebesta et al., 1981; Vafskov, 1978), a heat-stable adenine-nucleotide analog which inhibits DNA directed RNA-polymerase competing with ATP (Gohar and Perchat, 2001; Horská et al., 1976; Šebesta and Horská, 1970). Although it is believed that the role of this low molecular weight molecule (700 Da) is to regulate the transcription of δ-endotoxins, its toxicity affects a wide range of animals, including mammals (McClintock et al., 1995). This feature impedes its inclusion Bt-based insecticidal formulations (Glare and O'Callaghan, 2000). Nowadays, improved high-performance liquid chromatography separation techniques are used for the detection and quantification of ß-exotoxin in *B. thuringiensis* culture supernatants (Hernández et al., 2003; Levinson et al., 1990).

3.3. Toxic factors produced during the stationary phase of growth

During the stationary phase the bacterium synthetize the δ-endotoxins, oligomers commonly known as Cry and Cyt proteins, that will accumulate and crystallize forming the parasporal crystal. This is the defining feature of Bt. The bacterium will lysate and this inclusion body will be released to the medium along with the spore after the cell wall disintegrates. In some strains the crystal is found within the exosporium (Aronson and Fitz-James, 1976), but usually these two structures are not physically connected.

3.3.1. Cry proteins

Parasporal crystals can be composed by a single or a combination of Cry proteins cross-linked by disulphide bonds. The shape of the crystal keeps a certain correlation with the type of proteins that make it up. Those formed by Cry1 are

bipyramidal, Cry2 produce cubic forms, Cry3 form flat-rectangular crystals, those composed of Cry4 are spherical and Cry11 proteins form rhomboidal crystals. (Schnepf et al., 1998). The most common shapes among natural isolates are mainly bipyramidal and spherical (Bernhard et al., 1997; Martin and Travers, 1989). Usually, *cry* genes are conferred to one or more plasmids, although copies of them can also be located in the bacterial chromosome (Kronstad et al., 1983). The expression of these insecticidal genes is controlled at the transcriptional, post-transcriptional and post-translational level, and represents a huge metabolic investment, as the crystal may represent up to 20-30% of the dry weight in the sporulated cell. Cry proteins report a high and specific toxicity to a wide range of insect species, including different insect orders, such as Lepidoptera, Coleoptera, Diptera, Hemiptera, Hymenoptera, Orthoptera, Mallophaga, (Schnepf et al., 1998; Van Frankenhuyzen, 2009) but also nematodes, mites and protozoa (Iatsenko et al., 2014). However, there are many Bt strains obtained from diverse environments which have no known invertebrate target (Abulreesh et al., 2012) and are referred to as parasporins for exhibiting strong and cytocidal activity against human cancer cell lines of various origins (Okassov et al., 2015). This parasporin group of Cry proteins have both, Cry and Parasporin designations: parasporin-1 (PS1 or Cry31), parasporin-2 (PS2 or Cry46), parasporin-3 (PS3 or Cry41), parasporin-4 (PS4 or Cry45), parasporin-5 (PS5 or Cry64) and parasporin-6 (PS6 or Cry63), respectively (Ohba et al., 2009). Although the toxicity effect of Bt strains resides in the inclusion crystal proteins, as these have proved effective against arthropods when tested alone, it is believed that the spore may act as a synergistic factor enhancing the toxicity effect (Burges et al., 1976). This has been confirmed by irradiating the spores in order to avoid germination, which resulted in a reduction of the synergistic effect as expected (Asano et al., 2002). Also spore-coat proteins have shown to be contributors for this matter (Du and Nickerson, 1996). Nevertheless, it does not seem to be an universal fact, as it depends on the strain, the spore, the toxin and the proportion of them in the mixture (Liu et al., 1998).

3.3.2. Cyt proteins

Cytolytic (Cyt) proteins are parasporal inclusion toxins also referred to as δ-endotoxins. The characteristic features of these toxins are their cytolytic activity *in*

vitro, against a range of cultured cells, and their haemolytic effect in erythrocytes (de Maagd et al., 2003; Thomas and Ellar, 1983). According to the Bt Toxin Nomenclature Committee (Crickmore et al., 2018), putative proteins with a high sequence similarity with other previously described Cyt toxins are also defined as cytolytic protein. No sequence similarities are found with Cry or Vip toxins. Until date, three *cyt* gene families have been described, including a total of 11 holotypes according to their toxin nomenclature (Soberón et al., 2013). These were first detected as low molecular proteins specific to Bt subsp. *israelensis* (Tyrell et al., 1981) and showed to be soluble in alkaline buffers in absence of reducing agents, unlike the well-known lepidopteran-specific toxic proteins (Thomas and Ellar, 1983). In contrast to Cry proteins, Cyt proteins are mostly found in Bt strains active against dipteran species, being able to kill mosquitoes and blackflies (de Maagd et al., 2003) although activity against some coleopteran insects has also been reported (Guerchicoff et al., 1997; Van Frankenhuyzen, 2009). An interesting feature about Cyt toxins is their ability to synergize the activity of Cry or Vip proteins reducing the resistance levels in some pest species (Becker, 2000; Guerchicoff et al., 1997).

3.4. Structure and function of delta-endotoxins

The δ-endotoxins belong to a class of bacterial toxins known as the pore-forming toxins (PFT). Generally, PFT-producing bacteria secrete their toxins which interact with specific receptors located in the membrane brush border. There are two main groups of PFT: (i) the α-helical toxins, in which α-helix regions form the trans-membrane pore (3-domain Cry proteins), and (ii) the β-barrel toxins, that insert into the membrane by forming a β-barrel composed of β-sheet hairpins from each monomer (Cyt proteins) (Parker and Feil, 2005). The crystal protein family should be divided into four distinct homology groups which are not phylogenetically related, a) the 3-Domain Cry family, b) the MTX-like toxins, related to *B. sphaericus,* c) the binary-like toxins, and d) the parasporins.

3.4.1. The three-domain proteins

The three-domain Cry toxins are globular molecules containing three differentiated domains connected by single linkers. The three-dimensional structure of Cry3Aa was the first to be resolved using X-ray crystallography (Li et al., 1991).

Since then, the structure of different Cry proteins, such as Cry1Aa (Grochulski et al., 1995), Cry2Aa (Morse et al., 2001), Cry3Bb1 (Galitsky et al., 2001), Cry4Aa (Boonserm et al., 2006), Cry4Ba (Boonserm et al., 2005) and Cry8Ea1 (Guo et al., 2009) has been determined and provided considerable insight into the mechanism of toxin function, elucidating differences in toxin specificity (Figure 3). Multiple alignments of the aminoacidic sequences of several Cry proteins have revealed a low sequence similarity among them, with over 50 different subgroups with more than 200 members. This high diversity of toxins is responsible for the wide insect range specificities, although a high similarity in the overall topology structure is observed (de Maagd et al., 2003). Despite this low sequence identity, five conserved blocks of amino acidic residues (1 – 5) located in the N-terminal of protoxins, are defined among diverse Cry toxins (Höfte and Whiteley, 1989; Schnepf et al., 1998). A particular feature concerning the three-domain Cry protoxins is their production during sporulation of the bacteria as protoxins of two different lengths: either 130-140 kDa or approximately 70 kDa. The main difference between these is that in large protoxins the C-terminal cleaved by proteases in the insect midgut that remove about 500 to 600 amino acid residues and about 20-25 at the N-terminal. Nevertheless, short protoxins are only processed at the N-terminal. This suggests that C-terminal extension found in 130 kDa protoxins is dispensable for toxicity. In both cases the resulting activated toxin constitutes a protease resistant core of 60 kDa that presents the three mentioned domains (de Maagd et al., 2001). The mentioned five conserved blocks are located along the toxic core sequence (Höfte and Whiteley, 1989), while three additional blocks (6 – 8) are distributed in the C-terminal halves of large protoxins, where an extra domain, not found in smaller proteins, is found.

Domain I (the N-terminal domain) was first described in Cry3Aa (Li et al., 1991). It comprises a bundle of seven α-helices, in which six of them (amphipathic) surround a central helix (α5) which is hydrophobic. Its structural similarity to the pore-forming domain of colicin (Parker et al., 1989), along with its proteolytic cleavage during toxin activation, led to the hypothesis that Domain I may be responsible for membrane insertion and pore formation in Cry proteins (Li et al., 1991; Xu et al., 2014). Domain I contain the conserved blocks 1 and 2 (the C-terminal half of helix α6 and all of α7) (Figure 3). Site-directed mutagenesis

experiments showed alterations in toxicity when amino acidic residues were altered, but interactions of the toxin with the brush border were not disturbed (Ahmad, 2003; Wu and Aronson, 1992). Nevertheless, substitutions or mutations of Domain I targeting block 2 suggested its importance in crystallization and stability (Park and Federici, 2004).

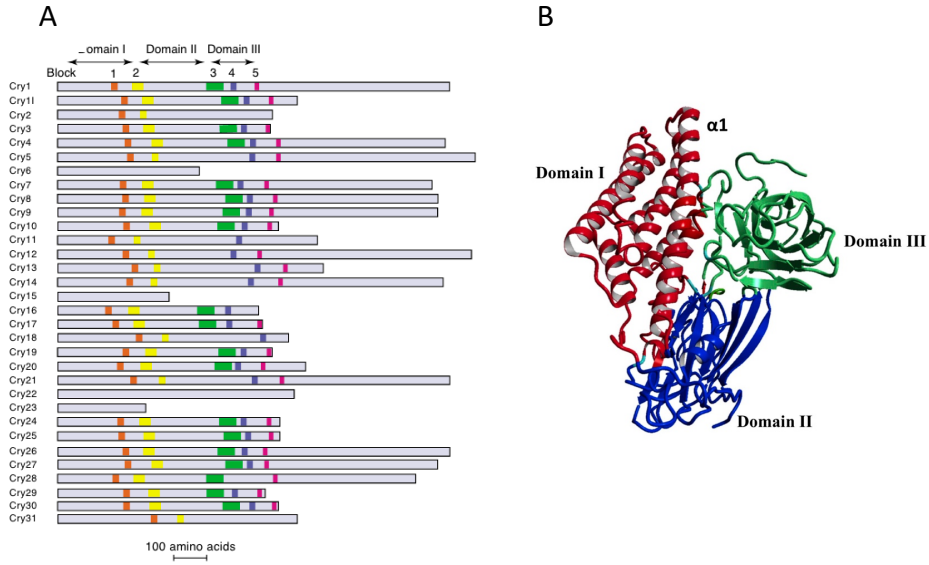

Figure 3. Primary and tertiary structure of Cry toxins. **A**. Position of the conserved five blocks, if present, among different Cry protoxins. The three domains are marked for protoxin Cry1. Domain I contains the conserved block 1 and part of block 2. Domain II contains the rest of the conserved block 2 and part of block 3. Domain III contains the rest of the conserved blocks 3, 4 and 5. The rest of the protoxins follow a similar structure with an N-terminal (20-40 amino acidic residues) before the conserved block 1 and a C-terminal followed by block 2 in long protoxins, which is digested when activated in the midgut of the insect. **B**. Three-dimensional structure of a Cry1A protein. Three domains can be differentiated: domain I (red) is involved in membrane insertion and pore formation; domains II and III (blue and green, respectively) are involved in specific recognition and receptor binding. (de Maagd *et al.,* 2001).

Domain II consists of three anti-parallel β-sheets, forming a β-prism, with exposed loop regions. It is the most variable of the toxin domains and plays an important role in toxin-receptor interactions which is determinant for host specificity (Boonserm et al., 2006; Li et al., 1991). Domain II contains part of the conserved blocks 2 (the first β-strand) and 3 (the last β-strand). Mutations in loops of this domain have revealed their role in specific interaction with host receptors, suggesting that toxins with high sequence similarity in this region may share some

binding sites in the epithelial cells of the midgut (Hernández and Ferré, 2005; Jurat-Fuentes and Adang, 2001). Domain III is a two antiparallel β-sheet sandwich, each of them composed by five strands (Li et al., 1991). It is proteolytically cleaved in some three-domain Cry proteins and is also involved in receptor binding, pore formation and host range definition, although it shows less structural variability than Domain II (Schnepf et al., 1998). Domain III contains the conserved blocks 3 (the N-terminal segment of the first β-strand), 4 (the second β-strand) and 5, located at the C-terminus of the domain (Jurat-Fuentes and Jackson, 2012). Mutations in block 4 in Cry1Aa revealed alterations in toxicity by forming truncated channels in brush border membrane vesicles (Chen et al., 1993; Wolfersberger et al., 1996), while block 5 showed to be involved in toxin stability in Cry4A, providing higher protection against protease digestion (Nishimoto et al., 1994).

3.4.2. Cyt proteins

Cyt proteins have a single α-β domain that comprises two outer layers of α-helix hairpins wrapped around a β-sheet. The three-dimensional structures of three Cyt proteins (Cyt1Aa, Cyt2Aa and Cyt2Ba) have been solved by crystallography showing similar topology. Although the mechanism of action of Cry and Cyt families is thought to be the same, no similarities are shared between them (Li et al., 1996, 1991). Both toxins produce a 1-2 mm pore in the membrane of epithelial insect cells that lead to an osmotic decompensation (Knowles and Ellar, 1987). After solubilization, the proteolytic activation of Cyt protoxins is required for toxicity. Insect gut enzymes cleave the C and N-terminal of the protoxin (27 kDa) and the active toxin (24 kDa) (Thomas and Ellar, 1983) is released as a monomer that interacts directly with membrane lipids forming a pore (Promdonkoy and Ellar, 2005, 2000) or by a detergent-like interaction (Butko, 2003).

3.5. Models of action of Cry proteins in lepidopteran species

Bt insecticidal proteins exhibit insecticidal activity against a wide range of different insect orders like Lepidoptera, Coleoptera, Diptera, Hemiptera and also other non-insect organisms such as mites (Van Frankenhuyzen, 2013) and nematodes (Wei et al., 2003). The mode of action of Cry proteins has been deeply studied during the last decade mainly focused on lepidopteran species. The

knowledge of how these insecticidal toxins interact with the host producing a lethal effect is of crucial interest for pest control and avoidance of resistance appearance. Crystal proteins sharing molecular elements in their mode of action may promote the premature selection of resistant biotypes in the insect population. Three different models for the mode of action of crystal proteins have been proposed.

3.5.1. The classical model

The “classical” model mode of action (Bravo et al., 2007) proposes that when the inert crystal proteins are ingested by a susceptible host the alkaline conditions of the insect midgut promotes its solubilization, releasing them in their protoxin form. These are then proteolytically converted to smaller protease-resistant toxic polypeptides, either by host proteases, spontaneous proteolysis or by the intervention of proteases associated to the spore (Cannon, 1996), yielding the active toxin. Solubilization and protease activation are two interactive processes, where the first one promotes a structural change in the insecticidal molecule facilitating the access of proteases to the target residues. Regardless of the size of the protoxin (130kDa for Cry1 and 70 kDa for Cry2 and Cry3 proteins) the active crystal proteins usually present a size of 60 kDa, so in some cases, these enzymes remove about half of the crystallized toxin in a process believed to activate it (Bravo et al., 2002). The activated toxin subsequently binds to specific receptors located on the brush border membrane of the midgut epithelium columnar cells before inserting into the membrane. Toxin subunits oligomerize to form pore structures in the microvilli of apical cell membranes allowing permeability to inorganic ions, amino acids and sugars (Carroll and Ellar, 1993; Kirouac et al., 2002). Such pores cause ion leakage that lead to cell lysis due to massive influx of solutes from the midgut lumen (Knowles and Ellar, 1987) and midgut disarrangements ending in insect death (Figure 4).

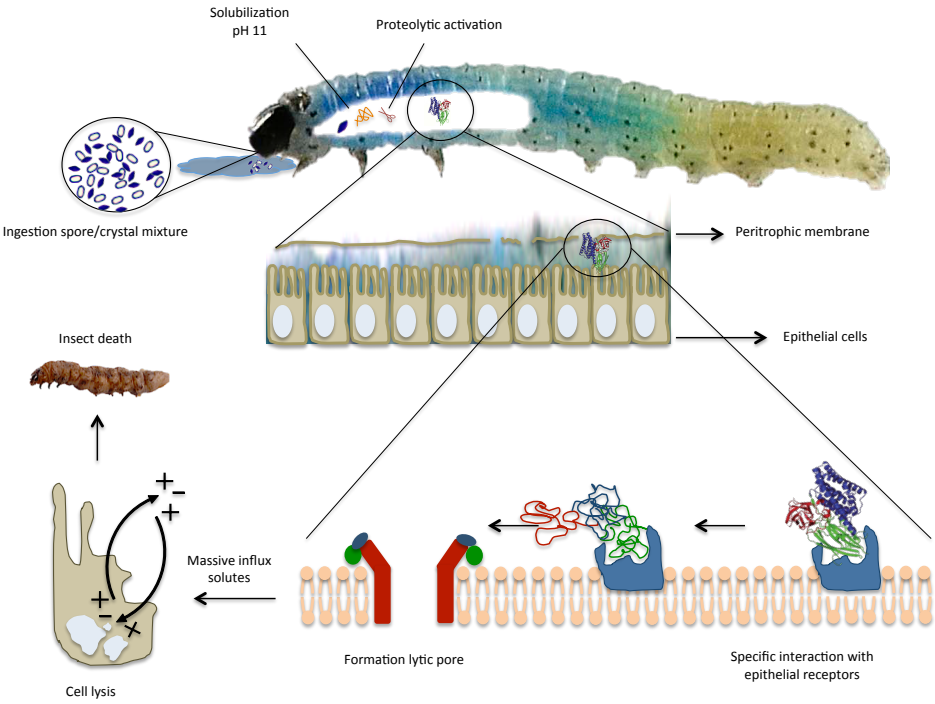

Figure 4. Mode of action of Cry toxins. After ingestion the parasporal crystal is dissolved in the insect gut due to alkaline conditions. A proteolytic activation of the protoxin clips off the C-terminal of long proteins (130 kDa) and a small fraction of the N-terminal. The toxin core binds to specific receptor located in the brush border of epithelial cells where domain II and III are involved. Finally, domain I forms a lytic pore that cause ion disarrangements resulting in cell lysis and insect death.

However, there are some details including the assembly of the toxins and the structure of the pore that still need to be clarified. In contrast, some advances have been made in the toxin-receptor recognition process and resistance to toxins (Gómez et al., 2007; Likitvivatanavong et al., 2011). In some occasions spores may colonize the hemocoel where they may germinate, replicate and cause a septicaemia killing the insect (Höfte and Whiteley, 1989; Schnepf et al., 1998). These mentioned conditions are responsible for the narrow spectrum of the microorganism and its safety for mammals.

3.5.2. The sequential binding model

The sequential binding model attempts to explain the pore formation event as a consequence of a multiple receptor contribution (Bravo et al., 2004; Gómez et al., 2002; Pacheco et al., 2009). The solubilization and proteolytic activation of the toxin would follow the same steps as the "classical" model. However, the first interaction takes place between the activated toxin and two specific receptors located on the luminal membrane of midgut epithelial cells. The toxin binds to a cadherin-like protein (receptor 1) that promotes a conformational change in the insecticidal molecule that favours a proteolytic cleavage of the N-terminal of the pore-forming domain of the toxin. This leads to removal of helix α1 and oligomerization of the toxic molecule that will form a pre-pore structure (Gómez et al., 2002). The oligomer then binds to a glycosyl-phosphati-dylinositol (GPI)-anchored aminopeptidase N (receptor 2), showing a greater affinity to it than the monomeric form of the toxin (Bravo et al., 2004). Finally, insertion into the membrane results in the formation of a pore structure that leads to a major permeability of the epithelial cells. This model suggest that alteration in the expression of either of the two mentioned receptors, cadherins or aminopeptidase N (Gahan et al., 2010; Morin et al., 2003; Xu et al., 2005; Zhang et al., 2009), can cause resistance events to Cry proteins, without undergoing any structural modification.

3.5.3. The signalling pathway model

The signalling pathway model of Bt mode of action, however, discards the importance of pore formation in toxicity as a cause but as a consequence (Zhang et al., 2006, 2005). This model is partially based on the observation that insect cell cultures expressing the BT-R_1 cadherin are sensible to Cry1Ab binding that activates a magnesium-dependent signalling pathway associated with cell death.

4. BIOASSAYS OF *B. thuringiensis* AGAINST LEPIDOPTERAN PESTS

A bioassay is a technique used to measure the amount of a substance, such as a toxin, needed to cause an effect on a living organism (Robertson et al., 2017). Bioassays are performed for several purposes, such as assessment of the activity of formulated and non-formulated pathogens, to serve as quality control of commercial products or to determine the mode of action of the active ingredients. The experimental design depends on some key parameters including the active ingredient, food source, treatment supply, host range, speed of kill and activity against different larval instars. In order to estimate the pathogenicity of the active ingredient of interest a range of 5-6 concentrations are usually applied to precisely estimate the amount of toxin required to cause mortality in larval insects. Single discriminating concentrations of the active ingredient are recommended in order to fix the appropriate range of dilutions (Navon, 2000).

4.1. Artificial diet

The purpose to use an artificial diet is to accurately evaluate the activity of, for example, a mixture of spores and crystals in absence of the chemical and physical barriers that are present in a host plant. A large number of insects, preferably reared with low cost and high performance (Zou et al., 2015), are employed for laboratory toxicity studies. Diet-based assays overcome the need to maintain a constant supply of plants in the greenhouse or in the field. In order to determine if an artificial diet is appropriate for the production of insects in the laboratory, it should supply the insect all nutrients needed, be innocuous to use and easy to prepare, have a long term shelf storage, and at least a total insect viability above 75% should be reached (Singh, 1983). A first diet was proposed to determine the activity of the French E-61 standard Bt subsp. *thuringiensis* against *Trichoplusia ni* or *Ephestia küehniella* (Dulmage et al., 1971). However, the increasing interest to characterize new Bt isolates against the wider possible insect range required the development of new bioassay diets specific to each pest. Thus, some authors proposed standardized diet recipes for rearing a wider range of lepidopteran insect species (Han et al., 2012; Navon et al., 1990; Singh, 1983).

4.2. Natural food

The main difference with dietary bioassays is the presence of chemical and physical barriers that provide the crop a particular defence. The plant generate an alkaline environment on the leaf surface due to the exudation of magnesium and potassium cations (Ellman and Entwistle, 1982; Navon et al., 1988) that may negatively affect the activity of δ-endotoxins if the pH rises above 10 (Behle et al., 1997). This feature opens the opportunity to test different adjuvants, that can be included in formulation recipes, in order to determine their effect on the potency of Bt and the insect feeding behaviour. Cotton is one of the most common hosts used as natural food, as several lepidopteran species can feed on it. Plants also grow fast, so within a few weeks the size and the number of leaves may be appropriate for either leaf or plotted-plant experiments.

4.3. Toxin supply

4.3.1. Superficial contamination of diet

Knowledge about the feeding behaviour of the target insect is crucial in order to determine the most suitable way to supply the toxin. For example, *Spodoptera exigua, Helicoverpa armigera* or *Mamestra brassicae*, feed by grazing across the surface, so superficial contamination of the synthetic diet seems to be the most suitable option. An important factor to take into account is that the active ingredient used must be stable in the diet, as many recipes include antimicrobial agents that could affect the activity of bacteria. This is the reason why a conscientious screening to determine the role of diet ingredients in the activity of *B. thuringiensis* is mandatory. In order to conduct this type of bioassay a known volume of the aqueous toxin solution is added to cover the semi-synthetic diet, contained in plastic cups, followed by agitation to ensure even coverage. The dosage is thus expressed in ng/cm^2. Larvae are then added individually one per well (if gregarious, mass feeding can be employed) and fed on the diet for a certain period of time. This method can be used either to calculate the median lethal dose (LD_{50}) or the median lethal concentration (LC_{50}), depending on whether the amount of active ingredient ingested by the treated larva is known or not. In the first case the entire amount of diet, which must be ascertained by trial and error, is consumed in a known short

time. In the second case a known concentration of the treatment is applied on the surface of the diet, but it is not entirely consumed (Evans and Shapiro, 1997).

4.3.2. Leaf contamination

Some insects cannot be reared on artificial diets so natural food plant should be used instead. Leaf discs, submerged in a treatment aqueous suspension, can be also used as source of food for the insect during the experiment. Individuals will ingest the toxin with the plant material, which will determine mortality. If the target insect shows cannibalistic tendencies, it is advisable to offer a leaf disk to individually confined larvae, however, an alternative using the whole leaf is available for lepidopteran species that can be grouped together without eating each other. For leaf disc bioassays a standardized size is used, and discs are submerged in an aqueous concentration of toxin to be evaluated (Iriarte et al., 1998). A wetting agent (0.1% concentration), is generally used to avoid the formation of drops by reducing surface tension and to improve the correct and homogeneous impregnation of the surface. These are then dried at room temperature and transferred to individual wells of 2 cm^2 containing a layer of 1.5-3 % agar (w/v) to maintain moisture of the leaf tissue. Larvae are individualized in wells by means of a camel-hair brush, and boxes are maintained at controlled conditions during the appropriate period of time before mortality is registered. For whole leaf bioassays a similar procedure is followed. The serial aqueous solutions (0.1% wetter) are poured over the leaf tissue and allowed to dry. The petiole is cut at 2 cm distance from the leaf and inserted into the agar layer. Then a certain number of neonate larvae are carefully placed on the leaf and mortality is registered after 3 days (Navon and Ascher, 2000).

4.3.3. Plotted-plant and field bioassays

Whole plants can be used to conduct a toxicity assay by caging first, second or third instars on leaves of potted plants. Several factors, including percentage of mortality, leaf consumption and feeding inhibition under greenhouse conditions (Navon et al., 1987) can be evaluated. Important advantages over leaf bioassays can be highlighted, such as; testing the activity of *B. thuringiensis* on intact plants, spraying or dusting the plants with the biological product with a higher concentration accuracy than in the field, extending the bioassay period as long as

the plant is consumed by the pest larvae and assaying the possible residual effect of the Bt-based product. Cages consist of a plastic cylinder, that surrounds the whole plant, closed by a plastic or metal mesh. Hatching eggs are placed in the plant and neonate larvae are allowed to stablish along the leaves. Then the corresponding treatment is sprayed over the plant and is allowed to dry. The cage is closed until mortality registration. These bioassays are usually handled as a previous step to field bioassays, which are conducted under natural and environmental conditions. A natural or artificial infestation can be used in order to stablish a population in the crop and a certain spraying volume is determined per hectare. Usually, mortality in neonate larvae is high due to natural entomopathogenic microorganisms or natural enemies. For this reason, second and third instar larvae are often evaluated. Portable meteorological stations can be useful to measure temperature and humidity conditions during the bioassay period (McGuire et al., 1997; Navon and Ascher, 2000).

4.3.4. Diet incorporation bioassays

The choice of diet incorporation relates to the behavior of the test insect. There are some lepidopteran species, such as *Cydia pomonella*, that bore into the diet spending very little time on the surface, reducing the chances to ingest the desired amount of toxin (Stará and Kocourek, 2007). In these cases, the incorporation of the toxin directly into the diet can be a better approach, where accuracy increases as the active ingredient is more evenly distributed through the food. To perform this bioassay, plastic, uniform and clean containers able to hold larvae individually, are preferred. Spore and crystal mixtures or the crystal suspensions should be prepared and diluted in sterile distilled water (supplied with 2.5ml of a 1% Tween 80 solution). Before adding the treatment to the diet, it should be cooled down enough (45°C) to avoid thermal inactivation of the proteins, but still be liquid to be poured into the containers. Larvae must be then carefully transferred individually to cups, which may be checked after 4-7 days for mortality record. This bioassay method is most conveniently employed to estimate the LC_{50} in lepidopteran species with gregarious feeding habits. Nevertheless, LD_{50} studies can be performed if diet plugs of known volume are removed and let to be entirely

consumed by individual larvae (Evans and Shapiro, 1997; Martignoni and Ignoffo, 1980).

4.3.5. Droplet feeding method

The droplet feeding method (Hughes et al., 1986; Hughes and Wood, 1981) turns to be a simple and fast assay with high precision and accuracy results that can be conducted with lepidopteran species that drink liquids on surfaces, especially after a short period of starvation. The most characteristic feature of this bioassay method is that treatments are prepared containing 5% sucrose (w/v) and 20% of food colouring Fluorella Blue (Figure 5).

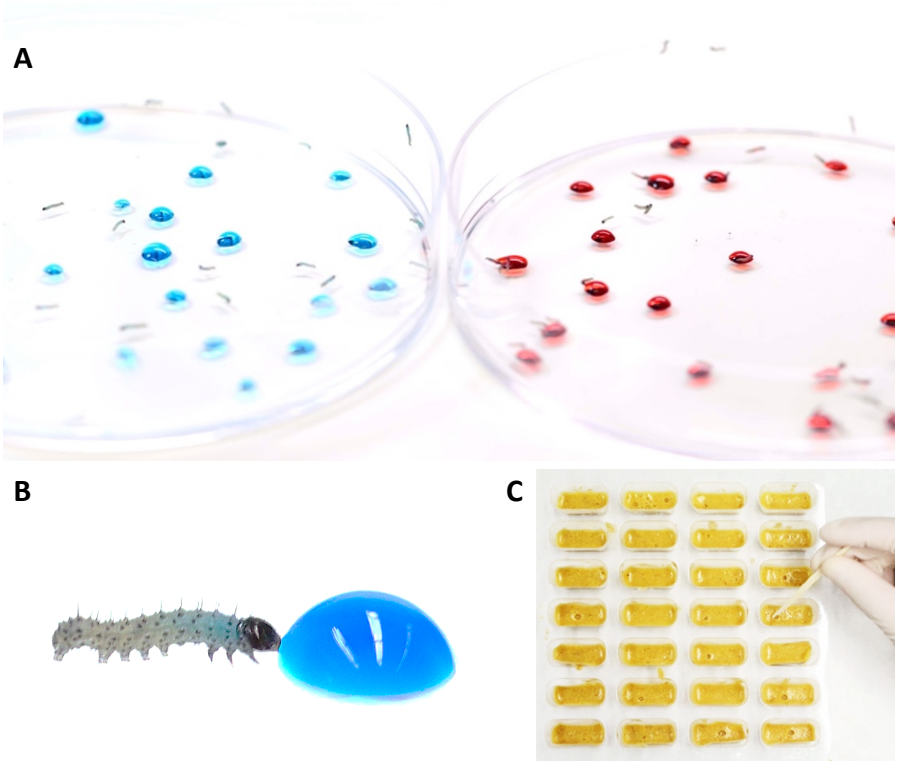

Figure 5. Droplet feeding method bioassay. **A.** *S. frugiperda* second instar larvae feeding on drops containing a known concentration of Bt toxin. **B.** Detailed *S. frugiperda* larvae staining blue while ingesting a colored protein suspension with Fluorella Blue. **C.** Bioassay tray containing artificial diet where larvae are placed after the treatment.

Newly moulted starved larvae are carefully placed on a surface, by using a camel-hair brush, and numerous tiny droplets (0.2-0.8 µl) of the aqueous suspensions are supplied using a pipette. If larvae are trapped in the droplets due to the surface tension of the water, a perforated parafilm could be used to feed them. Larvae immediately move to the droplets and feed within the first 10 min, dispersing then to the edges and not returning to imbibe a second time. Larvae that have ingested the sufficient dose, according to the colorant appearance of their tegument, are individually transferred to containers supplied with synthetic diet (Figure 5, C). Assays are conducted under controlled conditions until mortality is registered (Evans and Shapiro, 1997).

4.4. Types of bioassays

4.4.1. Concentration-mortality response

The goal of the bioassay is to determine either the mean lethal concentration (LC_{50}), concentration required to kill 50% of the target pest, or the mean lethal dose (LD_{50}), dose required to kill 50% of insects. LC_{50} is a standard measure to express virulence. The standard approach is to use five or more serial dilutions of the microbe of interest and register the mortality after a single period of time, depending on the larval instar. Establishing concentrations above and below the LC_{50} is considered a good practice. However, it is recommended to conduct a preliminary assay using two concentrations, one high and one low, to ensure a correct range of dilutions. For neonate larvae the stablished period for counting the biological effect is limited to 2-4 days, while this range comes up to 7 or 8 days for third instars. In order to determine the potency of an active ingredient, the mortality is referenced against the international standard, which is expressed in international units per mg (IU mg^{-1}) (Dulmage et al., 1971; Navon and Ascher, 2000).

4.4.2. Mortality-response

This type of bioassay is reserved to determine the mean lethal time (LT_{50}), which is defined as the period needed by the entomopathogenic agent to kill 50% of the insect population. It is suitable for second and third instar larvae, as neonate larvae are too sensible to the treatments and mortality occurs too quickly to obtain

time-mortality slopes. Hatching individuals are not able to survive without feeding, whereas third instars may starve for several days owing to the fat body, which is used as an energy reserve. Mortality should be counted every 6 to 8 hours on successive days allowing estimation of the LT_{50} with good slopes (Navon and Ascher, 2000). Alternatively, the mean time to death (MTD) may be calculated.

4.4.3. Feeding inhibition assays

Bioassays conducted with this goal estimate the concentration of the active ingredient needed to cause a 50% reduction in larval weigh of the treated individuals compared to the control larvae. The feeding inhibition assays are generally conducted with second and third instars (Navon and Ascher, 2000).

5. FORMULATION

As well as chemical products, microbial pesticides need to be formulated before application in the field. Formulation is the process by which the technical product that contains the active ingredient (e.g. mixture of spores and crystals in Bt-based products) is converted into the final product by the addition of adjuvants aimed at increasing the effectivity. Formulation must be achieved in a cost-effective manner to have a competitive price in the market. Commercial formulations are mainly liquid (aqueous liquid suspensions), dry solid (wettable powders, water dispersible granules, dusts or pellets) or oil emulsifiable suspensions. (Angus and Lüthy, 1971; Charles et al., 2000; Frey, 2001).

Bt products generally contain spores, crystals, vegetative insecticidal proteins, enzymes (such as chitinases, proteases and phospholipases), unknown virulent factors and inert materials. Ingredients used in formulation must be approved by the appropriate regulatory authorities that recognize them as safe. Addition of formulation additives to improve performance in plant coverage and field stability have been of major concern to optimize the effectiveness of microbial insecticides.

5.1. Additives that counter foliage factors

As mentioned previously (see section 4.2), the alkaline environment found in the surface of some plants, may alter the efficacy of Bt toxin as pH values as high

as 11 dissolve the crystal toxins. Protection agents must be used to retain the structure of insecticidal proteins, and thus, maintain their toxicity at extreme alkalinity conditions (Behle et al., 1997). The presence of allelochemicals (e.g. tannins and nicotine), produced by plants as part of their natural defence against herbivores, inactivate the toxins depressing the activity of Bt. They are also responsible for a decreasing effect in the feeding of insects, which is directly linked to a decrease in the amount of ingested toxin (Morris et al., 1995; Navon, 1993, 1992). Encapsulation of spores and crystals in an insoluble polymer (McGuire et al., 1996) or formulation in oil may overcome extreme pH conditions in the surface of the phylloplane. This negative effect due to antibacterial or allelochemical substances may be partially counteracted by the use of other formulation ingredients such as stickers or phagostimulants.

5.2. Wetters

Wetters are adjuvants that facilitate plant coverage by reducing the interfacial tension and surface tension of water (Burges and Jones, 1998). Mainly the concentration at which wetters should be applied vary from 0.01 to 0.5% in tank mixes. The most preferable components are Tweens, Tritons and non-ionic wetters. Tween 80 and Triton X-100 have been successfully used in spray contaminations. Surfactants do not seem to harm microbials when exposed during short periods of time, but they may cause deterioration when included in formulation and stored. In practice, wetters are best incorporated in the tank mixer before application in quantities that assure good dispersion and cover (Burges and Jones, 1998).

5.3. Stickers

Several authors have reported spores to last between 0.5 to 3 days depending on the addition of adjuvants (Ignoffo et al., 1974). In the field meteorological factors such as rain, must be taken into account, as the product can be rapidly removed from the target zone. The use of sticker (0.1-2%) improve the adherence of sprayed products to foliage and enhance the potency of the product, as it remains in the target zone during a larger period of time for its potential ingestion (Burges and Jones, 1998).

5.4. Phagostimulants

Bt-contaminated surfaces usually repel insects from feeding, unlike the untreated control, hindering the appropriate intake of a lethal dose (Glare and O'Callaghan, 2000). Phagostimulants are mainly added as adjuvants in commercial formulations (Burges and Jones, 1998) for the insect to ingest a lethal amount of toxin before it loses its insecticidal activity due to adverse environmental factors. Phagostimulants are mainly derives from host plants of target pests and they have not shown evidence of incompatibility with entomopathogenic agents. Feeding stimulants such as molasses or sugar, are used to facilitate larval intake of spores and crystals (Burges and Jones, 1998).

5.5. Sunscreens

Sunlight is the most destructive environmental factor for microbial insecticides (Ignoffo et al., 1977; Pozsgay et al., 1987). Potency of Bt is drastically reduced after UV light exposure and negatively contributes to its persistence on the phylloplane. Sunscreens absorb, block or reflect the sunlight, converting damaging UV light to harmless wavelengths (Burges and Jones, 1998).

5.6. Synergists

No matter the mode of action, a synergist is a substance that increases somehow the mortality produced by solely the active ingredient. Many of the additives used as synergists are inexpensive (Morris et al., 1995), however they may have a huge economic impact due to their performance (Salama et al., 1986). Combination of two or more synergists may also have a synergistic effect. Concentration of these substances may vary from 0.01 to 0.5% (w/w) (Burges and Jones, 1998).

6. COMMERCIAL PRODUCTS BASED ON *Bacillus thuringiensis*

6.1. Bt-based insecticides

Back in the 1920s, *B. thuringiensis* was already used by farmers for pest control in Hungary, and also in Yugoslavia at the beginning of the 1930s, as synthetic chemicals were not yet developed (Husz, 1928; Vouk and Klas, 1931).

The first commercial product was manufactured in France in 1938 by Laboratoire Libec (Aronson et al., 1986), under the name Sporeine® (Lambert and Peferoen, 1992), for the control of flour moths, but also other lepidopteran species (Milner, 1994). Thuricide®, based on Bt subsp. *thuringiensis,* was launched to the market in 1958, setting the starting point of Bt-based products commercialization in the United States. In 1961 Bt was first registered by the US Environmental Protection Agency (EPA) (Starnes et al., 1993). Ever since, efforts in the identification of strains with greater potency, whether due to the presence of novel insecticidal genes, new insecticidal gene combinations or higher gene expression levels, have been intensified for controlling different ranges of pests (Kuo and Chak, 1996; Porcar and Juárez-Pérez, 2003).

Bt-based products can be classified according to their active ingredient (AI). First generation products are those which AI is comprised by a mixture of spores and crystals of a natural strain (Kaur, 2000) (Figure 6). This generation products are fairly represented by several Bt-based preparations. In the 1970s Dulmage isolated the Bt HDI strain from *Pectinophora gossypiella* (Saunders) larvae (Dulmage, 1970), which resulted to be a new subspecies of Bt according to the flagellar serotyping, and so named *kurstaki* (de Barjac and Lemille, 1970). This new strain expressed four proteins, Cry1Aa, Cry1Ab, Cry1Ac (130-40 kDa) and Cry2Ab (65-70kDa), responsible for its host range, covering more than 167 species, including some of the most important species of noctuids (Lepidoptera; Noctuidae) such as *Trichoplusia ni, Helicoverpa zea, Helicoverpa armigera* among others (Glare and O'Callaghan, 2000; Hall et al., 1977; Sharpe and Baker, 1979; Yamamoto and McLaughlin, 1981). A significant activity against mosquito larvae has also been reported (Hall et al., 1977). Several commercial products based on Bt subsp. *kurstaki* have entered the market under different trademarks, including DiPel®, Bactospeine® and Biobit®(Abbot Laboratories) or Thuricide®(Thermo Trilogy) (Kaur, 2000; Starnes et al., 1993). In 1962 Keio Aizawa of Kyushu University in

Japan discovered a new isolate that belonged to a new subspecies, which was named after the researcher (Aizawa and Lida, 1963).

Figure 6. Commercial products based on a mixture of spores and crystals from natural Bt strains. The most popular products for the control of lepidopteran species are DiPel® and XenTari®, VectoBac® for dipteran species and Novodor® for coleopteran species.

Susbspecies *aizawai* reported to be very effective against *Galleria melonella* and several species of the genus *Spodoptera*, including *S. exempta*, *S. exigua*, and *S. littoralis* (Basedow et al., 2008; Van Frankenhuyzen, 2009). This effectivity was attributed to the presence of a plasmid in the Bt strain (Zakharyan et al., 1976) carrying genes responsible for the expression of Cry1Aa, Cry1Ab, Cry1C and Cry1D proteins (130-140 kDa) (Gonçalves et al., 2018). Different formulations based on Bt subsp. *aizawai* are commercialized, such as XenTari® or Florbac® (Abbott Laboratories) (Kaur, 2000). The discovery of Bt subsp. *israelensis* strain H-14 (Israel 1976) showed to be effective against dipteran larvae including mosquitoes and blackflies (Das and Dominic Amalraj, 1997; Goldberg and Margalit, 1977). The proteins responsible for the insecticidal activity are the Cry proteins, Cry4Aa, Cry4Ba, Cry10Aa and Cry11Aa, and the two cytolytic proteins Cyt1Aa and Cyt2Ba which are located in a mega plasmid called pBtoxis (Berry et al., 2002). Formulations

based on these Bt strains have been applied to water courses and stagnant pools in order to control the human disease vector such as malaria, yellow fever or dengue, including *Aedes aegypti ,Culex species* and *Anopheles gambiae* (Becker, 2000; Margalith and Ben-Dov, 2000; Pardo-López et al., 2013; Poncet et al., 1997). Some of these preparations are commercialized under different trademarks, such as VectoBac®, Skeetal® and Bactimos® (Abbott Laboratories).

Bt subsp. *tenebrionis* was isolated in 1983 and showed activity against coleopteran species (Krieg et al., 1983) including the Colorado potato beetle (CPB), *L. decemlineata*. This strain produces a flat and rectangular parasporal crystal with a major component of 65-70 kDa that corresponds with the Cry3 protein (Sekar et al., 1987). Some formulations have been prepared for producers to protect their crops, such as Novodor® (Abbott Laboratories).

Second generation products are those which AI is comprised by a Bt strain that has been modified by conjugation or transformation in order to widen the host range or delay resistance events. A few Bt-based products are commercialized worldwide, including Foil® which express Cry1Ac and the imported Cry3A, Crymax® which express Cry1Ac, Cry2A and the imported Cry1C, or Turex® a Bt subsp. *aizawai* which has been engineered with Cry1Ac form Bt subsp. *kurstaki* (Baum et al., 1999; Carlton and Gawron-Burke, 1993). Third generation products are designed to overcome limitations in open field by encapsulation of Bt strains into dead bacteria of *Pseudomonas fluorescens* (MVP®, Mycogen) (Stone et al., 1989).

6.2. Insecticidal potency of a Bt-based product

Since the appearance of the first Bt-based products, methods for comparing them among different countries were developed. Before 1970s the standardization procedure was through spore count. However, there was no relationship between number of spores and activity of the strain, as toxicity varied with crystal count as noted by previous authors (Angus, 1954; Beegle et al., 1991). The need for standardization increased as it served as a guarantee of the product for consumers. (Martouret, 1975).

This led to the determination of certain Bt strains as “standards” to which compare the toxicity of tested strains. The first standardized methodology to assess the potency of Bt formulated products involved the use of an “International Unit” (IU)

of potency (Dulmage, 1981). In order to calculate the potency of an isolate, a reference strain, which has been assigned a random value of IU/mg, and a reference susceptible insect, are required. In France, the strain E-61 was selected as reference standard for the commercial product Bactospeine® and assigned a potency of 1,000 IU/mg against *E. küehniella* larvae (Burges, 1967). However, in the United States Dulmage proposed HD-1-S1971 as standard strain which was assigned a potency of 18,000 IU/mg against *Trichoplusia ni* on the basis of assays compared to E-61 (Dulmage, 1981; Dulmage et al., 1971). The HD-968-1983 strain was selected as standard for Bt subsp. *israelensis* H-14 and assigned a potency of 4,740 IU/mg against *Aedes aegypti* larvae (Dulmage et al., 1985). Potency is obtained by comparing the estimated LC_{50} of a tested strain or formulation to that of a standard material and expressed in IU/mg (Dulmage, 1981). Potency is calculated as follows:

$$\frac{LC_{50} \text{ of a standard}}{LC_{50} \text{ of a sample}} \text{ x potency of the standard = Potency of the sample (IU/mg)}$$

Although standard protocols have been suggested (McLaughlin et al., 1984), fluctuations between laboratories still occur, even insects from the same population reared within a laboratory may show variations in susceptibility (Skovmand et al., 1998). Various authors have reviewed the existing Bt standards for lepidopteran species (Tompkins et al., 1990). Nowadays, IU are used to estimate the potency of Bt-based products according to the strain used as active ingredient: DiPel® (Bt ser. *kurstaki* ABTS-351) is assigned 32,000 IU/mg, XenTari® (Bt ser. *aizawai* ABTS-1857) 15,000 IU/mg (Figure 7), VectoBac® (Bt ser. *israelensis* H-14 AM65-52) 1,200 IU/mg and Novodor® (Bt ser. *tenebrionis* NB176) 15,000 IU/mg (www.kenogard.com). However, sometimes potency is not registered in the label and companies do not assure a good performance of the product because the potency measurements are not standardized.

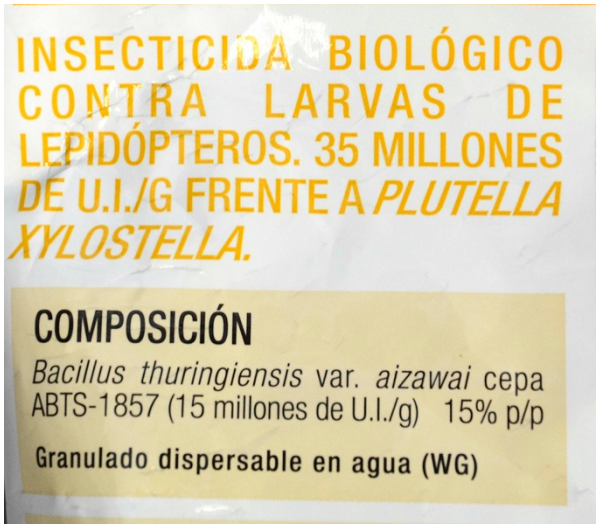

Figure 7. Detailed label of the Bt-based product XenTari® which active ingredient is the Bt subsp. *aizawai* strain ABTS-1857. The potency of the product has been estimated using *Plutella xylostella* as reference insect and assigned 15 million IU/g.

Several methods have been applied in order to fully characterize a Bt strain in order to determine its true potential. Immunoassay and high pressure liquid chromatography (HPLC) have been used by several authors in order to determine the composition of the parasporal crystal of *B. thuringiensis* (Chestukhina et al., 1994; Pang and Mathieson, 1991; Yamamoto, 2001). Purified crystals are solubilized followed by trypsin activation and chromatographic files are analysed by measuring time-retention peaks (Masson et al., 1998). However, to date no biotechnological method has been developed to determine the potency of a product according to the composition of its active ingredient. This task stills rely on insect bioassays (Beegle and Yamamoto, 1992) although these have resulted to be complicate and difficult to compare between laboratories. The relative abundance of crystal proteins in a Bt strain is crucial for the insecticidal activity. The knowledge of the proportion of each toxin would allow researcher to predict the potential toxicity of a certain product against a pest of interest, based on the interactions of toxins present in the inclusion body (BenFarhat-Touzri et al., 2018; Liao et al., 2002; Masson et al., 1998). The applications of such knowledge would have a huge impact

in the development of new products in order to avoid resistance using strains with different protein abundance.

6.3. Transgenic crops

6.3.1. Bt plants resistant to lepidopteran species

The so-called “Bt crops” are plants that have been genetically transformed and endogenously express insecticidal toxins from *B. thuringiensis*. The plant tissues express a soluble truncated form of the Bt protein, similar to the toxin found in the insect gut after enzyme cleavage, rendering these plants resistance to several insect pests (de Maagd et al., 1999). The first crops to be engineered with an insecticidal gene were tomato and tobacco plants (Barton et al., 1987; Vaeck et al., 1987). Since then, many other crops expressing truncated delta-endotoxins have been tested, providing excellent protection against lepidopteran species among others. One of the major benefits of their use is the decreased application of wide spectrum pesticides in the field (Carpenter et al., 2002) which render a positive impact on the natural enemies of insect pests as well as other beneficial insects including plant pollinators (Head et al., 2001). When compared to Bt sprayable products, transgenic crops show a longer persistence and higher resistance rates to environmental conditions, including UV light or rain wash off. They constitute a very successful delivery system with an easy application and high efficacy, showing a better cost-effectiveness ratio in the short term than Bt-based insecticides (Walker et al., 2003).The use of Bt crops has dramatically increased since 1996 and up to 189.8 million ha have been recorded in 2017 being soybean, maize, cotton and canola the most significant ones. United States leads as the country with larger hectares planted with transgenic crops (75 million ha), followed by Brazil (50.2 million ha) and Argentina (23.6 million ha) (James, 2017).

6.3.2. Bt toxicity factors used in Bt plants

The development of insect-resistant plants expressing Cry proteins has permitted the control of pests of economic importance (Jouzani et al., 2017; Melo et al., 2016; Salehi Jouzani et al., 2008), including plant pathogenic nematodes (Iatsenko et al., 2014).

The first genetically modified plants with the insecticidal capacity of Bt were obtained in 1986 in the United States and France. Tobacco plants expressing the Cry1Ab protein from Bt subsp. *kurstaki* strain HD-73, under the control of the constitutive *Cauliflower mosaic* virus 35S promoter, showed promising results against *Helicoverpa zea* (Hoffmann et al., 1992) and *Ostrinia nubilalis* (Vaeck et al., 1987). However, the expression levels of protein in plants were not enough for pest control (De la Riva and Adang, 1996; Koziel et al., 1993). In order to increase the levels of Cry proteins produced by Bt plants, coding sequences of *cry* genes were modified for optimal codon usage in plants and subsequent enhanced production (Perlak et al., 1991). Transgenic potato plants expressing *cry3A,* from Bt susbsp. *tenebrionis,* were the first to be commercialized to control *L. decemlineata* (Wierenga et al., 1996), performing better than sprayable Bt products (Perlak et al., 1993).

Since 1996 several transgenic Bt plants have been commercialized worldwide and allowed farmers to protect their crops from pests of economic importance (Christou et al., 2006). Single-gene cotton varieties which express Cry1Ac (e.g. Bollgard®, Monsanto) prove effective control of the tobacco budworm (*Heliothis virescens*) and the bollworm (*Pectinophora gossypiella*) pests (Jackson et al., 2003; Liao et al., 2002; MacIntosh et al., 1990; Padidam, 1992; Tabashnik et al., 2000). Besides, single-gene maize hybrids, which express Cry1Ab provide efficient for the control of the European corn borer (*O. nubilalis*) and the Southwestern corn borer (*Diatraea grandiosella*) (Archer et al., 2001; Castro et al., 2004). Bt corn expressing the Cry3Bb1 toxin (e.g. YieldGard® Rootworm, Monsanto) has shown resistance against *D. virgifera virgifera* (Jakka et al., 2016; Tabashnik and Gould, 2012), the most economical important pest of maize in the United States (Gray et al., 2009). However, applications of pyrethroids have been necessary in some fields to control other stablished pests including fall armyworm (*Spodoptera frugiperda),* black cutworm (*Agrotis ipsilon*), Western bean cutworm (*Richia albicosta*) and corn earworm (*Helicoverpa zea*) (Bacheler *et al.*, 1997; Buntin *et al.*, 2001; Burd *et al.*, 1999; Smith, 1998) in maize, and bollworm (*P. gossypiella*) in cotton, among others (Gore et al., 2001; Stewart et al., 2001). A new transgenic Bt maize variety that expresses the Cry1F toxin (e.g. Herculex®), as well as being effective against the damage of corn stem borers also confers resistance against

other secondary lepidopteran pests in this crop (Catangui and Berg, 2006; Sanahuja et al., 2011; Siebert et al., 2008).

One of the major concerns attributed to the systematic use of Bt plants has been the high probability of selection of insect biotypes that overcome the resistance offered by the transgenic crop. In order to delay the selection of insects resistant to Bt toxins and also to increase the range of controlled pests, a second generation of Bt plants that simultaneously express two Bt toxins has been developed. A Bt cotton (e.g. Bollgard® II, Monsanto) was launched in 2002 expressing two truncated genes (*cry1Ac* and *cry2Ab2)* simultaneously provided better control and expanded host range (Chitkowski *et al.*, 2003; Gore *et al.*, 2003; Hagerty *et al.*, 2005; Stewart *et al.*, 2001). No negative interaction in the expression of Cry1Ac was observed when introducing Cry2Ab2 (Adamczyk et al., 2001). Bt corn expressing Cry1Ac along with Cry1F (e.g. Smartmax®, Monsanto) were also commercialized in 2005. The addition of an extra protein to Cry1Ac provided better control of secondary pests (Greenberg and Adamczyk, 2007; Stewart et al., 2001). YielGard® Plus (Monsanto), a Bt corn hybrid that expresses Cry1Ab1 and Cry3Bb1, was the first transgenic plant to be engineered from two already existing cotton lines in the market (Sanahuja et al., 2011). In 2008, Bt cotton and Bt corn varieties expressing Vip proteins were registered in the United States. A third generation of Bt cotton that produced Cry1Ab, Cry2Ab and Vip3Aa (e.g. Bollgard® 3, Monsanto) was introduced in 2017 with the aim of increasing the longevity of the technology as the binding sites are different and expected cross-resistance events are low (Tabashnik and Carrière, 2017). Vip1 and Vip2 proteins have shown high toxicity levels against *D. virgifera virgifera*, however their expression in plants has not been yet possible due to the cytotoxicity of Vip2 proteins (Chakroun et al., 2016).

Transgenic soybean that expresses Cry1Ac and Cry1F provide excellent control of velvetbean caterpillar (*Anticarsia gemmatalis*), soybean looper (*Pseudoplusia includens*) and secondary pests such as tobacco budworm (*Heliothis virescens*) in Brazil (Bernardi et al., 2014; Marques et al., 2017). Bt rice expressing Cry1Ab for the control of several lepidopteran pests in China (Shu et al., 2000) and another expressing a chimeric gene *cry1Ab/cry1Ac* against the rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Crambidae) and the yellow stem borer;

Scirpophaga incertulas (Walker) (Lepidoptera:Crambidae), have been developed (Tu et al., 2000).

7. INSECT RESISTANCE TO *Bacillus thuringiensis*

Bacillus thuringiensis is a high potential entomopathogen for the control of pests in the field. Although development of resistance was considered, at once unlikely, many pests have become resistant to Bt toxins. Resistance events have been documented for lepidopteran, coleopteran and dipteran species (Ferré and Van Rie, 2002), but also for nematodes (Marroquin et al., 2000). The first evidence of resistance to Bt was observed in 1985 in *Plodia interpunctella* feeding on grain stores treated with DiPel® (Btk) (McGaughey, 1985). Since then, several cases have been reported in laboratory populations (Ferre´& van Rie 2008, Chaufaux *et al.*, 1997; Gould *et al.*, 1995; Rahardja and Whalon, 1995; Tabashnik, 1994). The first, and yet only ,report in open-field was in the diamondback moth, *Plutella xylostella,* in cruciferous plants in Asia and United States sprayed with Bt *kurstaki* (Ferré et al., 2008; Liu and Tabashnik, 1997; Tabashnik et al., 1990).

Appearance of resistance is directly related to constant exposure to insecticides or sometimes to the ecology of the pest. On the one hand, *Helicoverpa punctigera* is a polyphagous and migratory pest, so the chance of breeding between different populations dilutes resistant alleles, decreasing the possibilities to evolve resistance (Forrester, 1994). On the other hand, *P. xylostella* is not a migration pest, so no matting with individuals of other location occur and the pressure of selection of more virulent biotypes results in high levels of resistance in the field (Ferré et al., 1991; Gelernter, 1997).

7.1. Mechanisms of resistance

Alterations in any of the steps of the mode of action of Bt toxins could result in the selection of resistant insects. Several modes of resistance of insects to Bt toxins have been identified under laboratory conditions: reduction of binding of toxin to receptors, lack of solubilization of protoxins, alteration in the proteolytic processing of the toxin, binding site modifications, toxin degradation or precipitation by proteases, cell damage regeneration (Bruce et al., 2007; Ferré and Van Rie, 2002). Many of these mechanisms of resistance have been studied, but only

alteration in protease gut activity, reduced binding affinity and rapid cell replacement have been proved.

Alteration in the proteolytic processing of the protoxins may be a consequence of absence of certain proteases in the midgut of the insect that result in a decreased activation rate. A resistant colony of *P. interpunctella* to a Btk strain showed resistance to Cry1Ac protoxin due to the lack of activation of the protein (Oppert et al., 1996) caused by absence of a major trypsin-like midgut protease (Oppert et al., 1997). This observation was corroborated as the insect colony showed higher levels of resistance to the inactivated protoxin than to the activated toxin (Herrero et al., 2001).

Mutations in binding sites is the most common resistance mechanism responsible for the so-called “cross-resistance”. Modifications in the binding sites may result in different events of resistance. A decreased affinity of the toxin with the binding site (Ferré et al., 1991) or in a total absence of its interaction (Van Rie et al., 1990). Also a decreased event in the number of binding sites may occur (Herrero et al., 2001). A resistant colony of *P. interpunctella* showed a decreased binding affinity (K_d) of Cry1Ab to its receptor in the midgut of the insect, although the number of binding sites (R_t) remained as in a susceptible colony (Van Rie et al., 1990). However, mutations in the binding sites not always affect the binding affinity. It is the case of Cry1Ac, which did not show affinity alterations in a resistant colony of *P. interpunctella,* although sharing at least one binding site with Cry1Ab. Post-binding events, like membrane insertion, may be responsible for decreased susceptibility of the insect to Cry1Ab (Herrero et al., 2001). In some cases, mutations in a single binding site may affect the affinity interactions of several proteins at once (Lee et al., 1995). A colony of *H. virescens* showed high levels of resistance to Cry1Ac, although no binding alterations were observed. Cry1Aa, Cry1Ab and Cry1Ac shared common binding sites in the midgut of the insect, and modifications in the binding site of Cry1Aa resulted in resistance to Cry1Ac (Van Rie et al., 1989).

Resistant colonies of *H. virescens* to Cry1Ac showed similar histopathological changes in columnar gut cells (Forcada et al., 1999; Martínez-Ramírez et al., 1999). However, it is suggested that resistant populations are able to replace or regenerate damaged cells faster than susceptible insect lines.

7.2. Cross-resistance

The term “cross-resistance” is used to define the phenomenon by which a resistant insect to a certain toxin shows resistance to other toxins to which it has never been exposed (Pereira et al., 2008; Xu et al., 2010). Resistance affects both, Bt sprayable products and transgenic crops, as the mode of action of proteins is the same no matter the delivery system. That how, cases of cross-resistance in any of the two systems may occur, although the incidence is low.

This phenomenon is likely to happen between insects that belong to the same subspecies, due to similarities in midgut receptors. Populations of *O. nubilalis* and *Ostrinia furnicalis* (Mutuura and Munroe, 1970), showed great similarity in susceptibility to Cry1 proteins, suggesting shared binding sites and mechanism of action of toxins (Tan et al., 2011). However, cross-resistance is not always apparent. This is the case of Cry1Ab and Cry1F, which although being similar, show high affinity for different receptors (Tan et al., 2013). Notwithstanding, studies have revealed that a lack of cross-resistance is expected between not-related insects or for Bt proteins that do not share the same binding sites, such as Cry1, Cry2 and Vip3 toxins (Gouffon et al., 2011; Sena et al., 2009).

In order to study cross-resistance events, selection of resistant biotypes among an insect population has been achieved by feeding them on contaminated artificial diets (Tabashnik et al., 1992). Cross-resistance may occur between insecticidal proteins, usually upon the same family (Ferré et al., 1991), or even between Bt products. In the first case, some studies revealed that *P. interpunctella* acquired resistance to Cry1Ab and Cry1Ac after reared on Btk strain HD-1, but it remained susceptible to Cry1Aa, Cry1B, Cry1C and Cry2 single proteins. However, a wider cross-resistance event was reported when reared on Bt *aizawai*, including Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C and Cry2. The most surprising result was the developed resistance to Cry2, as neither of the mentioned strains produced it (McGaughey and Johnson, 1994). This event, although unusual, has been reported also elsewhere (Gould et al., 1995).

A number of reports on the selection of resistant *H. virescens* biotypes to Cry1Ac also described the acquisition of resistance to Cry1Aa, Cry1Ab, Cry1F and Cry2A (Gould et al., 1995, 1992; Heckel et al., 1997; Mi Kyong Lee et al., 1995),

and a moderate tolerance to Cry1B and Cry1C (Gould et al., 1995). Interesting studies have been performed on *P. xylostella* using commercial Bt products that express a battery of proteins.(Ferré and Van Rie, 2002). Although based on the same active ingredient (Btk) a cross-resistance event was observed towards Thuricide®, but not to DiPel®. However, a 200-fold cross-resistance to Cry1F, which is expressed in some Bt *aizawai* (Bta) strains, was detected (Tabashnik, 1994). No evidence of resistance was reported against XenTari® (Bta), as expected (Sarnthoy et al., 1997). Populations of *H. zea* cultured on artificial diet containing Cry1Ac developed resistance to MVP® formulations (Mycogen Corporation, San Diego, CA), which active ingredient is composed by that single protein, but not to DiPel® or XenTari®, that produce several toxins (Akhurst et al., 2003).

An important issue is that no cross-resistance has been reported between chemical pesticides and Bt toxins as their mode of action differ (Sarnthoy et al., 1997). Effectivity of Bt products was not altered when tested against insect populations that had developed resistance to chemicals (Jespersen et al., 1990).

7.3. Strategies to evolve resistance in transgenic plants

The ability of insects to adapt to insecticides and the rapid evolution of resistance endangers the success of Bt crops (Gould, 1998; Tabashnik et al., 2013). In populations of insect pests, associated with Bt corn crops, resistant biotypes have been selected for a number of species including, including *B. fusca, D. virgifera virgifera* and *S. frugiperda* to Cry1Ab, Cry3Bb and Cry1F, respectively, and *H. zea* and *P. gossypiella* to Cry1Ac in cotton (Storer et al., 2010; Tabashnik et al., 2013). In order to overcome this potential problem, the Bt crop industry implemented some strategies. The first adopted strategy to delay the evolution of resistance to insecticides, was the refuge strategy (Comins, 1977; Curtis et al., 1978), which consist in growing a refuge of non-Bt crops in association with Bt crops in order to dilute the resistance alleles in the insect population by encouraging the breeding between resistant and non-resistant biotypes (Georghiou and Taylor, 1977). Although this strategy seems to be the main responsible fact for the lack of resistance cases for the past decades, several alternative approaches have been proposed to evolve resistance, such as a refuge combined with high-dose expression of toxins. The aim of this strategy is to kill all of the offspring from

mattings of susceptible and resistant insects, due to the immense quantity of expressed protein (Gould, 1998, 1994). Nevertheless, the most robust one seems to be the pyramiding strategy, in which crops express two or more insecticidal toxins that act independently using different mechanisms of action against the same target pest (Roush, 1998; Zhao et al., 2003). The base of this strategy is that the chances of two simultaneous mutations in the receptors for two independently acting toxins is much lower than this event happening against a single protein (Soberón et al., 2009; Zhao et al., 2010). This strategy is supported by observations in the field where in 2009 pink bollworm developed resistance to cotton varieties expressing Cry1Ac but turned susceptible to another variety expressing also Cry2Ab (Monsanto, 2010). In this case refuges are also recommended, but the required proportion in the field is smaller (Shelton et al., 2002). In the past decade the expression of fusion proteins has become a promising alternative. A certain approach, by combining toxins with fragments of their specific receptors, allowed toxins to directly assemble for immediate pore formation, enhancing the activity of the protein (Chen et al., 2007). This new tendency allowed to broaden the spectrum of hosts by using a truncated version of two proteins. In order to confer protection to potato plants against various pests of different orders, Cry3Ba was fused with domain II of Cry1Ia. The resulting fusion protein was efficient for the control of *Leptinotarsa decemlineate* (Coleoptera; Chrysomelidae), *Phthorimaea operculella* (Lepidoptera; Gelechiidae) and *O. nubilalis*, simultaneously (Naimov et al., 2003). Research on the interaction of proteins and their binding sites showed no activity for individual toxins unless applied together. It is the case of Cry3A and Cry1Ab, which are not suitable for the control of *D. virgifera virgifera* as they show no toxicity to the pest when supplied as single proteins. However, the construction of a hybrid toxin that combined both of them, the so-called eCry3.1Ab, surprisingly resulted to be very effective against these coleopteran species (Walters et al., 2010). Although Bt gene expression in GM crops have resulted in multiple benefits in terms of cultivar yields and pest control, it is important to assure long-term usefulness of Bt crops, deploying appropriate strategies to delay resistance.

8. INSECT TARGET PESTS

8.1. Taxonomic position and geographical distribution of *Spodoptera* species

The beet armyworm, *Spodoptera exigua,* (Hübner, 1808), the cotton leaf worm *Spodoptera littoralis* (Boisduval, 1833) and the fall armyworm, *Spodoptera frugiperda* (J.E. Smith 1797) are among the most economically important species in the genus *Spodoptera* (Lepidoptera, Noctuidae). Most species of this genus (e.g. *S. exigua* and *S. frugiperda*) can make migratory flights of thousands of kilometers, which explains their wide geographical distribution (Zheng et al., 2011a). *S. exigua* is found in temperate zones and subtropical regions, extending all over the Asian countries, Europe, South and Central America and New Zeland (Brown and Dewhurst, 1975; Zheng et al., 2011b). *S. littoralis* is present in practically the entire African continent and the countries that make up the Mediterranean coast, with a limited northern distribution attributed to low winter temperatures (Miller, 1976; Sidibe and Lauge, 1977). Traditionally, the distribution area of *S. frugiperda* has been limited to the western hemisphere, all over the United States, north to southern and eastern Canada and south to Argentina (Pogue, 2002). Unexpectedly, this pest has recently invaded the African continent, where outbreaks were recorder for the first time in 2016 (Goergen et al., 2016). Currently, it is also present in India and China and all the favourable conditions are present for it to be able to invade and establish itself in the countries of the European Union.

8.2. Biology of *Spodoptera* species

Species of the genus *Spodoptera* have a complex metamorphosis (holometabolous) and complete four stages of development during their biological cycle: adult, egg, larva and pupa. Under optimal environmental and nutritional conditions, most of these species complete their biological cycle in approximately 4 weeks. All of them are multi-volt species so, each year, they can complete up to 6-8 generations in the most favourable natural conditions.

8.2.1. Egg

Spodoptera species lay their eggs on the lower surface of the leaves in clusters usually distributed in different plants to ensure the survival of newly hatched

larvae. Eggs of *S. exigua* and *S. frugiperda* measure about 0.45 and 0.35 mm in width and height, respectively, and are usually laid in groups forming one or two layers. However, *S. littoralis* females lay spherical flattened eggs of 0.6 mm in diameter. Eggs are usually covered with brownish-yellow hairs and scales that protect the eggs from natural enemies. Normal egg production is about 300-600 per female in *S. exigua,* from 1000 to 3500 in *S. littoralis* and about 1500-2000 in the case of *S. frugiperda.* The newly laid eggs are light colored and darken as the embryonic development progresses which is completed in 2-3 days under optimal conditions of temperature (26 ± 1 °C) and relative humidity (70 ± 5%) (Peterson, 1964; Pinhey, 1975).

8.2.2. Larval stage

Spodoptera larvae develop from five (*S. exigua*) to six (*S. littoralis* and *S. frugiperda*) instars. The passage from one larval instar to the next is carried out by replacing the cuticle with a new one, which is a process regulated by the juvenile hormone and ecdysone. The different larval stages, of a specific species, can also be recognized by the biometric characteristics of their cephalic capsule. First instars present a gregarious behaviour, while mature individuals are mainly solitary. *S. exigua* larvae normally have five instars, although an additional instar is sometimes reported, that vary in colour from pale green to yellow. As they mature larvae become darker and develop dorsal stripes. Neonates are 1.5 mm in size and grow up to 25-30 mm in the fifth instar (Figure 8).

Figure 8. Fifth instars of *S. exigua* (A), *S. littoralis* (B) and *S. frugiperda* (C)

No spines or hairs are adverted in their body. *S. littoralis* larvae normally have six instars which are variable in colour, from blackish-grey to dark green. A longitudinal dark and light stripe is developed in latter instars (Bishara, 1934; Brown and Dewhurst, 1975), where individuals can grow up to 40-45 mm in length (EPPO Bulletin, 2015). *S. frugiperda* individuals normally have six instars and vary in colour form light green with black lines and spots to dark brown with black dorsal and longitudinal stripes. An inverted yellow Y-shape on the head and four black spots on the last abdomen segment are differential characteristics in latter instars, which can reach up to 45 mm in size (Crumb, 1956; Levy and Habeck, 1976).

8.2.3. Pupa stage

Once larvae reach an appropriate length form a pupa. Pupae of *Spodoptera* species are shorter than larvae with a cylindrical shape, measuring about 15-22 mm, and usually have a dark reddish-brown color. All three species present a cremaster of 2 spines in the last segment, with two short extra hooks in the case of *S. exigua* (Figure 9). However, morphological similarities between species do not permit to differentiate *S. exigua, S. littoralis* and *S.frugiperda* until emergence of adults. The development period of this stage lasts between 6 to 9 days during warm weather but can extend up to 30 days during cooler seasons. Pupation normally occur in the soil (Pinhey, 1975; Pitre and Hogg, 1983; Zheng et al., 2011a).

Figure 9. Pupa individuals of *S. exigua* (A), *S. littoralis* (B) and *S. frugiperda* (C).

8.2.4. Adult

When adults emerge depending on the season they may migrate to warm regions for breeding (Feng et al., 2003). The adults of *S. exigua* are greyish moths with a wing span of 25-30 mm (Figure 10). Hindwings are white with dark veins. *S. littoralis* are grey-brown moths with a wing span of 30-38 mm and greyish-white hindwings with lack dark veins. In the case of *S. frugiperda* moths have a wing span of 37-38 and silver-white hindwings (Figure 10) (EPPO Bulletin, 2015). Adults life is estimated between 10 to 20 days in average (Sparks, 1979).

Figure 10. Adult individuals of *S. exigua* (A), *S. littoralis* (B) and *S. frugiperda* (C).

8.3. Economic damage

Spodoptera species are naturally polyphagous pests that are primary and secondary pests of a huge number of crops such as tomato, corn, cotton, tobacco, strawberry, cabbage or alfalfa. Among the different stages of the pest it is the larvae the ones that cause the damage, and the greater the instar the greater the economic impact. The beet armyworm *S. exigua* is a major pest of vegetables and flower crops worldwide that can have an important economic effect on crop production. It feeds on more than 50 crops including tomato (*Solanum lycopersicum*), lettuce (*Lactuca sativa*), cotton (*Gossypium* spp.) or tobacco (*Nicotiana tabacum*). It is a major pest of greenhouse sweet pepper (*Capsicum annuum*), aubergene (*Solanum melongena*) and melon (*Cucumis melo*) in Spain, among others (Moreno et al., 1992). This pest may cause holes in lettuce and tomatoes that lead to rotten producing a negative influence in crop yields. The cotton leafworm *S. littoralis,* affects more than 84 crops of economic importance (Salama et al., 1970). This pest

causes a considerable damage on cotton plants by feeding on the leaves and flower buds, but also affects tomato, maize, alfalfa and vegetables. Nowadays it causes important economic damages in Italy, Spain, Malta, Morocco, Cyprus, Egypt, North Africa and Israel. The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) is the most destructive pest of maize (*Zea mays*) in America (Barros et al., 2010). This polyphagous pest also damages sorghum (*Sorghum bicolor*), rice (*Oryza sativa L.*), soybean (*Glycine max L.*) and causes defoliation of cotton plants (*Gossypium* spp.) (Ali and Luttrell, 1990). Severe infestations occur in Brazilian crops as this species may have up to eight generations per year (Fitt et al., 2006; Pogue, 2002). In Brazil *S. frugiperda* outbreaks result in a huge investment in insecticides and yield losses of more than $250 millions every year. Its recent invasion of Africa has caused greater crop damages than any other African *Spodoptera* species (IITA, 2016).

8.4. Control Methods

Several methods have been used to control *Spodoptera* pests in the field. Application of conventional chemical insecticides, including methomyl, cypermethrin and carbaryl have been used to regulate the population density of the *Spodoptera* species that habitually or occasionally exceed the economic threshold of damage in the different crops in which they occur (Liburd et al., 2000). However, the repetitive use of the latter products have exerted tremendous selective pressure on insect populations which has led to the selection of resistant biotypes in many of the natural populations of these three species of *Spodoptera* (Devine and Furlong, 2007). *B. thuringiensis* toxins are one of the best alternative control agent to chemical insecticides due to their high specificity (Roh et al., 2007; Schnepf et al., 1998). Bt insecticides are undoubtedly the most used control agent among insecticides of microbial origin for the control of these pests (Jain et al., 2008). Also the use of transgenic plants with the insecticidal capacity of Bt (Bt crops) currently represent the preferred alternative in the case of some pests (e.g. *S. frugiperda*) in extensive crops (e.g. maize and soybean). (Kumar et al., 2008).

Several studies have been carried out in order to determine which specific toxins are responsible for toxicity among *Spodoptera* species. Cry1A is the most common protein in natural Bt strains and has been widely used in transgenic plants, however it has reported low efficacy. Bt strains expressing Cry1Bb, Cry1Ca,

Cry1Da, Cry1Fa, Vip3Ab, Vip3Ae and Vip3Af have proven to be the best candidates in order to control the genus *Spodoptera* (Hernández-Martínez et al., 2013, 2008; Ruiz De Escudero et al., 2007; Van Frankenhuyzen, 2009). As a matter of fact, sprayable products based on *Bacillus thuringiensis* subsp. *aizawai* have been commercialized worldwide due to their effectiveness. Xentari® is a registered Bt-based biopesticide for the management of *Spodoptera* spp. whose active ingredient is composed by several Cry1 proteins (Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da) responsible for toxicity against the beet armyworm, the cotton leafworm and the fall armyworm, among others (Akhurst et al., 2003; Basedow et al., 2008; Bioscience, 2009; Gonçalves et al., 2018). Transgenic crops, like Bt maize or Bt cotton, have also been commercialized as a control method, and have provided promising results during the first years by expressing Cry1 toxins (BenFarhat-Touzri et al., 2016; Hernández-Martínez et al., 2008; Okumura et al., 2013; Sena et al., 2009; Sorgatto et al., 2015). However, low expression of proteins, along with reduced refuges and high rates of pressure of selection, have favoured the appearance of resistant populations (Farias et al., 2014; Horikoshi et al., 2019; Storer et al., 2010; Tabashnik and Carrière, 2017). The selection of resistant *S. frugiperda* biotypes and the increase of their relative proportion, in the populations of the insects exposed to transgenic crops, leads to a continuous loss of the protection originally offered by the Bt crop that ends up being ineffective to control the pest .The efficacy of Bt crops has decreased and cases of cross resistance among proteins expressed in plants, such as Cry1F, Cry1Ab, and Cry1Ac, and those contained in Bt commercial products have been reported (Burtet et al., 2017; Federici, 1998; Santos-Amaya et al., 2015).). These events reduce the options for organic farmers who usually use Bt products for pest control (Jakka et al., 2014) and cultivars yield would decrease, pushing farmers to return to broad spectrum chemical insecticides with fatal consequences (Federici, 1998).

9. AIMS OF THE THESIS

The main aims of this thesis are:

1. Development of a genomic and proteomic based method capable to determine the qualitative and quantitative composition of toxic factors present in the parasporal crystal of *Bacillus thuringiesis* strains and its application to commercial Bt-based products.

2. Design of new artificial mixtures containing the appropriate insecticidal components to increase the potency and expand the host range of the spore and crystal mixture.

In order to accomplish the main aims of the thesis, the following specific aims will be addressed:

1. Development of an analytical method to determine the composition of delta-endotoxins (Cry and Cyt) that make up the parasporal crystal of the Bt strains used as active ingredients in commercial Bt products.

2. Generate knowledge concerning the effect of each of the insecticidal components of a Bt strain on phytophagous species of agricultural importance.

3. Determine the relative proportion of crystal components that allow the improvement of the insecticidal potency for one or more phytophagous lepidopteran species that cause pests.

10. REFERENCES

Abulreesh, H.H., Osman, G.E.H., Assaeedi, A.S.A., 2012. Characterization of insecticidal genes of *Bacillus thuringiensis* strains isolated from arid environments. Indian J. Microbiol. 52, 500–3. https://doi.org/10.1007/s12088-012-0257-z

Adamczyk, J.J., Adams, L.C., Hardee, D.D., 2001. Field Efficacy and Seasonal Expression Profiles for Terminal Leaves of Single and Double Bacillus thuringiensis Toxin Cotton Genotypes. J. Econ. Entomol. 94, 1589–1593. https://doi.org/10.1603/0022-0493-94.6.1589

Agaisse, H., Lereclus, D., 1995. How does Bacillus thuringiensis produce so much insecticidal crystal protein? J. Bacteriol. 177, 6027–6032.

Ahmad, W., 2003. Directed mutagenesis of selected regions of a bacillus thuringiensis entomocidal protein. FEMS Microbiol. Lett. 68, 97–104. https://doi.org/10.1016/0378-1097(90)90132-a

Aizawa, K., Iida, S., 1963. Nucleic acids extracted from the virus polyhedra of the silkworm, *Bombyx mori* (Linnaeus). J. Invertebr. Pathol. 5, 344–348.

Akhurst, R.J., James, W., Bird, L.J., Beard, C., 2003. Resistance to the Cry1Ac delta-endotoxin of *Bacillus thuringiensis* in the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Econ Entomol 96, 1290–1299.

Alewu B, N.C., 2018. Pesticides and human health. Intech open 2, 64. https://doi.org/10.5772/32009

Ali, A., Luttrell, R.G., 1990. Survival of Fall Armyworm (Lepidoptera: Noctuidae) Immatures on Cotton. Florida Entomol. 73, 459. https://doi.org/10.2307/3495462

Angus, T., Lüthy, P., 1971. Formulation of microbial insecticides, in: Burges HD & Hussey NW (Ed), Microbial Control of Insects and Mites, Academic Press. pp. 623–628.

Angus, T.A., 1956. Association of toxicity with protein-crystalline inclusions of *Bacillus sotto* Ishiwata. Can. J. Microbiol. 2, 122–131. https://doi.org/10.1139/m56-017

Angus, T.A., 1954. A bacterial toxin paralyzing silkworm larvae. Nature 173, 545–546.

Archer, T.L., Patrick, C., Schuster, G., Cronholm, E.D., Bynum, J., Morrison, W.P., 2001. Ear and shank damage by corn borers and corn earworms to four events of Bacillus thuriengiensis transgenic maize. Crop Prot. 20, 139–144.

Aronson, A.I., Beckman, W., Dunn, P., 1986. *Bacillus thuringiensis* and related insect pathogens. Microbiol. Rev. 50, 1–24.

Aronson, A.I., Fitz-James, P., 1976. Structure and morphogenesis of the bacterial spore coat. Bacteriol. Rev. 40, 360–402.

Asano, S., Ogiwara, K., Indrasith, L.S., Takahashi, M., Suzuki, N., Hori, H., 2002. Synergism of the spore on insecticidal activity of δ-endotoxin of *Bacillus thuringiensis* against the diamondback moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae) is not observed at late stage in bioassay. Appl. Entomol. Zool. 35, 583–590. https://doi.org/10.1303/aez.2000.583

Bacheler, J., Mott, D., Morrison, D.E., 1997. Efficacy of grower-managed Bt cotton in North Carolina. Proceedings, Beltwide Cott. Conf. 2, 858–861.

Barros, E.M., Torres, J.B., Ruberson, J.R., Oliveira, M.D., 2010. Development of *Spodoptera frugiperda* on different hosts and damage to reproductive structures in cotton. Entomol. Exp. Appl. 137, 237–245. https://doi.org/10.1111/j.1570-7458.2010.01058.x

Barth, H., Aktories, K., Popoff, M., Stiles, B., 2004. Binary bacterial toxins: biochemistry, biology, and applications of common *Clostridium* and *Bacillus* proteins. Microbiol. Mol. Biol. Rev. 68, 373–402.

Barton, K.A., Whiteley, H.R., Yang, N.-S., 1987. Bacillus thuringiensis δ-endotoxin expressed in transgenic Nicotiana tabacum provides resistance to Lepidopteran insects. Plant Physiol. 85, 1103–1109. https://doi.org/10.1104/pp.85.4.1103

Basedow, T., Ahmad, M., Tadesse, B., El Shafie, H., 2008. Galleria mellonella (L.) (Pyralidae) and Spodoptera exigua (Hubner) (Noctuidae): Differences in effects of XenTari (R) (Bacillus thuringiensis aizawai), NeemAzal T/S (R) and their combinations on survival. Mitteilungen Der Dtsch. Gesellschaft Fur Allg. Und Angew. Entomol. Vol 16 16, 365–368.

Baum, J., Johnson, T., Carlton, B., 1999. *Bacillus thuringiensis*: natural and recombinant bioinsecticide products. Biopesticides: use and delivery. FR Hall & JJ Menn. Methods Biotechnol. 5, 189–209.

Baum, J.A., Gonzalez, J.M., 1992. Mode of replication, size and distribution of naturally occurring plasmids in *Bacillus thuringiensis*. FEMS Microbiol. Lett. 96, 143–148. https://doi.org/10.1016/0378-1097(92)90394-4

Baumann, L., Okamoto, K., Unterman, B.M., Lynch, M.J., Baumann, P., 1984. Phenotypic characterization of *Bacillus thuringiensis* and *Bacillus cereus*. J. Invertebr. Pathol. 44, 329–341. https://doi.org/10.1016/0022-2011(84)90031-4

Bechtel, D.B., Bulla, L.A., 1982. Ultrastructural analysis of membrane development during *Bacillus thuringiensis* sporulation. J. Ultrastruct. Res. 79, 121–132. https://doi.org/10.1016/S0022-5320(82)90024-7

Becker, N., 2000. Bacterial control of vector-mosquitoes and black flies, in: Entomopathogenic Bacteria: From Laboratory to Field Application. Springer Netherlands, Dordrecht, pp. 383–398. https://doi.org/10.1007/978-94-017-1429-7_21

Beegle, C.C., Rose, R.I., Ziniu, Y., 1991. Mass production of *Bacillus thuringiensis* and *B. sphaericus* for microbial control of insect pests, in: Biotechnology for Biological Control of Pests and Vectors. pp. 195–216. https://doi.org/10.1201/9781351070300

Beegle, C.C., Yamamoto, T., 1992. History of *Bacillus thuringiensis* Berliner research and development. Can. Entomol. 124, 587–616. https://doi.org/10.4039/Ent124587-4

Behle, R.W., Mcguire, M.R., Gillespie, R.L., Shasha, B.S., 1997. Effects of alkaline gluten on the insecticidal activity of *Bacillus thuringiensis*. J. Econ. Entomol. 90, 354–360. https://doi.org/10.1093/jee/90.2.354

BenFarhat-Touzri, D., Driss, F., Jemli, S., Tounsi, S., 2018. Molecular characterization of Cry1D-133 toxin from *Bacillus thuringiensis* strain HD133 and its toxicity against *Spodoptera littoralis*. Int. J. Biol. Macromol. 112, 1–6. https://doi.org/10.1016/J.IJBIOMAC.2018.01.081

BenFarhat-Touzri, D., Driss, F., Tounsi, S., 2016. A promising HD133-like strain of *Bacillus thuringiensis* with dual efficiency to the two Lepidopteran pests: *Spodoptera littoralis* (Noctuidae) and *Ephestia kühniella* (Pyralidae). Toxicon 118, 112–120. https://doi.org/10.1016/J.TOXICON.2016.04.045

Bernardi, O., Dourado, P.M., Carvalho, R.A., Martinelli, S., Berger, G.U., Head, G.P., Omoto, C., 2014. High levels of biological activity of Cry1Ac protein expressed on MON 87701 × MON 89788 soybean against *Heliothis virescens* (Lepidoptera: Noctuidae). Pest Manag. Sci. 70, 588–594. https://doi.org/10.1002/ps.3581

Bernhard, K., Jarrett, P., Meadows, M., Butt, J., Ellis, D.J., Roberts, G.M., Pauli, S., Rodgers, P., Burges, H.D., 1997. Natural isolates of *Bacillus thuringiensis*: worldwide distribution, characterization, and activity against insect pests. J. Invertebr. Pathol. 70, 59–68.

Bernhard, K., Utz, R., 1993. Production of Bacillus thuringiensis insecticides for experimental and commercial uses, in: *Bacillus Thuringiensis*, an Environmental Biopesticide: Theory and Practice. pp. 255–267.

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D., Zaritsky, A., Parkhill, J., 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68, 5082–5095. https://doi.org/10.1128/AEM.68.10.5082-5095.2002

Bi, Y., Zhang, Y., Shu, C., Crickmore, N., Wang, Q., Du, L., Song, F., Zhang, J., 2015. Genomic sequencing identifies novel *Bacillus thuringiensis* Vip1/Vip2 binary and Cry8 toxins that have

high toxicity to *Scarabaeoidea* larvae. Appl. Microbiol. Biotechnol. 99, 753–760. https://doi.org/10.1007/s00253-014-5966-2

Bioscience, V., 2009. XenTari protoxin blend technical bulletin [WWW Document]. URL https://scholar.google.es/scholar?hl=es&as_sdt=0%2C5&q=Valent+Biosciences%2C+2009.+XenTari+protoxin+blend+technical+bulletin.+%3Chttp%3A%2F%2Fmicrobials.+valentbiosciences.com%2Fdocs%2Fag-microbials-resources-library%2Fxentari-protoxin-blend-+technical-bu (accessed 6.21.19).

Bishara, I., 1934. The cotton worm, *Prodenia litura* F., in Egypt. Bull. la Soc. Entomol. d'Egypte 18, 288-420 pp.

Boonserm, P., Davis, P., Ellar, D.J., Li, J., 2005. Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J. Mol. Biol. 348, 363–382. https://doi.org/10.1016/j.jmb.2005.02.013

Boonserm, P., Mo, M., Angsuthanasombat, C., Lescar, J., 2006. Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. J. Bacteriol. 188, 3391–401. https://doi.org/10.1128/JB.188.9.3391-3401.2006

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon 49, 423–435. https://doi.org/10.1016/j.toxicon.2006.11.022

Bravo, A., Gómez, I., Conde, J., Muñoz-Garay, C., Sánchez, J., Miranda, R., Zhuang, M., Gill, S.S., Soberón, M., 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochim. Biophys. Acta - Biomembr. 1667, 38–46. https://doi.org/10.1016/j.bbamem.2004.08.013

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. https://doi.org/10.1016/j.ibmb.2011.02.006

Bravo, A., Sánchez, J., Kouskoura, T., Crickmore, N., 2002. N-terminal activation is an essential early step in the mechanism of action of the *Bacillus thuringiensis* Cry1Ac insecticidal toxin. J. Biol. Chem. 277, 23985–23987. https://doi.org/10.1074/jbc.C200263200

Brown, E.S., Dewhurst, C.F., 1975. The genus Spodoptera (Lepidoptera, Noctuidae) in Africa and the Near East. Bull. Entomol. Res. 65, 221–262. https://doi.org/10.1017/S0007485300005939

Bruce, M., Gatsi, R., Crickmore, N., 2007. Mechanisms of resistance to *Bacillus thuringiensis* in the Diamondback Moth. Biopestic. Int. 3, 1–12.

Bulla, L.A., Bechtel, D.B., Kramer, K.J., Shethna, Y.I., Aronson, A.I., Fitz-James, P.C., 1980. Ultrastructure, physiology, and biochemistry of *Bacillus thuringiensis*. CRC Crit. Rev. Microbiol. 8, 147–204. https://doi.org/10.3109/10408418009081124

Buntin, G.D., Lee, R.., Wilson, D.M., McPherson, R.M., 2001. Evaluation of YieldGard transgenic resistance for control of fall armyworm and corn earnworm (Lepidoptera: Noctuidae) on corn. Florida Entomol. 84, 37–42.

Burd, T., Bradley, J.R., Van Duyn, J.W., 1999. Performance of selected Bt cotton genotypes against bollworm in North Carolina, in: Dugger and D. Richter (Eds.), Proceedings Beltwide Cotton Conferences. National Cotton Council, Memphis, TN. pp. 931–934.

Burges, H.D., 1967. Standardization of *Bacillus thuringiensis* products: homology of the standard. Nature 215, 664–665.

Burges, H.D., Jones, K.A., 1998. Formulation of bacteria, viruses and protozoa to control insects, in: Formulation of Microbial Biopesticides. Springer Netherlands, Dordrecht, pp. 33–127. https://doi.org/10.1007/978-94-011-4926-6_3

Burges, H.D., Thomson, E.M., Latchford, R.A., 1976. Importance of spores and δ-endotoxin protein

crystals of *Bacillus thuringiensis* in *Galleria mellonella*. J. Invertebr. Pathol. 27, 87–94. https://doi.org/10.1016/0022-2011(76)90032-X

Burtet, L.M., Bernardi, O., Melo, A.A., Pes, M.P., Strahl, T.T., Guedes, J.V., 2017. Managing fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), with Bt maize and insecticides in southern Brazil. Pest Manag. Sci. 73, 2569–2577. https://doi.org/10.1002/ps.4660

Butko, P., 2003. Cytolytic toxin Cyt1A and its mechanism of membrane damage: data and hypotheses. Appl. Environ. Microbiol. 69, 2415–22.

Cannon, R.J.C., 1996. *Bacillus thuringiensis* use in agriculture: a molecular perspective. Biol. Rev. 71, 561–636. https://doi.org/10.1111/j.1469-185x.1996.tb01285.x

Carlson, C.R., Caugant, D.A., Kolstø, A.B., 1994. Genotypic diversity among *Bacillus cereus* and *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 60, 1719–25.

Carlson, C.R., Johansen, T., Lecadet, M.M., Kolstø, A.B., 1996. Genomic organization of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *berliner* 1715. Microbiology 142, 1625–1634. https://doi.org/10.1099/13500872-142-7-1625

Carlton, B.C., Gawron-Burke, C., 1993. Genetic improvement of *Bacillus thuringiensis* for biopesticide development, in: Advanced Engineered Biopesticides (Kim, L., Ed). pp. 43–61.

Carpenter, J., Felsot, A., Goode, T., M Hammig, Onstad, D., Sankula, S., 2002. Comparative environmental impacts of biotechnology-derived and traditional soybean , corn and cotton crops, Council for Agricultural Science and Technology.

Carroll, J., Ellar, D.J., 1993. An analysis of *Bacillus thuringiensis* δ-endotoxin action on insect-midgut-membrane permeability using a light-scattering assay. Eur. J. Biochem. 214, 771–778. https://doi.org/10.1111/j.1432-1033.1993.tb17979.x

Carson, R., Wilson, E.O., Lear, L.J., 2002. Silent Spring. Houghton Mifflin.

Castro, B.A., Leonard, B.R., Riley, T.J., 2004. Management of feeding damage and survival of Southwestern corn borer and sugarcane borer (Lepidoptera: Crambidae) with *Bacillus thuringiensis* transgenic field corn. J. Econ. Entomol. 97, 2106–2116. https://doi.org/10.1603/0022-0493-97.6.2106

Catangui, M.A., Berg, R.K., 2006. Western bean cutworm, *Striacosta albicosta* (Smith) (Lepidoptera: Noctuidae), as a potential pest of transgenic Cry1Ab *Bacillus thuringiensis* corn hybrids in South Dakota. Environ. Entomol. 35, 1439–1452. https://doi.org/10.1603/0046-225x(2006)35[1439:wbcsas]2.0.co;2

Chakroun, M., Banyuls, N., Bel, Y., Escriche, B., Ferré, J., 2016. Bacterial vegetative insecticidal proteins (Vip) from entomopathogenic bacteria. Microbiol. Mol. Biol. Rev. 80, 329–350.

Chandler, D., Bailey, A.S., Mark Tatchell, G., Davidson, G., Greaves, J., Grant, W.P., 2011. The development, regulation and use of biopesticides for integrated pest management. Philos. Trans. R. Soc. B Biol. Sci. 366, 1987–1998. https://doi.org/10.1098/rstb.2010.0390

Charles, J.-F., Delécluse, A., Nielsen-Le Roux, C., 2000. Entomopathogenic bacteria : from laboratory to field application.

Chaufaux, J., MÛller-Cohn, J., Buisson, C., Sanchis, V., Lereclus, D., Pasteur, N., 1997. Inheritance of resistance to the *Bacillus thuringiensis* CrylC toxin in *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 90, 873–878. https://doi.org/10.1093/jee/90.4.873

Chen, J., Hua, G., Jurat-Fuentes, J.L., Abdullah, M.A., Adang, M.J., 2007. Synergism of *Bacillus thuringiensis* toxins by a fragment of a toxin-binding cadherin. Proc. Natl. Acad. Sci. 104, 13901–13906. https://doi.org/10.1073/pnas.0706011104

Chen, X.J., Lee, M.K., Dean, D.H., 1993. Site-directed mutations in a highly conserved region of *Bacillus thuringiensis* delta-endotoxin affect inhibition of short circuit current across *Bombyx mori* midguts. Proc. Natl. Acad. Sci. 90, 9041–9045. https://doi.org/10.1073/pnas.90.19.9041

Chestukhina, G.G., Kostina, L.I., Zalunin, I.A., Revina, L.P., Mikhailova, A.L., Stepanov, V.M., 1994.

Production of multiple δ-endotoxins by *Bacillus thuringiensis*: δ-endotoxins produced by strains of the subspecies *galleriae* and *wuhanensis*. Can. J. Microbiol. 40, 1026–1034. https://doi.org/10.1139/m94-163

Chitkowski, R.L., Turnipseed, S.G., Sullivan, M.J., Bridges, W.C., 2003. Field and laboratory evaluations of transgenic cottons expressing one or two *Bacillus thuringiensis* var. *kurstaki* Berliner proteins for management of Noctuid (Lepidoptera) pests. J. Econ. Entomol. 96, 755–762. https://doi.org/10.1603/0022-0493-96.3.755

Christou, P., Capell, T., Kohli, A., Gatehouse, J.A., Gatehouse, A.M.R., 2006. Recent developments and future prospects in insect pest control in transgenic crops. Trends Plant Sci. https://doi.org/10.1016/j.tplants.2006.04.001

Claus, D., Fritze, D., 1989. Taxonomy of *Bacillus*, in: Bacillus. Springer US, Boston, MA, pp. 5–26. https://doi.org/10.1007/978-1-4899-3502-1_2

Comins, H.N., 1977. The development of insecticide resistance in the presence of migration. J. Theor. Biol. 64, 177–197. https://doi.org/10.1016/0022-5193(77)90119-9

Crickmore, N., 2013. The diversity of *Bacillus thuringiensis* δ-endotoxins. Entomopathog. Bact. from Lab. to F. Appl. 65–79. https://doi.org/10.1007/978-94-017-1429-7_4

Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., Schnepf, E., Sun, M., Zeigler, D.R., 2018. *Bacillus thuringiensis* toxin nomenclature [WWW Document]. URL http://www.btnomenclature.info/

Crickmore, N., Zeigler, D.R., Feitelson, J., Schnepf, E., Van Rie, J., Lereclus, D., Baum, J., Dean, D.H., 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 807–13.

Crumb, S.E., 1956. The larvae of the Phalaenidae. Tech. Bull. No. 1135. Washingt. DC, USA United States Dep. Agric.

Curtis, C.F., Cook, L.M., Wood, R.J., 1978. Selection for and against insecticide resistance and possible methods of inhibiting the evolution of resistance in mosquitoes. Ecol. Entomol. 3, 273–287. https://doi.org/10.1111/j.1365-2311.1978.tb00928.x

Das, P.K., Dominic Amalraj, D., 1997. Biological control of malaria vectors. Indian J. Med. Res. 106, 174–197.

Das, S.K., 2013. Mode of action of pesticides and the novel trends a critical review. Int. Res. J. Agric. Soil Sci. 3. https://doi.org/10.14303/irjas.2013.127

de Barjac, H., Bonnefoi, A., 1962. Essai de classification biochimique et sérologique de 24 souches de *Bacillus* du type *B. thuringiensis*. Entomophaga 7, 5–31. https://doi.org/10.1007/BF02375988

de Barjac, H., Frachon, E., 1990. Classification of *Bacillus thuringiensis* strains. Entomophaga 35, 233–240. https://doi.org/10.1007/BF02374798

de Barjac, H., Lemille, F., 1970. Presence of flagellar antigenic subfactors in Serotype 3 of *Bacillus thuringiensis*. J. Invertebr. Pathol. 15, 139–140.

De la Riva, G.A., Adang, M.J., 1996. Expression of *Bacillus thuringiensis* delta-endotoxin genes in transgenic plants. Biotecnol. Apl. 13, 251–260.

de Maagd, R.., Bosch, D., Stiekema, W., 1999. Toxin-mediated insect resistance in plants. Trends Plant Sci. 4, 9–13.

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, H.E., 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu. Rev. Genet. 37, 409–433. https://doi.org/10.1146/annurev.genet.37.110801.143042

de Maagd, R.A., Bravo, A., Crickmore, N., 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet. 17, 193–199. https://doi.org/10.1016/S0168-9525(01)02237-5

Devine, G.J., Furlong, M.J., 2007. Insecticide use: Contexts and ecological consequences. Agric. Human Values 24, 281–306. https://doi.org/10.1007/s10460-007-9067-z

Donovan, W.P., Donovan, J.C., Engleman, J.T., 2001. Gene knockout demonstrates that *vip3A* contributes to the pathogenesis of Bacillus thuringiensis toward Agrotis ipsilon and Spodoptera exigua. J. Invertebr. Pathol. 78, 45–51. https://doi.org/10.1006/JIPA.2001.5037

Donovan, W.P., Engleman, J.T., Donovan, J.C., Baum, J.A., Bunkers, G.J., Chi, D.J., Clinton, W.P., English, L., Heck, G.R., Ilagan, O.M., Krasomil-Osterfeld, K.C., Pitkin, J.W., Roberts, J.K., Walters, M.R., 2006. Discovery and characterization of Sip1A: A novel secreted protein from *Bacillus thuringiensis* with activity against coleopteran larvae. Appl. Microbiol. Biotechnol. 72, 713–719. https://doi.org/10.1007/s00253-006-0332-7

Drobniewski, F.A., 1993. *Bacillus cereus* and related species. Clin. Microbiol. Rev. 6, 324–38. https://doi.org/10.1128/CMR.6.4.324

Du, C., Nickerson, K.W., 1996. *Bacillus thuringiensis* HD-73 spores have surface-localized Cry1Ac toxin: physiological and pathogenic consequences. Appl. Environ. Microbiol. 62, 3722–6.

Dulmage, H.T., 1981. Insecticidal activity of isolated of *Bacillus thuringiensis* and their potential for pest control. Microb. Control pests plant Dis. 1970–1980, Ed.H.D Burges, Academic Press, London.

Dulmage, H.T., 1970. Insecticidal activity of HD-1, a new isolate of *Bacillus thuringiensis* var. *alesti*. J. Invertebr. Pathol. 15, 232–239. https://doi.org/10.1016/0022-2011(70)90240-5

Dulmage, H.T., Boening, O.P., Rehnborg, C.S., Hansen, G.D., 1971. A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit. J. Invertebr. Pathol. 18, 240–245. https://doi.org/10.1016/0022-2011(71)90151-0

Dulmage, H.T., McLaughlin, R.E., Lacey, L.A., Couch, T.L., Alls, R.T., Rose, R.I., 1985. HD-968-S-1983, a proposed U.S. standard for bioassays of preparations of *Bacillus thuringiensis* subsp. *israelensis*-H-14. Bull. Entomol. Soc. Am. 31, 31–34. https://doi.org/10.1093/besa/31.2.31

Eilenberg, J., Damgaard, P.H., Hansen, B.M., Pedersen, J.C., Bresciani, J., Larsson, R., 2000. Natural coprevalence of *Strongwellsea castrans, Cystosporogenes deliaradicae,* and *Bacillus thuringiensis* in the host, *Delia radicum*. J. Invertebr. Pathol. 75, 69–75. https://doi.org/10.1006/jipa.1999.4892

Ellman, C., Entwistle, P., 1982. A study of glands on cotton responsible for the high pH and cation concentration of the leaf surface. Annu. Appl. Biol. 100, 553–558.

EPPO Bulletin, 2015. PM 7/124 (1) Spodoptera littoralis, Spodoptera litura, Spodoptera frugiperda, Spodoptera eridania. EPPO Bull. 45, 410–444. https://doi.org/10.1111/epp.12258

Estruch, J.J., Warren, G.W., Mullins, M.A., Nye, G.J., Craig, J.A., Koziel, M.G., 1996. Vip3A, a novel *Bacillus thuringiensis* vegetative insecticidal protein with a wide spectrum of activities against lepidopteran insects. Proc. Natl. Acad. Sci. 93, 5389–5394.

Evans, H., Shapiro, M., 1997. Viruses, in: Manual of Techniques in Insect Pathology. Academic Press, pp. 17–53. https://doi.org/10.1016/B978-012432555-5/50005-1

Farias, J.R., Andow, D.A., Horikoshi, R.J., Sorgatto, R.J., Fresia, P., dos Santos, A.C., Omoto, C., 2014. Field-evolved resistance to Cry1F maize by *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Brazil. Crop Prot. 64, 150–158. https://doi.org/10.1016/j.cropro.2014.06.019

Federici, B.A., 1998. Transgenic Bt crops and resistance: Broadscale use of pest-killing plants to be true test. Calif. Agric. 52, 14–20. https://doi.org/10.3733/ca.v052n06p14

Feng, H., Wu, K., Cheng, D., Guo, Y.-Y., 2003. Radar observations of the autumn migration of the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) and other moths in northern China. Bull. Entomol. Res. 93, 115–124. https://doi.org/10.1079/ber2002221

Ferré, J., Real, M.D., Van Rie, J., Jansens, S., Peferoen, M., 1991. Resistance to the *Bacillus thuringiensis* bioinsecticide in a field population of *Plutella xylostella* is due to a change in a

midgut membrane receptor. Proc. Natl. Acad. Sci. 88, 5119–5123. https://doi.org/10.1073/pnas.88.12.5119

Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47, 501–553.

Ferré, J., Van Rie, J., Macintosh, S.C., 2008. Insecticidal Genetically Modified Crops and Insect Resistance Management (IRM), in: Integration of Insect-Resistant Genetically Modified Crops within IPM Programs. Springer Netherlands, Dordrecht, pp. 41–85. https://doi.org/10.1007/978-1-4020-8373-0_3

Fishel, F.M., 2016. Pest management and pesticides : A historical early uses of pesticides and other insect-vectored diseases. IFAS extension-UF 2–6.

Fitt, G.P., Omoto, C., Maia, A.H., Waquil, J.M., Caprio, M., Okech, M.A., Cia, E., Nguyen, H., Andow, D.A., 2006. Resistance risks of Bt cotton and their management in Brazil., in: Environmental Risk Assessment of Genetically Modified Organisms. Volume 2: Methodologies for Assessing Bt Cotton in Brazil. pp. 300–345. https://doi.org/10.1079/9781845930004.0300

Forcada, C., Alcácer, E., Garcerá, M.D., Tato, A., Martínez, R., 1999. Resistance to *Bacillus thuringiensis* Cry1Ac toxin in three strains of *Heliothis virescens*: Proteolytic and SEM study of the larval midgut. Arch. Insect Biochem. Physiol. 42, 51–63. https://doi.org/10.1002/(SICI)1520-6327(199909)42:1<51::AID-ARCH6>3.0.CO;2-6

Forrester, N.W., 1994. Resistance management options for conventional *Bacillus thuringiensis* and transgenic plants in Australian summer field crops. Biocontrol Sci. Technol. 4, 549–553. https://doi.org/10.1080/09583159409355368

Forsyth, G., Logan, N.A., 2000. Isolation of *Bacillus thuringiensis* from Northern Victoria Land, Antarctica. Lett. Appl. Microbiol. 30, 263–6.

Frey, P.M., 2001. Biocontrol agents in the age of molecular biology. Trends Biotechnol. 19, 432–433. https://doi.org/10.1016/s0167-7799(01)01770-x

Gahan, L.J., Gould, F., Heckel, D.G., 2010. Identification of a gene associated with Bt resistance. science.sciencemag.org 293, 857–860.

Galitsky, N., Cody, V., Wojtczak, A., Ghosh, D., Luft, J.R., Pangborn, W., English, L., 2001. Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. Acta Crystallogr. Sect. D Biol. Crystallogr. 57, 1101–1109. https://doi.org/10.1107/S0907444901008186

Garbeva, P., Van Veen, J.A., Van Elsas, J.D., 2003. Predominant *Bacillus spp.* in agricultural soil under different management regimes detected via PCR-DGGE. Microb. Ecol. 45, 302–316. https://doi.org/10.1007/s00248-002-2034-8

Gelernter, W.., 1997. Resistance to microbial insecticides: the scale of the problem and how to manage it, in: BCPC Symposium Proceedings No. 68: Microbial Insecticides: Novelty or Necessity? pp. 201–213.

Georghiou, G.P., Taylor, C.E., 1977. Operational influences in the evolution of insecticide resistance. J. Econ. Entomol. 70, 653–658. https://doi.org/10.1093/jee/70.5.653

Glare, T., O'Callaghan, M., 2000. *Bacillus thuringiensis*: biology, ecology and safety. Chichester, UK: John Wiley & Sons.

Goergen, G., Kumar, P.L., Sankung, S.B., Togola, A., Tamò, M., 2016. First report of outbreaks of the fall armyworm *Spodoptera frugiperda* (J E Smith) (Lepidoptera, Noctuidae), a new alien invasive pest in West and Central Africa. PLoS One 11, 1–9. https://doi.org/10.1371/journal.pone.0165632

Gohar, M., Perchat, S., 2001. Sample preparation for β-exotoxin determination in *Bacillus thuringiensis* cultures by reversed-phase high-performance liquid chromatography. Anal. Biochem. 298, 112–117. https://doi.org/10.1006/abio.2001.5373

Goldberg, L.J., Margalit, J., 1977. A bacterial spore demonstrating rapid larvicidal activity against

Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti and *Culex pipiens* . Mosq. News 37, 355–358.

Gómez, I., Pardo-López, L., Muñoz-Garay, C., Fernandez, L.E., Pérez, C., Sánchez, J., Soberón, M., Bravo, A., 2007. Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides 28, 169–173. https://doi.org/10.1016/j.peptides.2006.06.013

Gómez, I., Sánchez, J., Miranda, R., Bravo, A., Soberón, M., 2002. Cadherin-like receptor binding facilitates proteolytic cleavage of helix α-1 in domain I and oligomer pre-pore formation of *Bacillus thuringiensis* Cry1Ab toxin. FEBS Lett. 513, 242–246. https://doi.org/10.1016/S0014-5793(02)02321-9

Gonçalves, K.C., Boiça Júnior, A.L., Duarte, R.T., Moreira, L.F., do Nascimento, J., Polanczyk, R.A., 2018. *Spodoptera albula* susceptibility to *Bacillus thuringiensis*-based biopesticides. J. Invertebr. Pathol. 157, 147–149. https://doi.org/10.1016/j.jip.2018.05.008

González, J.M., Carlton, B.C., 1984. A large transmissible plasmid is required for crystal toxin production in *Bacillus thuringiensis* variety *israelensis*. Plasmid 11, 28–38. https://doi.org/10.1016/0147-619X(84)90004-0

Gore, J., Leonard, B.R., Adamczyk, J.J., 2001. Bollworm (Lepidoptera: Noctuidae) survival on 'Bollgard' and 'Bollgard II' cotton flower bud and flower components. J. Econ. Entomol. 94, 1445–1451. https://doi.org/10.1603/0022-0493-94.6.1445

Gore, J., Leonard, B.R., Gable, R.H., 2003. Distribution of Bollworm, *Helicoverpa zea* (Boddie), injured reproductive structures on genetically engineered *Bacillus thuringiensis* var. *kurstaki* Berliner cotton. J. Econ. Entomol. 96, 699–705. https://doi.org/10.1603/0022-0493-96.3.699

Gouffon, C., Van Vliet, A., Van Rie, J., Jansens, S., Jurat-Fuentes, J.L., 2011. Binding sites for *Bacillus thuringiensis* Cry2Ae toxin on *Heliothine* brush border membrane vesicles are not shared with Cry1A, Cry1F, or Vip3A toxin. Appl. Environ. Microbiol. 77, 3182–3188. https://doi.org/10.1128/aem.02791-10

Gould, F., 1998. Sustainability of transgenic insecticidal cultivars: integrating pest genetics and ecology. Annu. Rev. Entomol. 43, 701–726. https://doi.org/10.1146/annurev.ento.43.1.701

Gould, F., 1994. Potential and problems with high-dose strategies for pesticidal engineered crops. Biocontrol Sci. Technol. 4, 451–461. https://doi.org/10.1080/09583159409355357

Gould, F., Anderson, A., Reynolds, A., Bumgarner, L., Moar, W., 1995. Selection and genetic analysis of a *Heliothis virescens* (Lepidoptera: Noctuidae) strain with high levels of resistance to *Bacillus thuringiensis* toxins. J. Econ. Entomol. 88, 1545–1559. https://doi.org/10.1093/jee/88.6.1545

Gould, F., Martinez-Ramirez, A., Anderson, A., Ferre, J., Silva, F.J., Moar, W.J., 1992. Broad-spectrum resistance to *Bacillus thuringiensis* toxins in *Heliothis virescens*. Proc. Natl. Acad. Sci. 89, 7986–7990. https://doi.org/10.1073/pnas.89.17.7986

Gray, M.E., Sappington, T.W., Miller, N.J., Moeser, J., Bohn, M.O., 2009. Adaptation and invasiveness of Western Corn Rootworm: Intensifying research on a worsening pest. Annu. Rev. Entomol. 54, 303–321. https://doi.org/10.1146/annurev.ento.54.110807.090434

Greenberg, S.M., Adamczyk, J.J., 2007. Noctuid survivorship and damage in Widestrike, Bollgard, and Bollgard II cottons in the lower Rio Grande valley of Texas., in: P. Dugger and D. Richter (Eds.), Proc. Beltwide Cotton Conf., 9-12 January 2007, New Orleans, LA. National Cotton Council, Memphis, TN. pp. 316–320.

Grochulski, P., Masson, L., Borisova, S., Puzstai-Carey, M., Schwartz, J.L., Brousseau, R., Cygler, M., 1995. *Bacillus thuringiensis* CrylA(a) insecticidal toxin: crystal structure and channel formation. J. Mol. Biol. 254, 447–464.

Guerchicoff, A., Ugalde, R.A., Rubinstein, C.P., 1997. Identification and characterization of a previously undescribed cyt gene in *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 2716–2721.

Gunnell, D., Eddleston, M., Phillips, M.R., Konradsen, F., 2007. The global distribution of fatal pesticide self-poisoning: Systematic review. BMC Public Health 7, 1–15. https://doi.org/10.1186/1471-2458-7-357

Guo, S., Ye, S., Liu, Y., Wei, L., Xue, J., Wu, H., Song, F., Zhang, J., Wu, X., Huang, D., Rao, Z., 2009. Crystal structure of *Bacillus thuringiensis* Cry8Ea1: an insecticidal toxin toxic to underground pests, the larvae of *Holotrichia parallela*. J. Struct. Biol. 168, 259–266.

Hagerty, A.M., Kilpatrick, A.L., Turnipseed, S.G., Sullivan, M.J., Bridges, W.C., 2005. Predaceous arthropods and Lepidopteran pests on conventional, Bollgard, and Bollgard Ii cotton under untreated and disrupted conditions. Environ. Entomol. 34, 105–114. https://doi.org/10.1603/0046-225x-34.1.105

Hall, I.M., Arakawa, K.Y., Dulmage, H.T., Correa, J.A., 1977. The pathogenicity of strains of *Bacillus thuringiensis* to larvae of *aedes* and to *culex* mosquitoes. Mosq. News 37, 246–251.

Han, L., Li, S., Liu, P., Peng, Y., Hou, M., 2012. New artificial diet for continuous rearing of *Chilo suppressalis* (Lepidoptera: Crambidae). Ann. Entomol. Soc. Am. 105, 253–258. https://doi.org/10.1603/an10170

Hansen, B.M., Salamitou, S., 2000. Virulence of *Bacillus thuringiensis*, in: Entomopathogenic Bacteria: From Laboratory to Field Application. Springer Netherlands, Dordrecht, pp. 41–64. https://doi.org/10.1007/978-94-017-1429-7_3

Head, G., Freeman, B., Mina, J., Moar, W., Ruberso, J., Turnipseed, S., 2001. Natural enemy abundance in commercial Bollgard and conventional cotton fields. Proc. Beltwide Cott. Conf. Memphis, TN Natl. Cott. Counc. 2, 796–798.

Heckel, D.G., Gahan, L.C., Gould, F., Anderson, A., 1997. Identification of a linkage group with a major effect on resistance to *Bacillus thuringiensis* Cry1Ac endotoxin in the tobacco budworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 90, 75–86. https://doi.org/10.1093/jee/90.1.75

Heimpel, M., 1966. A critical review of *Bacillus thuringi-ensis* var. *thuringiensis* Berliner and other crystalliferous bacterin.

Helgason, E., Økstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolstø, A.B., 2000. *Bacillus anthracis, Bacillus cereus,* and *Bacillus thuringiensis*- One species on the basis of genetic evidence. Appl. Environ. Microbiol. 66, 2627–2630. https://doi.org/10.1128/AEM.66.6.2627-2630.2000

Hendriksen, N.B., Hansen, B.M., 2003. Long-term survival and germination of *Bacillus thuringiensis* var. *kurstaki* in a field trial. Can. J. Microbiol. 48, 256–261. https://doi.org/10.1139/w02-009

Hernández-Martínez, P., Ferré, J., Escriche, B., 2008. Susceptibility of *Spodoptera exigua* to 9 toxins from *Bacillus thuringiensis*. J. Invertebr. Pathol. 97, 245–250. https://doi.org/10.1016/J.JIP.2007.11.001

Hernández-Martínez, P., Hernández-Rodríguez, C.S., Rie, J. Van, Escriche, B., Ferré, J., 2013. Insecticidal activity of Vip3Aa, Vip3Ad, Vip3Ae, and Vip3Af from *Bacillus thuringiensis* against lepidopteran corn pests. J. Invertebr. Pathol. 113, 78–81. https://doi.org/10.1016/J.JIP.2013.02.001

Hernández, C.S., Ferré, J., 2005. Common receptor for *Bacillus thuringiensis* toxins Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera, Helicoverpa zea*, and *Spodoptera exigua*. Appl. Environ. Microbiol. 71, 5627–5629. https://doi.org/10.1128/AEM.71.9.5627-5629.2005

Hernández, C.S., Martínez, C., Porcar, M., Caballero, P., Ferré, J., 2003. Correlation between serovars of *Bacillus thuringiensis* and type I β-exotoxin production. J. Invertebr. Pathol. 82, 57–62. https://doi.org/10.1016/S0022-2011(02)00199-4

Herrero, S., Oppert, B., Ferré, J., 2001. Different mechanisms of resistance to *Bacillus thuringiensis* toxins in the Indianmeal moth. Appl. Environ. Microbiol. 67, 1085–1089.

Hoffmann, M.P., Zalom, F.G., Wilson, L.T., Smilanick, J.M., Malyj, L.D., Kiser, J., Hilder, V.A., Barnes, W.M., 1992. Field evaluation of transgenic tobacco containing genes encoding

Bacillus thuringiensis ∂-endotoxin or cowpea trypsin inhibitor: efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). J. Econ. Entomol. 85, 2516–2522. https://doi.org/10.1093/jee/85.6.2516

Höfte, H., Whiteley, H.R., 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol. Rev. 53, 242–55.

Hokkanen, H.M.T., Hajek, A., 2003. Environmental impacts of microbial insecticides: Need and methods for risk assessment. Kluwer Academic.

Horikoshi, R.J., Bernardi, O., Amaral, F.S. de A. e A., Miraldo, L.L., Durigan, M.R., Bernardi, D., Silva, S.S., Omoto, C., 2019. Lack of relevant cross-resistance to Bt insecticide XenTari in strains of *Spodoptera frugiperda* (J. E. Smith) resistant to Bt maize. J. Invertebr. Pathol. 161, 1–6. https://doi.org/10.1016/J.JIP.2018.12.008

Horská, K., Kalvoda, L., Šebesta, K., 1976. Inhibition of DNA-dependent RNA polymerase by derivatives and analogues of thuringiensin. Collect. Czechoslov. Chem. Commun. 41, 3837–3841. https://doi.org/10.1135/cccc19763837

Hughes, P.R., van Beek, N.A.M., Wood, H.A., 1986. A modified droplet feeding method for rapid assay of *Bacillus thuringiensis* and baculoviruses in noctuid larvae. J. Invertebr. Pathol. 48, 187–192. https://doi.org/10.1016/0022-2011(86)90122-9

Hughes, P.R., Wood, H.A., 1981. A synchronous peroral technique for the bioassay of insect viruses. J. Invertebr. Pathol. 37, 154–159. https://doi.org/10.1016/0022-2011(81)90069-0

Husz, B., 1928. *Bacillus thuringiensis* Berliner, a bacterium pathogenic to Corn Borer larvae. A preliminary report., in: International Corn Borer Investigations. Scientific Reports. pp. 191–193.

Iatsenko, I., Boichenko, I., Sommer, R.J., 2014. *Bacillus thuringiensis* DB27 produces two novel protoxins, Cry21Fa1 and Cry21Ha1, which act synergistically against nematodes. Appl. Environ. Microbiol. 80, 3266–75. https://doi.org/10.1128/AEM.00464-14

Ignoffo, C., Hostetter, D., Pinnell, R., 1974. Stability of *Bacillus thuringiensis* and Baculovirus heliothis on soybean foliage. Environ. Entomol. 3, 117–119.

Ignoffo, C.M., Hostetter, D.L., Sikorowski, P.P., Sutter, G., Brooks, W.M., 1977. Inactivation of representative species of entomopathogenic viruses, a bacterium, fungus, and protozoan by an ultraviolet light source. Environ. Entomol. 6, 411–415. https://doi.org/10.1093/ee/6.3.411

IITA, 2016. First report of outbreaks of the Fall Armyworm on the African continent. IITA Bull. No 2330 4.

Iriarte, J., Bel, Y., Ferrandis, M.D., Andrew, R., Murillo, J., Ferré, J., Caballero, P., 1998. Environmental distribution and diversity of *Bacillus thuringiensis* in Spain. Syst. Appl. Microbiol. 21, 97–106. https://doi.org/10.1016/S0723-2020(98)80012-X

Iriarte, J., Dumanoir, V.C., Bel, Y., Porcar, M., Ferrandis, M.D., Lecadet, M., Ferré, J., Caballero, P., 2000. Characterization of *Bacillus thuringiensis* ser. *balearica* (Serotype H48) and ser. *navarrensis* (serotype H50): two novel serovars isolated in Spain. Curr. Microbiol. 40, 17–22.

Jackson, R.E., Boddie, H., Duyn, J.W. Van, 2003. Field performance of transgenic cottons expressing one or two *Bacillus thuringiensis* endotoxins against Bollworm, *Helicoverpa zea* (Boddie). J. Cott. Sci. 64, 57–64.

Jadamus, A., Vahjen, W., Simon, O., 2001. Growth behaviour of a spore forming probiotic strain in the gastrointestinal tract of broiler chicken and piglets. Arch. Anim. Nutr. 54, 1–17. https://doi.org/10.1080/17450390109381962

Jain, S., Ayres, C.F., de Oliveira, C.M.F., Lucena, W.A., de Souza, J.D.A., 2008. Toxicity of a *Bacillus thuringiensis israelensis*-like strain against *Spodoptera frugiperda*. BioControl 54, 467–473. https://doi.org/10.1007/s10526-008-9191-8

Jakka, S.R.K., Knight, V.R., Jurat-Fuentes, J.L., 2014. *Spodoptera frugiperda* (J.E. Smith) with field-

evolved resistance to Bt maize are susceptible to Bt pesticides. J. Invertebr. Pathol. 122, 52–4. https://doi.org/10.1016/j.jip.2014.08.009

Jakka, S.R.K., Shrestha, R.B., Gassmann, A.J., 2016. Broad-spectrum resistance to *Bacillus thuringiensis* toxins by western corn rootworm (*Diabrotica virgifera virgifera*). Sci. Rep. 6, 1–9. https://doi.org/10.1038/srep27860

James, C., 2017. Global Status of Commercialized Biotech/GM Crops in 2017: Biotech Crop Adoption Surges as Economic Benefits Accumulate in 22 years. Isaaa 127. https://doi.org/10.1017/S0014479706343797

Jarman, W.M., Ballschmiter, K., 2012. From coal to DDT: The history of the development of the pesticide DDT from synthetic dyes till Silent Spring. Endeavour. https://doi.org/10.1016/j.endeavour.2012.10.003

Jensen, G.B., Larsen, P., Jacobsen, B.L., Madsen, B., Smidt, L., Andrup, L., 2002. *Bacillus thuringiensis* in fecal samples from greenhouse workers after exposure to *B. thuringiensis*-based pesticides. Appl. Environ. Microbiol. 68, 4900–4905. https://doi.org/10.1128/AEM.68.10.4900-4905.2002

Jespersen, J., Affecting, J.K.-B. of arthropods, 1990, U., 1990. The effect of *Bacillus thuringiensis* var. *thuringiensis* on *Musca domestica* L. larvae resistant to insecticides. Biocontrol Arthropods Affect. Livest. Poult. (eds. D.A. Rutz R.S Patterson), Westview Press. Inc.; Boulder, Color. USA 215–229.

Jouzani, G.S., Valijanian, E., Sharafi, R., 2017. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-017-8175-y

Jurat-Fuentes, J.L., Adang, M.J., 2001. Importance of Cry1 δ-endotoxin domain II loops for binding specificity in *Heliothis virescens* (L.). Appl. Environ. Microbiol. 67, 323–329. https://doi.org/10.1128/AEM.67.1.323-329.2001

Jurat-Fuentes, J.L., Jackson, T.A., 2012. Bacterial Entomopathogens, Insect Pathology. https://doi.org/10.1016/b978-0-12-384984-7.00008-7

Kaur, S., 2000. Molecular approaches towards development of novel *Bacillus thuringiensis* biopesticides. World J. Microbiol. Biotechnol. 16, 781–793. https://doi.org/10.1023/A:1008931207374

Kirouac, M., Vachon, V., Noël, J.F., Girard, F., Schwartz, J.L., Laprade, R., 2002. Amino acid and divalent ion permeability of the pores formed by the *Bacillus thuringiensis* toxins Cry1Aa and Cry1Ac in insect midgut brush border membrane vesicles. Biochim. Biophys. Acta - Biomembr. 1561, 171–179. https://doi.org/10.1016/S0005-2736(02)00342-5

Knowles, B.H., Ellar, D.J., 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. BBA - Gen. Subj. 924, 509–518. https://doi.org/10.1016/0304-4165(87)90167-X

Kortenkamp, A., 2007. Ten years of mixing cocktails: A review of combination effects of endocrine-disrupting chemicals. Environ. Health Perspect. 115, 98–105. https://doi.org/10.1289/ehp.9357

Koziel, M.G., Beland, G.L., Bowman, C., Carozzi, N.B., Crenshaw, R., Crossland, L., Dawson, J., Desai, N., Hill, M., Kadwell, S., Launis, K., Lewis, K., Maddox, D., McPherson, K., Meghji, M.R., Merlin, E., Rhodes, R., Warren, G.W., Wright, M., Evola, S. V., 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Nat. Biotechnol. 11, 194–200. https://doi.org/10.1038/nbt0293-194

Krieg, A., Huger, A.M., Langenbruch, G.A., Schnetter, W., 1983. *Bacillus thuringiensis* var. *tenebrionis*, a new pathotype effective against larvae of Coleoptera. J. Appl. Entomol. 96, 500–508.

Kronstad, J.W., Schnepf, H.E., Whiteley, H.R., 1983. Diversity of locations for *Bacillus thuringiensis* crystal protein genes. J. Bacteriol. 154, 419–428.

Kumar, S., Chandra, A., Pandey, K.., 2008. *Bacillus thuringiensis* (Bt) transgenic crop: An environment friendly insect-pest management strategy. J. Environ. Biol. 29, 641–653. https://doi.org/10.1016/S0040-4039(98)02395-8

Kuo, W.S., Chak, K.F., 1996. Identification of novel cry-type genes from *Bacillus thuringiensis* strains on the basis of restriction fragment length polymorphism of the PCR-amplified DNA. Appl. Envir. Microbiol. 62, 1369–1377.

Lacey, L.A., 2016. Entomopathogens Used as Microbial Control Agents, Microbial Control of Insect and Mite Pests: From Theory to Practice. Elsevier Inc. https://doi.org/10.1016/B978-0-12-803527-6.00001-9

Lacey, L.A., Frutos, R., Kaya, H.K., Vail, P., 2001. Insect pathogens as biological control agents: Do they have a future? Biol. Control 21, 230–248. https://doi.org/10.1006/BCON.2001.0938

Lambert, B., Peferoen, M., 1992. Insecticidal Promise of *Bacillus thuringiensis*. Bioscience 42, 112–122. https://doi.org/10.2307/1311652

Leaber, M., Orlik, F., Schiffler, B., Sickmann, A., Benz, R., 2006. Vegetative insecticidal protein (Vip1Ac) of *Bacillus thuringiensis* HD201: evidence for oligomer and channel formation. Biochemistry 45, 283–288.

Lecadet, M.-M., Frachon, E., Dumanoir, V.C., Ripouteau, H., Hamon, S., Laurent, P., Thiery, I., 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. J. Appl. Microbiol. 86, 660–672. https://doi.org/10.1046/j.1365-2672.1999.00710.x

Lee, M.-K., Rajamohan, F., Gould, F., Dean, D.H., 1995. Resistance to *Bacillus thuringiensis* CryIA δ-endotoxins in a laboratory- selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. 61, 3836–3842.

Lee, M.K., Miles, P., Chen, J.-S., 2006. Brush border membrane binding properties of *Bacillus thuringiensis* Vip3A toxin to *Heliothis virescens* and *Helicoverpa zea* midguts. Biochem. Biophys. Res. Commun. 339, 1043–1047. https://doi.org/10.1016/J.BBRC.2005.11.112

Lee, M.K., Walters, F.S., Hart, H., Palekar, N., Chen, J.S., 2003. The mode of action of the *Bacillus thuringiensis* vegetative insecticidal protein Vip3A differs from that of Cry1Ab δ-endotoxin. Appl. Environ. Microbiol. 69, 4648–4657. https://doi.org/10.1128/AEM.69.8.4648-4657.2003

Lereclus, D., Lecadet, M.-M., Ribier, J., Dedonder, R., 1982. Molecular relationships among plasmids of *Bacillus thuringiensis*: Conserved sequences through 11 crystalliferous strains. MGG Mol. Gen. Genet. 186, 391–398. https://doi.org/10.1007/BF00729459

Levinson, B.L., Kasyan, K.J., Chiu, S.S., Currier, T.C., Gonzalez, J.M., 1990. Identification of β-exotoxin production, plasmids encoding β-exotoxin, and a new exotoxin in *Bacillus thuringiensis* by using high-performance liquid chromatography. J. Bacteriol. 172, 3172–3179. https://doi.org/10.1128/JB.172.6.3172-3179.1990

Levy, R., Habeck, D.H., 1976. Descriptions of the larvae of *Spodoptera sunia* and *S. latifascia* with a key to the mature *Spodoptera* larvae of the Eastern United States (Lepidoptera: Noctuidae)1. Ann. Entomol. Soc. Am. 69, 585–588. https://doi.org/10.1093/aesa/69.4.585

Li, J., Koni, P.A., Ellar, D.J., 1996. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. 257, 129–152. https://doi.org/10.1006/JMBI.1996.0152

Li, J.D., Carroll, J., Ellar, D.J., 1991. Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature 353, 815–821.

Li, Q., Xu, L.Z., Zou, T., Ai, P., Huang, G.H., Li, P., Zheng, A.P., 2015. Complete genome sequence of *Bacillus thuringiensis* strain HD521. Stand. Genomic Sci. 10, 62. https://doi.org/10.1186/s40793-015-0058-1

Liao, C., Heckel, D.G., Akhurst, R., 2002. Toxicity of *Bacillus thuringiensis* insecticidal proteins for *Helicoverpa armigera* and *Helicoverpa punctigera* (Lepidoptera: Noctuidae), major pests of cotton. J. Invertebr. Pathol. 80, 55–63.

Liburd, O.E., Funderburk, J.E., Olson, S.M., 2000. Effect of biological and chemical insecticides on *Spodoptera* species (Lep., Noctuidae) and marketable yields of tomatoes. J. Appl. Entomol. 124, 19–25. https://doi.org/10.1046/j.1439-0418.2000.00418.x

Likitvivatanavong, S., Chen, J., Evans, A.M., Bravo, A., Soberon, M., Gill, S.S., 2011. Multiple receptors as targets of Cry toxins in mosquitoes. J. Agric. Food Chem. 59, 2829–2838. https://doi.org/10.1021/jf1036189

Liu, J.W., Porter, A.G., Wee, B.Y., Thanabalu, T., 1996. New gene from nine *Bacillus sphaericus* strains encoding highly conserved 35.8-kilodalton mosquitocidal toxins. Appl. Environ. Microbiol. 62, 2174–6.

Liu, Y.-B., Tabashnik, B.E., 1997. Experimental evidence that refuges delay insect adaptation to *Bacillus thuringiensis*. Proc. R. Soc. London. Ser. B Biol. Sci. 264, 605–610. https://doi.org/10.1098/rspb.1997.0086

Liu, Y.B., Tabashnik, B.E., Moar, W.J., Smith, R.A., 1998. Synergism between *Bacillus thuringiensis* spores and toxins against resistant and susceptible Diamondback moths (*Plutella xylostella*). Appl. Environ. Microbiol. 64, 1385–1389.

MacIntosh, S.C., Stone, T.B., Sims, S.R., Hunst, P.L., Greenplate, J.T., Marrone, P.G., Perlak, F.J., Fischhoff, D.A., Fuchs, R.L., 1990. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. 56, 258–266. https://doi.org/10.1016/0022-2011(90)90109-J

Madshus, I.H., Stenmark, H., 1992. Entry of ADP-ribosylating toxins into cells. Curr. Top. Microbiol. Immunol. 175, 1–26. https://doi.org/10.1007/978-3-642-76966-5_1

Maeda, M., Mizuki, E., Nakamura, Y., Hatano, T., Ohba, M., 2000. Recovery of *Bacillus thuringiensis* from marine sediments of Japan. Curr. Microbiol. 40, 418–22.

Margalith, Y., Ben-Dov, E., 2000. Biological control by *Bacillus thuringiensis* subsp *israelensis*, in: Rechcigl JE, Rechcigl NA, Editors. Insect Pest Management: Techniques for Environmental Protection. CRC Press. p. 243.

Marques, L.H., Santos, A.C., Castro, B.A., Moscardini, V.F., Rossetto, J., Silva, O.A.N., Zobiole, L.H.S., Valverde-Garcia, P., Babcock, J.M., Storer, N.P., Rule, D.M., Fernandes, O.A., 2017. Field evaluation of soybean transgenic event DAS-81419-2 expressing Cry1F and Cry1Ac proteins for the control of secondary lepidopteran pests in Brazil. Crop Prot. 96, 109–115. https://doi.org/10.1016/j.cropro.2017.02.014

Marroquin, L., Elyassnia, D., Griffitts, J.., Feitelson, J.., Aroian, R.., 2000. *Bacillus thuringiensis* (Bt) toxin susceptibility and isolation of resistance mutants in the nematode *Caenorhabditis elegans*. Genet. Soc Am. 155, 1693–1699.

Martignoni, M., Ignoffo, C., 1980. Biological activity of Baculovirus preparations: in vivo assay, in: Characterization, Production and Utilization of Entomopathogenic Viruses (Eds C.M. Ignoffo, M. E. Martignoni & J. L. Vaughn), Pp. 138 American Society for Microbiology and National Science Foundation. Washington DC.

Martin, P.A.W., Travers, R.S., 1989. Worldwide abundance and distribution of *Bacillus thuringiensis* isolates. Appl. Environ. Microbiol. 55, 2437–2442.

Martínez-Ramírez, A.C., Gould, F., Ferré, J., 1999. Histopathological effects and growth reduction in a susceptible and a resistant strain of *Heliothis virescens* (Lepidoptera: Noctuidae) caused by sublethal doses of pure Cry1A crystal proteins from *Bacillus thuringiensis*. Biocontrol Sci. Technol. 9, 239–246. https://doi.org/10.1080/09583159929811

Martouret, D., 1975. Remarques sur le titre des formules commerciales a base de *Bacillus thuringiensis*. Entomophaga 20, 3–9. https://doi.org/10.1007/BF02373445

Masson, L., Erlandson, M., Puzstai-Carey, M., Brousseau, R., Juárez-Pérez, V., Frutos, R., 1998. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 64, 4782–8.

McClintock, J.T., Schaffer, C.R., Sjoblad, R.D., 1995. A comparative review of the mammalian toxicity of *Bacillus thuringiensis*-based pesticides. Pestic. Sci. https://doi.org/10.1002/ps.2780450202

McGaughey, W.., 1985. Insect resistance to the biological insecticide *Bacillus thuringiensis*. Science (80-.). 229, 193–195. https://doi.org/10.1126/science.229.4709.193

McGaughey, W.H., Johnson, D.E., 1994. Influence of crystal protein composition of *Bacillus thuringiensis* strains on cross-resistance in Indianmeal moths (Lepidoptera: Pyralidae). J. Econ. Entomol. 87, 535–540. https://doi.org/10.1093/jee/87.3.535

McGuire, M.R., Galan-Wong, L.J., Tamez-Guerra, P., 1997. Bacteria: Bioassay of *Bacillus thuringiensis* against lepidopteran larvae, in: Manual of Techniques in Insect Pathology. Academic Press, pp. 91–99. https://doi.org/10.1016/B978-012432555-5/50008-7

McGuire, M.R., Shasha, B.S., Eastman, C.E., Oloumi-Sadeghi, H., 1996. Starch- and flour-based sprayable formulations: Effect on rainfastness and solar stability of *Bacillus thuringiensis*. J. Econ. Entomol. 89, 863–869. https://doi.org/10.1093/jee/89.4.863

McLaughlin, R.E., Dulmage, H.T., Alls, R., Couch, T.L., Dame, D.A., Hall, I.M., Rose, R.I., Versoi, P.L., 1984. U.S. Standard bioassay for the potency assessment of *Bacillus thuringiensis* serotype H-14 against mosquito larvae. Bull. Entomol. Soc. Am. 30, 26–29. https://doi.org/10.1093/besa/30.1.26

Meher, S.M., Bodhankar, S.L., Kumar, A., Dhuley, J.N., Khodape, D.J., Naik, S.R., 2002. Toxicity studies of microbial insecticide *Bacillus thuringiensis* var. *kenyae* in rats, rabbits, and fish. Int. J. Toxicol. 21, 99–105. https://doi.org/10.1080/10915810252866079

Melo, A.L. de A., Soccol, V.T., Soccol, C.R., 2016. *Bacillus thuringiensis*: mechanism of action, resistance, and new applications: a review. Crit. Rev. Biotechnol. 36, 317–26. https://doi.org/10.3109/07388551.2014.960793

Metcalf, R.L., 1973. A Century of DDT. J. Agric. Food Chem. 21, 511–519. https://doi.org/10.1021/jf60188a040

Mi Kyong Lee, Rajamohan, F., Gould, F., Dean, D.H., 1995. Resistance to *Bacillus thuringiensis* CryIA δ-endotoxins in a laboratory- selected *Heliothis virescens* strain is related to receptor alteration. Appl. Environ. Microbiol. 61, 3836–3842.

Mikesell, P., Vodkin, M., 1985. Plasmids of *Bacillus anthracis*. Microbiol. Rev. 52–55.

Miller, G.W., 1976. Cold Storage as a quarantine treatment to prevent the introduction of *Spodoptem littoralis* (Boisd.) into glasshouses in the U.K. Plant Pathol. 25, 193–196. https://doi.org/10.1111/j.1365-3059.1976.tb01957.x

Milner, R.J., 1994. History of *Bacillus thuringiensis*. Agric. Ecosyst. Environ. 49, 9–13. https://doi.org/10.1016/0167-8809(94)90014-0

Mock, M., Fouet, A., 2001. Anthrax. Annu. Rev. Microbiol. 55, 647–671. https://doi.org/10.1146/annurev.micro.55.1.647

Monsanto, C., 2010. Monsanto. Press release, 5th March 2010. Available online http//www.monsanto.com/newsviews/Pages/India-pink-bollworm.aspx.

Montesinos, E., 2003. Development, registration and commercialization of microbial pesticides for plant protection. Int. Microbiol. https://doi.org/10.1007/s10123-003-0144-x

Moreno, R., Téllez, M.M., Benítez, E., Gomez, J., Rodriguez, M.D., Sáez, E., Belda, J.E., Cañero, R., Cabello, T., 1992. Lucha integrada: aplicación en los cultivos bajo plástico del sur de España. Horticultura 1, 41–54.

Morin, S., Biggs, R.W., Sisterson, M.S., Shriver, L., Ellers-Kirk, C., Higginson, D., Holley, D., Gahan, L.J., Heckel, D.G., Carriere, Y., Dennehy, T.J., Brown, J.K., Tabashnik, B.E., 2003. Three cadherin alleles associated with resistance to *Bacillus thuringiensis* in pink bollworm. Proc. Natl. Acad. Sci. 100, 5004–5009. https://doi.org/10.1073/pnas.0831036100

Morris, O.N., Converse, V., Kanagaratnam, P., 1995. Chemical additive effects on the efficacy of *Bacillus thuringiensis* Berliner subsp. *kurstaki* against *Mamestra configurata* (Lepidoptera: Noctuidae). J. Econ. Entomol. 88, 815–824. https://doi.org/10.1093/jee/88.4.815

Morse, R.J., Yamamoto, T., Stroud, R.M., 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9, 409–417. https://doi.org/10.1016/S0969-2126(01)00601-3

Mutuura, A., Munroe, E., 1970. Taxonomy and distribution of the European Corn Borer and allied species: genus Ostrinia (Lepidoptera: Pyralidae). Mem. Entomol. Soc. Canada 102, 1–112. https://doi.org/10.4039/entm10271fv

Naimov, S., Dukiandjiev, S., De Maagd, R.A., 2003. A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato. Plant Biotechnol. J. 1, 51–57. https://doi.org/10.1046/j.1467-7652.2003.00005.x

Navon, A., 2000. Bioassays of *Bacillus thuringiensis* products used against agricultural pests., in: Bioassays of Entomopathogenic Microbes and Nematodes. pp. 1–72. https://doi.org/10.1079/9780851994222.0001

Navon, A., 1993. Control of lepidopteran pests with *Bacillus thuringiensis*, in *Bacillus thuringiensis*, an Environmental Biopesticide: Theory and Practice (PF Entwistle, JS Cory, M.J. Bailey and S. Higgs), Wiley, Chichester 125–46.

Navon, A., 1992. Interactions among herbivores, microbial insecticides and crop plants. Phytoparasitica 20, 21–5. https://doi.org/10.1007/BF02980403

Navon, A., Ascher, K.R.S., 2000. Bioassays of entomopathogenic microbes and nematodes. CABI Pub.

Navon, A., Klein, M., Braun, S., 1990. *Bacillus thuringiensis* potency bioassays against *Heliothis armigera, Earias insulana,* and *Spodoptera littoralis* larvae based on standardized diets. J. Invertebr. Pathol. 55, 387–393. https://doi.org/10.1016/0022-2011(90)90082-H

Navon, A., Meisner, J., Ascher, K., 1987. Feeding stimulant mixtures for *Spodoptera littoralis* (Lepidoptera: Noctuidae). J. Econ. Entomol. 80, 990–993.

Navon, A., Zur, M., Arcan, L., 1988. Effects of cotton leaf surface alkalinity on feeding of *Spodoptera littoralis* larvae. J. Chem. Ecol. 14, 839–844.

Nicolopoulou-Stamati, P., Maipas, S., Kotampasi, C., Stamatis, P., Hens, L., 2016. Chemical pesticides and human health: the urgent need for a new concept in agriculture. Front. Public Heal. 4, 1–8. https://doi.org/10.3389/fpubh.2016.00148

Nielsen-LeRoux, C., Gaudriault, S., Ramarao, N., Lereclus, D., Givaudan, A., 2012. How the insect pathogen bacteria *Bacillus thuringiensis* and *Xenorhabdus/Photorhabdus* occupy their hosts. Curr. Opin. Microbiol. 15, 220–231. https://doi.org/10.1016/j.mib.2012.04.006

Nishimoto, T., Yoshisue, H., Ihara, K., Sakai, H., Komano, T., 1994. Functional analysis of block 5, one of the highly conserved amino acid sequences in the 130-kDa CryIVA protein produced by *Bacillus thuringiensis* subsp. *israelensis*. FEBS Lett. 348, 249–254. https://doi.org/10.1016/0014-5793(94)00604-0

Ohba, M., Mizuki, E., Uemori, A., 2009. Parasporin, a new anticancer protein group from *Bacillus thuringiensis*. Anticancer Res. 29, 427–33.

Okassov, A., Nersesyan, A., Kitada, S., Ilin, A., 2015. Parasporins as new natural anticancer agents: a review. J. BUON. 20, 5–16.

Okumura, R.S., Mariano, D.D.C., Dallacort, R., 2013. Agronomic efficiency of *Bacillus thuringiensis* (Bt) maize hybrids in pests control on Lucas do Rio Verde city , State of Mato Grosso , Brazil. African J. Agric. Res. 8, 2232–2239. https://doi.org/10.5897/AJAR12.2201

Oppert, B., Kramer, K.J., Beeman, R.W., Johnson, D., McGaughey, W.H., 1997. Proteinase-mediated insect resistance to *Bacillus thuringiensis* toxins. J. Biol. Chem. 272, 23473–23476.

https://doi.org/10.1074/jbc.272.38.23473

Oppert, B., Upton, S.J., McGaughey, W.H., Kramer, K.J., Johnson, D., 1996. Luminal proteinases from *Plodia interpunctella* and the hydrolysis of *Bacillus thuringiensis* CryIA(c) protoxin. Insect Biochem. Mol. Biol. 26, 571–583.

Osman, G.E.H., Already, R., Assaeedi, A.S.A., Organji, S.R., El-Ghareeb, D., Abulreesh, H.H., Althubiani, A.S., 2015. Bioinsecticide *Bacillus thuringiensis* a comprehensive review. Egypt. J. Biol. Pest Control 25, 271–288.

Pacheco, S., Gómez, I., Arenas, I., Saab-Rincon, G., Rodríguez-Almazán, C., Gill, S.S., Bravo, A., Soberón, M., 2009. Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "Ping Pong" binding mechanism with *Manduca sexta* aminopeptidase-N and cadherin receptors. J. Biol. Chem. 284, 32750–32757. https://doi.org/10.1074/jbc.M109.024968

Padidam, M., 1992. The insecticidal crystal protein CrylA (c) from *Bacillus thuringiensis* is highly toxic for *Heliothis armigera*. J. Invertebr. Pathol. 59, 109–111.

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., 2014. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins (Basel). https://doi.org/10.3390/toxins6123296

Pang, A.S.D., Mathieson, B., 1991. Peptide mapping of different *Bacillus thuringiensis* toxin gene products by CNBr cleavage in SDS-PAGE gels. J. Invertebr. Pathol. 57, 82–93. https://doi.org/10.1016/0022-2011(91)90044-Q

Pardo-López, L., Soberón, M., Bravo, A., 2013. *Bacillus thuringiensis* insecticidal three-domain Cry toxins: Mode of action, insect resistance and consequences for crop protection. FEMS Microbiol. Rev. 37, 3–22. https://doi.org/10.1111/j.1574-6976.2012.00341.x

Park, H.-W., Federici, B.A., 2004. Effect of specific mutations in helix α7 of domain I on the stability and crystallization of Cry3A in *Bacillus thuringiensis*. Mol. Biotechnol. 27, 089–100. https://doi.org/10.1385/mb:27:2:089

Parker, M.W., Feil, S.C., 2005. Pore-forming protein toxins: from structure to function. Prog. Biophys. Mol. Biol. 88, 91–142. https://doi.org/10.1016/J.PBIOMOLBIO.2004.01.009

Parker, M.W., Pattus, F., Tucker, A.D., Tsernoglou, D., 1989. Structure of the membrane-pore-forming fragment of colicin A. Nature 337, 93–96. https://doi.org/10.1038/337093a0

Pereira, E.J.G., Lang, B.A., Storer, N.P., Siegfried, B.D., 2008. Selection for Cry1F resistance in the European corn borer and cross-resistance to other Cry toxins. Entomol. Exp. Appl. 126, 115–121. https://doi.org/10.1111/j.1570-7458.2007.00642.x

Perlak, F.J., Fuchs, R.L., Dean, D.A., McPherson, S.L., Fischhoff, D.A., 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. 88, 3324–3328. https://doi.org/10.1073/pnas.88.8.3324

Perlak, F.J., Stone, T.B., Muskopf, Y.M., Petersen, L.J., Parker, G.B., McPherson, S.A., Wyman, J., Love, S., Reed, G., Biever, D., Fischhoff, D.A., 1993. Genetically improved potatoes: protection from damage by Colorado potato beetles. Plant Mol. Biol. 22, 313–321. https://doi.org/10.1007/BF00014938

Peterson, A., 1964. Egg types among moths of the Noctuidae (Lepidoptera). Florida Entomol. 47, 71–91.

Pinhey, E.C.G., 1975. Moths of Southern Africa : descriptions and colour illustrations of 1183 species.

Pirsaheb, M., Limoee, M., Namdari, F., Khamutian, R., 2015. Organochlorine pesticides residue in breast milk: A systematic review. Med. J. Islam. Repub. Iran 29.

Pitre, H., Hogg, D., 1983. Development of the Fall Armyworm on cotton, soybean and corn. J. Georg. Entomkological Soc. 18, 187–194.

Pogue, M., 2002. A world revision of the genus *Spodoptera* Guenée (Lepidoptera: Noctuidae). Am. Entomol. Soc. 202.

Poncet, S., Bernard, C., Dervyn, E., Cayley, J., Klier, A., Rapoport, G., 1997. Improvement of *Bacillus sphaericus* toxicity against dipteran larvae by integration, via homologous recombination, of the Cry11A toxin gene from *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 63, 4413–4420.

Porcar, M., Juárez-Pérez, V., 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol. Rev. https://doi.org/10.1016/S0168-6445(02)00128-6

Pozsgay, M., Fast, P., Kaplan, H., Carey, P.R., 1987. The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var. *kurstaki* HD1 and NRD12: A Raman spectroscopic study. J. Invertebr. Pathol. 50, 246–253. https://doi.org/10.1016/0022-2011(87)90089-9

Promdonkoy, B., Ellar, D.J., 2005. Structure-function relationships of a membrane pore forming toxin revealed by reversion mutagenesis. Mol. Membr. Biol. 22, 327–337. https://doi.org/10.1080/09687860500166192

Promdonkoy, B., Ellar, D.J., 2000. Membrane pore architecture of a cytolytic toxin from *Bacillus thuringiensis*. Biochem. J. 350, 275–282. https://doi.org/10.1042/bj3500275

Rahardja, U., Whalon, M.E., 1995. Inheritance of resistance to *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA delta-endotoxin in Colorado potato beetle (Coleoptera: Chrysomelidae). J. Econ. Entomol. 88, 21–26. https://doi.org/10.1093/jee/88.1.21

Raymond, B., Wyres, K.L., Sheppard, S.K., Ellis, R.J., Bonsall, M.B., 2010. Environmental factors determining the epidemiology and population genetic structure of the *Bacillus cereus* group in the field. PLOS Pathog. 6, e1000905. https://doi.org/10.1371/journal.ppat.1000905

Robertson, J.L., Jones, M.M., Olguin, E., Alberts, B., 2017. Bioassays with arthropods, third edition, Bioassays with Arthropods, Third Edition. CRC Press, Third edition. | Boca Raton : CRC Press, 2017. https://doi.org/10.1201/9781315373775

Roh, J.Y., Choi, J.Y., Li, M.S., 2007. *Bacillus thuringiensis* as a specific, safe and effective tool for insect pest control. J Microbiol. Biotechnol. 17, 547–559.

Roush, R.T., 1998. Two-toxin strategies for management of insecticidal transgenic crops: Can pyramiding succeed where pesticide mixtures have not? Philos. Trans. R. Soc. B Biol. Sci. 353, 1777–1786. https://doi.org/10.1098/rstb.1998.0330

Ruiz De Escudero, I., Estela, A., Escriche, B., Caballero, P., 2007. Potential of the *Bacillus thuringiensis* toxin reservoir for the control of *Lobesia botrana* (Lepidoptera: Tortricidae), a major pest of grape plants. Appl. Environ. Microbiol. 73, 337–340. https://doi.org/10.1128/AEM.01511-06

Saharan, B.S., Nehra, V., BS Saharan, V.N., 2011. Plant growth promoting rhizobacteria : A critical review. Life Sci. Med. Res. 21, 1–30.

Salama, H.S., Dimetry, N.Z., Salem, S.A., 1970. On the host preference and biology of the Cotton Leaf Worm *Spodoptera littoralis* Bois. Zeitschrift für Angew. Entomol. 67, 261–266. https://doi.org/10.1111/j.1439-0418.1971.tb02122.x

Salama, H.S., Foda, M.S., Sharaby, A., 1986. Possible extension of the activity spectrum of *Bacillus thuringiensis* strains through chemical additives. J. Appl. Entomol. 101, 304–313. https://doi.org/10.1111/j.1439-0418.1986.tb00862.x

Saleh, S.M., Harris, R.F., Allen, O.N., 2010. Fate of *Bacillus thuringiensis* in soil: effect of soil pH and organic amendment. Can. J. Microbiol. 16, 677–680. https://doi.org/10.1139/m70-116

Salehi Jouzani, G., Goldenkova, I. V., Piruzian, E.S., 2008. Expression of hybrid cry3aM-licBM2 genes in transgenic potatoes (*Solanum tuberosum*). Plant Cell. Tissue Organ Cult. 92, 321–325. https://doi.org/10.1007/s11240-007-9333-1

Sanahuja, G., Banakar, R., Twyman, R.M., Capell, T., Christou, P., 2011. *Bacillus thuringiensis*: A century of research, development and commercial applications. Plant Biotechnol. J. 9, 283–300. https://doi.org/10.1111/j.1467-7652.2011.00595.x

Sánchez-Bayo, F., Goulson, D., Pennacchio, F., Nazzi, F., Goka, K., Desneux, N., 2016. Are bee diseases linked to pesticides? — A brief review. Environ. Int. 89–90, 7–11. https://doi.org/10.1016/J.ENVINT.2016.01.009

Sanchis, V., 2011. From microbial sprays to insect-resistant transgenic plants: history of the biospesticide *Bacillus thuringiensis* . A review. Agron. Sustain. Dev. 31, 217–231. https://doi.org/10.1051/agro/2010027

Sanchis, V., Chaufaux, J., Lereclus, D., 1996. Biotechnological improvement of *Bacillus thuringiensis*: Dangers and risks. Ann. l'Institut Pasteur/Actualites 7, 271–284. https://doi.org/10.1016/S0924-4204(97)86395-0

Santos-Amaya, O.F., Rodrigues, J.V.C., Souza, T.C., Tavares, C.S., Campos, S.O., Guedes, R.N.C., Pereira, E.J.G., 2015. Resistance to dual-gene Bt maize in *Spodoptera frugiperda*: Selection, inheritance, and cross-resistance to other transgenic events. Sci. Rep. 5. https://doi.org/10.1038/srep18243

Sarnthoy, O., Li, T., Keinmeesuke, P., Sinchaisri, N., Miyata, T., Saito, T., 1997. Cross-resistance of *Bacillus thuringiensis* resistant population of Diamondback moth *Plutella xylostella* (lepidoptera: Yponomeutidae). Resist. Pest Manag. 9, 11–13.

Sattar, S., Maiti, M.K., 2011. Molecular characterization of a novel vegetative insecticidal protein from *Bacillus thuringiensis* effective against sap-sucking insect pest. J. Microbiol. Biotechnol. 21, 937–946. https://doi.org/10.4014/jmb.1105.05030

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806. https://doi.org/1092-2172

Schnepf, H.E., Whiteley, H.R., 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 78, 2893–7. https://doi.org/10.1073/PNAS.78.5.2893

Šebesta, K., Farkas, J., Horska, K., Vankova, J., 1981. Thuringiensin, the beta-exotoxin of Bacillus thuringiensis, Microbial control of pests and plant diseases,1970-1980.

Šebesta, K., Horská, K., 1970. Mechanism of inhibition of DNA-dependent RNA polymerase by exotoxin of *Bacillus thuringiensis*. Biochim. Biophys. Acta - Nucleic Acids Protein Synth. 209, 357–367. https://doi.org/10.1016/0005-2787(70)90734-3

Sekar, V., Thompson, D. V., Maroney, M.J., Bookland, R.G., Adang, M.J., 1987. Molecular cloning and characterization of the insecticidal crystal protein gene of *Bacillus thuringiensis* var. *tenebrionis*. Proc. Natl. Acad. Sci. 84, 7036–7040. https://doi.org/10.1073/pnas.84.20.7036

Sena, J.A.D., Hernández-Rodríguez, C.S., Ferré, J., 2009. Interaction of *Bacillus thuringiensis* Cry1 and Vip3A proteins with *Spodoptera frugiperda* midgut binding sites. Appl. Environ. Microbiol. 75, 2236–7. https://doi.org/10.1128/AEM.02342-08

Sharpe, E.S., Baker, F.L., 1979. Ultrastructure of the unusual crystal of the HD-1 isolate of *Bacillus thuringiensis* var. *kurstaki*. J. Invertebr. Pathol. 34, 320–322. https://doi.org/10.1016/0022-2011(79)90084-3

Shelton, A.M., Zhao, J.-Z., Roush, R.T., 2002. Economic, ecological, food safety, and social consequences of the deployment of Bt transgenic plants. Annu. Rev. Entomol. 47, 845–881. https://doi.org/10.1146/annurev.ento.47.091201.145309

Shi, Y., Xu, W., Yuan, M., Tang, M., Chen, J., Pang, Y., 2004. Expression of vip1/vip2 genes in *Escherichia coli* and *Bacillus thuringiensis* and the analysis of their signal peptides. J. Appl. Microbiol. 97, 757–765. https://doi.org/10.1111/j.1365-2672.2004.02365.x

Shu, Q., Ye, G., Cui, H., Cheng, X., Xiang, Y., Wu, D., Gao, M., Xia, Y., Hu, C., Sardana, R., Altosaar, I., 2000. Transgenic rice plants with a synthetic cry1Ab gene from *Bacillus thuringiensis* were highly resistant to eight lepidopteran rice pest species. Mol. Breed. 6, 433–439. https://doi.org/10.1023/A:1009658024114

Sidibe, B., Lauge, G., 1977. Effect of warm periods and of constant temperatures on some biological criteria in *Spodoptera littoralis* Boisduval (Lepidoptera Noctuidae), Annales de la Societe Entomologique de France.

Siebert, M.W., Tindall, K. V., Leonard, B.R., Van Duyn, J.W., Babcock, J.M., 2008. Evaluation of corn hybrids expressing CrylF (Herculex® I insect protection) against Fall Armyworm (Lepidoptera: Noctuidae) in the Southern United States. J. Entomol. Sci. 43, 41–51. https://doi.org/10.18474/0749-8004-43.1.41

Singh, P., 1983. A general purpose laboratory diet mixture for rearing insects. Insect Sci. Appl. 4, 357–362.

Skovmand, O.L.E., Thiery, I., Benzon, G.L., Sinègre, G., Monteny, N., Becker, N., 1998. Potency of products based on *Bacillus thuringiensis* var. *israelensis*: interlaboratory variations. J. Am. Mosq. Control Assoc. 14, 298–304.

Smith, R.A., Couche, G.A., 1991. The phylloplane as a source of *Bacillus thuringiensis* variants. Appl. Environ. Microbiol. 57, 311–5.

Smith, R.H., 1998. Year two of Bollgard behind Boll Weevil eradication-Alabama observations. Proceedings, Beltwide Cott. Conf. 2, 965–966.

Soberón, M., Gill, S.S., Bravo, A., 2009. Signaling versus punching hole: How do *Bacillus thuringiensis* toxins kill insect midgut cells? Cell. Mol. Life Sci. 66, 1337–1349. https://doi.org/10.1007/s00018-00

Soberón, M., López-Díaz, J.A., Bravo, A., 2013. Cyt toxins produced by *Bacillus thuringiensis*: A protein fold conserved in several pathogenic microorganisms. Peptides. https://doi.org/10.1016/j.peptides.2012.05.023

Sorgatto, R.J., Bernardi, O., Omoto, C., 2015. Survival and development of *Spodoptera frugiperda* and *Chrysodeixis includens* (Lepidoptera: Noctuidae) on Bt cotton and implications for resistance management strategies in Brazil. Environ. Entomol. 44, 186–192. https://doi.org/10.1093/ee/nvu018

Soufiane, B., Côté, J.C., 2009. Discrimination among *Bacillus thuringiensis* H serotypes, serovars and strains based on 16S rRNA, *gyrB* and *aroE* gene sequence analyses. Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 95, 33–45. https://doi.org/10.1007/s10482-008-9285-4

Sparks, A.N., 1979. A review of the biology of the Fall Armyworm. Florida Entomol. 62, 82. https://doi.org/10.2307/3494083

Stará, J., Kocourek, F., 2007. Insecticidal resistance and cross-resistance in populations of *Cydia pomonella* (Lepidoptera: Tortricidae) in central Europe. J. Econ. Entomol. 100, 1587–95. https://doi.org/10.1093/jee/100.5.1587

Starnes, R.L., Liu, C.., Marrone, P.G., 1993. History, use and future of microbial insecticides. Am. Entomol. 39, 83–91. https://doi.org/10.1093/ae/39.2.83

Stewart, S.D., Adamczyk, J.J., Knighten, K.S., Davis, F.M., 2001. Impact of Bt cottons expressing one or two insecticidal proteins of *Bacillus thuringiensis* Berliner on growth and survival of Noctuid (Lepidoptera) larvae. J. Econ. Entomol. 94, 752–760. https://doi.org/10.1603/0022-0493-94.3.752

Stone, T.B., Sims, S.R., Marrone, P.G., 1989. Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. J. Invertebr. Pathol. 53, 228–234. https://doi.org/10.1016/0022-2011(89)90011-6

Storer, N.P., Babcock, J.M., Schlenz, M., Meade, T., Thompson, G.D., Bing, J.W., Huckaba, R.M., 2010. Discovery and characterization of field resistance to Bt maize: *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Puerto Rico. J. Econ. Entomol. 103, 1031–1038. https://doi.org/10.1603/ec10040

Swiecicka, I., Fiedoruk, K., Bednarz, G., 2002. The occurrence and properties of *Bacillus thuringiensis* isolated from free-living animals. Lett. Appl. Microbiol. 34, 194–198. https://doi.org/10.1046/j.1472-765x.2002.01070.x

Tabashnik, B., 1994. Evolution of resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 39, 47–79. https://doi.org/10.1146/annurev.ento.39.1.47

Tabashnik, B.E., 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. U. S. A. 105, 19029–30. https://doi.org/10.1073/pnas.0810763106

Tabashnik, B.E., Brévault, T., Carrière, Y., 2013. Insect resistance to Bt crops: Lessons from the first billion acres. Nat. Biotechnol. https://doi.org/10.1038/nbt.2597

Tabashnik, B.E., Carrière, Y., 2017. Surge in insect resistance to transgenic crops and prospects for sustainability. Nat. Biotechnol. 35, 926–935. https://doi.org/10.1038/nbt.3974

Tabashnik, B.E., Cushing, N.L., Finson, N., Johnson, M.W., 1990. Field development of resistance to *Bacillus thuringiensis* in Diamondback moth (Lepidoptera: Plutellidae). J. Enonomic Entomol. 83, 1671–1676. https://doi.org/10.1093/jee/83.5.1671

Tabashnik, B.E., Gould, F., 2012. Delaying corn rootworm resistance to Bt corn. J. Econ. Entomol. 105, 767–776. https://doi.org/10.1603/ec12080

Tabashnik, B.E., Patin, A.L., Dennehy, T.J., Liu, Y.-B., Carriere, Y., Sims, M.A., Antilla, L., 2000. Frequency of resistance to *Bacillus thuringiensis* in field populations of pink bollworm. Proc. Natl. Acad. Sci. 97, 12980–12984. https://doi.org/10.1073/pnas.97.24.12980

Tabashnik, B.E., Schwartz, J.M., Finson, N., Johnson, M.W., 1992. Inheritance of resistance to *Bacillus thuringiensis* in Diamondback moth (Lepidoptera: Plutellidae). J. Econ. Entomol. 85, 1046–1055. https://doi.org/10.1093/jee/85.4.1046

Tan, S.Y., Cayabyab, B.F., Alcantara, E.., Ibrahim, Y.B., Huang, F., Blankenship, E.E., Siegfried, B.D., 2011. *Ostrinia nubilalis and Diatraea saccharalis* (Lepidoptera: Crambidae) to *Bacillus thuringiensis* Cry1 toxins. Crop Prot. 30, 1184–1189.

Tan, S.Y., Cayabyab, B.F., Alcantara, E.P., Huang, F., He, K., Nickerson, K.W., Siegfried, B.D., 2013. Comparative binding of Cry1Ab and Cry1F *Bacillus thuringiensis* toxins to brush border membrane proteins from *Ostrinia nubilalis, Ostrinia furnacalis* and *Diatraea saccharalis* (Lepidoptera: Crambidae) midgut tissue. J. Invertebr. Pathol. 114, 234–240. https://doi.org/10.1016/j.jip.2013.08.007

Thomas, W.E., Ellar, D.J., 1983. *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J. Cell Sci. 60, 181–97.

Tompkins, G., Engler, R., Mendelsohn, M., Hutton, P., 1990. Historical aspects of the quantification of the active ingredient percentage for *Bacillus thuringiensis* products, in: Hickle LA & Fitch WL (Ed.), Analytical Chemistry of *Bacillus Thuringiensis* , American Chemical Society, Washington DC. pp. 9–13. https://doi.org/10.1021/bk-1990-0432.ch002

Tu, J., Zhang, G., Datta, K., Xu, C., He, Y., Zhang, Q., Khush, G.S., Datta, S.K., 2000. Field performance of transgenic elite commercial hybrid rice expressing *Bacillus thuringiensis* δ-endotoxin. Nat. Biotechnol. 18, 1101–1104. https://doi.org/10.1038/80310

Tyrell, D.J., Bulla, L.A., Andrews, R.E., Kramer, K.J., Davidson, L.I., Nordin, P., 1981. Comparative biochemistry of entomocidal parasporal crystals of selected *Bacillus thuringiensis* strains. J. Bacteriol. 145, 1052–1062.

Vaeck, M., Reynaerts, A., Hofte, S.J., De Beuckeleer, M., Dean, C., Zabeau, M., Van Montagu, M., Leemans, J., 1987. Transgenic plants protected from insect attack. Nature 328, 33–37.

Vafskov, J., 1978. The heat-stable exotoxin of *Bacillus thuringiensis*. Folia Microbiol. (Praha). 23, 162–174.

Van Frankenhuyzen, K., 2013. Cross-order and cross-phylum activity of *Bacillus thuringiensis* pesticidal proteins. J. Invertebr. Pathol. 114, 76–85. https://doi.org/10.1016/j.jip.2013.05.010

Van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J. Invertebr. Pathol. 101, 1–16. https://doi.org/10.1016/j.jip.2009.02.009

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. 186, 239–47.

Van Rie, J., McGaughey, W.H., Johnson, D.E., Barnett, B.D., Van Mellaert, H., 1990. Mechanism of insect resistance to the microbial insecticide *Bacillus thuringiensis*. Science (80-.). 247, 72–74. https://doi.org/10.1126/science.2294593

Vilas-Bôas, G.T., Lemos, M.V.F., 2004. Diversity of cry genes and genetic characterization of *Bacillus thuringiensis* isolated from Brazil. Can. J. Microbiol. 50, 605–613. https://doi.org/10.1139/w04-052

Vilas-Bôas, G.T., Peruca, A.P.S., Arantes, O.M.N., 2007. Biology and taxonomy of *Bacillus cereus , Bacillus anthracis* and *Bacillus thuringiensis*. Can. J. Microbiol. 53, 673–687. https://doi.org/10.1139/w07-029

Vouk, V., Klas, Z., 1931. Conditions influencing the growth of the insecticidal fungus *Metarrhizium anisopliae*. Int Corn Borer Invest Sci Rept 4, 24–45.

Walker, K., Mendelsohn, M., Matten, S., Alphin, M., Ave, D., 2003. The role of microbial Bt products in U.S. crop protection. J. New Seeds 5, 31–51. https://doi.org/10.1300/j153v05n01_03

Walters, F.S., Defontes, C.M., Hart, H., Warren, G.W., Chen, J.S., 2010. Lepidopteran-active variable-region sequence imparts coleopteran activity in eCry3.1Ab, an engineered *Bacillus thuringiensis* hybrid insecticidal protein. Appl. Environ. Microbiol. 76, 3082–3088. https://doi.org/10.1128/AEM.00155-10

Ward, E.S., Ridley, A.R., Ellar, D.J., Todd, J.A., 1986. *Bacillus thuringiensis* var. *israelensis* δ-endotoxin. Cloning and expression of the toxin in sporogenic and asporogenic strains of *Bacillus subtilis*. J. Mol. Biol. 191, 13–22. https://doi.org/10.1016/0022-2836(86)90418-3

WHO, 1990. World Health Organization & United Nations Environment Programme. (1990). Public health impact of pesticides used in agriculture. World Health Organization [WWW Document].

Wierenga, J.M., Norris, D.L., Whalon, M.E., 1996. Stage-specific mortality of Colorado potato beetle (Coleoptera: Chrysomelidae) feeding on transgenic potatoes. J. Econ. Entomol. 89, 1047–1052. https://doi.org/10.1093/jee/89.5.1047

Wolfersberger, M.G., Chen, X.J., Dean, D.H., 1996. Site-directed mutations in the third domain of *Bacillus thuringiensis* δ-endotoxin CryIAa affect its ability to increase the permeability of *Bombyx mori* midgut brush border membrane vesicles. Appl. Environ. Microbiol. 62, 279–282.

Wu, D., Aronson, A.I., 1992. Localized mutagenesis defines regions of the *Bacillus thuringiensis* δ-endotoxin involved in toxicity and specificity. J. Biol. Chem. 267, 2311–2317.

Xu, C., Wang, B.-C., Yu, Z., Sun, M., 2014. Structural insights into *Bacillus thuringiensis* Cry, Cyt and parasporin toxins. Toxins (Basel). 6, 2732–2770. https://doi.org/10.3390/toxins6092732

Xu, D., Côté, J.C., 2008. Sequence diversity of *Bacillus thuringiensis* flagellin (H antigen) protein at the intra-H serotype level. Appl. Environ. Microbiol. 74, 5524–5532. https://doi.org/10.1128/AEM.00951-08

Xu, L., Wang, Z., Zhang, J., He, K., Ferry, N., Gatehouse, A.M.R., 2010. Cross-resistance of Cry1Ab-selected Asian corn borer to other Cry toxins. J. Appl. Entomol. 134, 429–438. https://doi.org/10.1111/j.1439-0418.2010.01517.x

Xu, X., Yu, L., Wu, Y., 2005. Disruption of a cadherin gene associated with resistance to Cry1Ac δ-endotoxin of *Bacillus thuringiensis* in *Helicoverpa armigera*. Society 71, 948–954. https://doi.org/10.1128/AEM.71.2.948

Yamamoto, S., Harayama, S., 1995. PCR amplification and direct sequencing of *gyrB* genes with universal primers and their application to the detection and taxonomic analysis of *Pseudomonas putida* strains. Appl. Envir. Microbiol. 61, 1104–1109.

Yamamoto, T., 2001. One hundred years of *Bacillus thuringiensis* research and development: Discovery to transgenic crops. J. Insect Biotechnol. Sericology 70, 1–23. https://doi.org/10.1360/zd-2013-43-6-1064

Yamamoto, T., McLaughlin, R.., 1981. Isolation of a protein from the parasporal crystal of *Bacillus thuringiensis* var. *kurstaki* toxic to the mosquito larva, *Aedes taeniorhynchus*. Biochem. Biophys. Res. Commun. 103, 414–421.

Zakharyan, R.A., Agabalyan, A.S., Chil-Akopyan, L.A., Gasparyan, N.S., Bankunts, K.A., Tatevosyan, P.E., Afrikyan, E.K., 1976. About the possibility of extrachromosomal DNA in creation of the entomocidal endotoxin of *Bacillus thuringiensis*. Dokl Akad Nauk Arrn SSR 63, 42–47.

Zhang, S., Cheng, H., Gao, Y., Wang, G., Liang, G., Wu, K., 2009. Mutation of an aminopeptidase N gene is associated with *Helicoverpa armigera* resistance to *Bacillus thuringiensis* Cry1Ac toxin. Insect Biochem. Mol. Biol. 39, 421–429. https://doi.org/10.1016/j.ibmb.2009.04.003

Zhang, X., Candas, M., Griko, N.B., Rose-Young, L., Bulla, L.A., 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R1 expressed in insect cells. Cell Death Differ. 12, 1407–1416. https://doi.org/10.1038/sj.cdd.4401675

Zhang, X., Candas, M., Griko, N.B., Taussig, R., Bulla, L.A., 2006. A mechanism of cell death involving an adenylyl cyclase/PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proc. Natl. Acad. Sci. 103, 9897–9902. https://doi.org/10.1073/pnas.0604017103

Zhao, J., Jin, L., Yang, Y., Wu, Y., 2010. Diverse cadherin mutations conferring resistance to *Bacillus thuringiensis* toxin Cry1Ac in *Helicoverpa armigera*. Insect Biochem. Mol. Biol. 40, 113–118. https://doi.org/10.1016/j.ibmb.2010.01.001

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493–1497. https://doi.org/10.1038/nbt907

Zheng, X.L., Cong, X.P., Wang, X.P., Lei, C.L., 2011a. Pupation behaviour, depth, and site of *Spodoptera exigua*. Bull. Insectology 64, 209–214.

Zheng, X.L., Cong, X.P., Wang, X.P., Lei, C.L., 2011b. A review of geographic distribution, overwintering and migration in *Spodoptera exigua* Hubner (Lepidoptera: Noctuidae). J. Entomol. Res. Soc.

Zhu, C., Ruan, L., Peng, D., Yu, Z., Sun, M., 2006. Vegetative insecticidal protein enhancing the toxicity of *Bacillus thuringiensis* subsp *kurstaki* against *Spodoptera exigua*. Lett. Appl. Microbiol. 42, 109–114. https://doi.org/10.1111/j.1472-765X.2005.01817.x

Zou, D.Y., Coudron, T.A., Wu, H.H., Gu, X.S., Xu, W.H., Zhang, L.S., Chen, H.Y., 2015. Performance and cost comparisons for continuous rearing of *Arma chinensis* (Hemiptera: Pentatomidae: Asopinae) on a zoophytogenous artificial diet and a secondary prey. J. Econ. Entomol. 108, 454–461. https://doi.org/10.1093/jee/tov024

CHAPTER II

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Unravelling the composition of insecticidal crystal proteins in *Bacillus thuringiensis* by genome sequencing and mass spectrometry

Abstract

Bacillus thuringiensis (Bt) is the most widely used active ingredient of biological insecticides. The composition of δ-endotoxins (Cry and Cyt proteins) in the parasporal crystal determines the toxicity profile in each Bt strain. However, a reliable method for their identification and quantification has not been available, due to the high sequence identity of the genes coding for the δ-endotoxins and the toxins themselves. Here we have developed an accurate and reproducible mass spectrometry-based method (LC-MS/MS-MRM), using isotopically-labelled proteotypic peptides for each protein in a particular mixture, to determine the relative proportion of each δ-endotoxin within the crystal. To validate the method, artificial mixtures containing Cry1Aa, Cry2Aa and Cry6Aa were analyzed. Determination of the relative abundance of proteins (in molarity) with our method was in good agreement with the expected values. This method was then applied to the most common commercial Bt-based products: DiPel® DF, Xentari® GD, Vectobac® 12S and Novodor® in which between 3 and 6 δ-endotoxins were identified and quantified in each product. This novel approach is of great value for the characterization of Bt-based products, not only providing information on host range toxicity, but also for monitoring industrial crystal production and quality control of Bt-based insecticides.

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1. INTRODUCTION

For decades, the bacterium *Bacillus thuringiensis* (Berliner) has been the most important biological insecticide for crop protection. Its specificity and ecotoxicological profile have been key to its development as an alternative to synthetic pesticides. The world biopesticide market is currently valued at three billion dollars annually (Olson, 2015). Three-quarters of all biopesticides are *Bacillus thuringiensis* (Bt)-based products, which are among the safest and most environmentally-benign insecticides available (Raymond and Federici, 2017; Siegel, 2001).

Bt produces large parasporal crystals during sporulation. These crystals consist of various δ-endotoxins, mostly Cry proteins, and, in some Bt strains, cytotoxic Cyt proteins (Palma et al., 2014). The Cry proteins are cleaved in the insect gut by host proteinases, which give rise to 65–70-kDa activated toxins that form pores in the columnar cell brush border membranes, leading to the disruption of ion and metabolite transport, and insect death (Bravo et al., 2007). Cyt proteins also undergo activation of protoxins (27kDa) by proteolytic cleavage in the insect gut to produce an activated toxin of 25 kDa, which interacts directly with non-saturated membrane lipids (Li et al., 1996). In many Bt strains, Cry and Cyt proteins are very similar, with 86–90% shared amino acid identity in some cases, such as in the well-known Bt-*kurstaki* strain HD1. Nevertheless, small sequence differences in the critical regions of these proteins are responsible for pronounced differences in the insecticidal potency and effective range of target species (Bravo, 1997). A large number of *cry* and *cyt* genes have been cloned, expressed, and shown to encode proteins with specific insecticidal activity against pests from the orders Lepidoptera, Diptera, Coleoptera, and Hymenoptera, as well as other invertebrates, such as nematodes and mites (Van Frankenhuyzen, 2009).

Historically, Bt strains have been classified based on the serological characteristics associated with the bacterial flagellar antigen (H) into more than 60 serotypes (H serotypes) and over 80 serological varieties (serovars, also known as subspecies), with broadly different *cry* gene profiles and insecticidal activity spectra (Ibrahim et al., 2010). In the absence of any other classification method, the insecticidal spectrum of a Bt strain and its potential industrial applicability was

frequently presumed based on the serological characteristics. In the past, this classification was useful. For example, many Bt strains toxic to lepidopterans belong to serovar *kurstaki* or *aizawai* (Palma et al., 2014), strains toxic to mosquito larvae often belong to serovar *israelensis* (Floore, 2006), and strains active against coleopterans often belong to serovar *morrisoni* (Kati et al., 2007). However, many new Bt strains not belonging to these serovars have also shown to have insecticidal activity against these orders of insects. Moreover, classification into a specific serovar does not guarantee the presence of a specific set of *cry* genes or their expression (Porcar and Juárez-Pérez, 2003). In part because the serovar system does not provide reliable information on the insecticidal spectrum of a given strain, this classification system is now considered obsolete.

Advances in DNA technologies allow Bt strains to be easily characterized according to their *cry* and *cyt* gene content (Ye et al., 2012). Characterization of the δ-endotoxin gene content is useful for strain classification, but of limited predictive value, as it is the expression of these genes what determines the spectrum of activity of a given strain. There are many *cry* and *cyt* genes that are cryptic, or with insignificant levels of expression, that contribute little to the toxicity of a given strain. Therefore, identification of the insecticidal proteins that make up the parasporal crystal is essential to infer the insecticidal activity of a particular strain or to understand why production batches of a Bt-based insecticide vary in their toxicity characteristics.

To date, attempts to quantify the δ-endotoxin content of Bt strains have relied on reverse-phase high performance liquid chromatography (RP-HPLC) of urea-denatured and trypsinized proteins (Yamamoto, 1983), or ion-exchange liquid chromatography at constant pH applied to alkaline-digested peptides (Pusztai-Carey and Carey, 1994). However, neither of these techniques can be used to reliably distinguish proteins with similar amino acid sequences, such as some Cry proteins, or those present at low concentrations. An alternative approach involves HPLC analysis of semi-trypsinized crystal protein preparations (Masson et al., 1998). Nonetheless, the partial digestion used by these authors is not effective for differentiating among proteins with a high degree of similarity, commonly present in the crystals produced by many Bt strains.

Due to the difficulties faced in the identification and quantification of the different Cry and Cyt proteins in a mixture, commercial Bt-based bioinsecticides currently include no description of the composition of the active ingredients - their characterization is based entirely on their potency against a reference insect species, in comparison with an international Bt standard (Dulmage et al., 1971). A major disadvantage of such bioassay-based characterization is that the potency of a Bt preparation depends on the insect species tested, notwithstanding the individual and population variation in susceptibility that affect the results of bioassays. Furthermore, the susceptibility of an insect species cannot be extrapolated to other insect species. For example, within the lepidopteran family Noctuidae, heliothine species are very susceptible to the Cry1Ac protein, whereas species of the genus *Spodoptera* are usually very tolerant (Bernardi et al., 2014).

Recent advances in the field of proteomics now allow the quantitative characterization of proteins in a mixture. Specifically, the use of liquid chromatography coupled to mass spectrometry (LC-MS/MS), in combination with multiple reaction monitoring (MRM), allows the precise analysis of complex samples in which each component differs by one or more proteotypic peptides (peptides generated by a protease treatment and that are unique to a given protein), charge and hydrophobicity (Gallien et al., 2011). Indeed, the targeted nature of MRM, its high selectivity and wide dynamic range render this technique ideal for quantitative proteomics, especially when combined with known quantities of stable isotope-labelled (SIL) synthetic peptides (Holman et al., 2012; Lange et al., 2008). The process, by which a triple quadrupole mass spectrometer operates in an MRM assay to provide a highly sensitive, specific and cost-effective analysis, is shown schematically in Figure 1.

Here we describe a novel LC-MS/MS-MRM technique that, for the first time, allows reliable quantitative determination of the components of insecticidal crystals of Bt strains. Following verification using artificial mixtures of Cry proteins, we applied the technique to four Bt-based products (Dipel® DF, Xentari® GD, Vectobac® 12S and Novodor®) that are widely used for the control of lepidopteran, dipteran and coleopteran pests. The method is rapid, accurate and more reproducible than the previous analytical techniques developed for this purpose. This technique will also enable improved monitoring of the production processes,

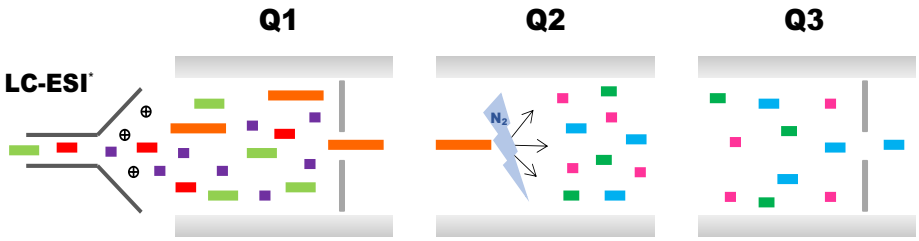

Figure 1. Diagram representing the operation of a triple quadrupole mass spectrometer in an MRM assay. First, peptides are filtered in the first quadrupole (Q1) according to the mass to charge ratio (*m/z*) of the precursor ion. In the collision cell (second quadrupole or Q2), peptides are fragmented by collision-induced dissociation using nitrogen as the collision gas. Finally, predefined peptide-specific fragments are selected in the second mass filter (third quadrupole or Q3), which is followed by measurement of the intensity of the transitions. Transitions are the precursor/product ion pairs, and several transitions are monitored over time for each peptide. *LC-ESI, liquid chromatography coupled to electrospray ionization.

particularly for the optimization of fermentation conditions, control of batch variation, and production of Bt-based products with high potency. It will also provide phytosanitary product registration authorities with precise information on the composition of Bt-based crop protection products.

2. MATERIAL AND METHODS

2.1 Bt recombinant strains expressing a single Cry protein

Three recombinant Bt BMB171 strains, each producing either Cry1Aa, Cry2Aa, or Cry6Aa, were obtained from Dr. Colin Berry, University of Cardiff, UK. Luria-Bertani (LB) medium was used to grow the samples that were incubated at 28 ºC overnight. A single colony of each strain was isolated and grown in 50 ml of CCY medium (Stewart et al., 1981), enriched with mineral salts and pH adjusted to 7.5. Erlenmeyer flasks were used for the 96 h incubation in a shaker-incubator (New Brunswick™ Innova 42R) at 28 ± 1 ºC and 200 rpm. NaCl-EDTA was added in order to obtain a 1 M - 10 mM suspension before centrifugation at 15,000×*g* for 10 min. The resulting pellet was washed twice in cold 1M NaCl, six times in cold Milli-Q water and finally resuspended in 1.5 ml of 10 mM KCl.

2.2 Purification of parasporal crystals

Crystals were purified from the mixture by ultracentrifugation in a discontinuous sucrose gradient, as previously described (Thomas and Ellar, 1983). Briefly, the spore-crystal mixture was sonicated for 20 s using a Soniprep 150 MSE apparatus (Curtin Matheson Scientific, Houston, TX), and immediately loaded onto a two-layer sucrose gradient composed of 16 ml of 67% (w/v) sucrose solution and 16 ml of 79% (w/v) sucrose solution. After centrifugation at 70,000×*g* for 16 h (Optima™ L-100 XP Beckman Coulter, rotor SW 32 Ti), the interphase containing the crystals was recovered using a Pasteur pipette, mixed with sterile Milli-Q water to a final volume of 50 ml, and centrifuged again (15,000×*g*, 15 min). This step was repeated twice, and the crystal pellet was finally resuspended in 1 ml of sterile Milli-Q water. Crystal purity was assessed using phase-contrast microscopy (Zeiss AX-10) under 1000× magnification using immersion oil (data not shown).

2.3 Polyacrylamide gel electrophoresis of Bt crystal proteins

A 100 μl volume of purified crystals was solubilized in 500 μl of a solution of 50 mM Na_2CO_3 (pH 11.3) and 10 mM dithiothreitol (DTT) by gentle agitation for 2 h at 28 ºC. Unsolubilized crystals were removed by centrifugation at 9000×*g* for 10 min at 4 °C. Aliquots (10 μl) of the solubilized proteins in the supernatant, and of the spore and crystal mixture, were then resolved by 10% SDS-PAGE (100:1 acrylamide/bis-acrylamide ratio) at 50 mA for 1 h using a mini-Protean III apparatus (Bio-Rad, Hercules, CA). The gels were stained with 50% (v/v) ethanol, 10% (v/v) acetic acid, and 0.1% (w/v) Coomassie brilliant blue R250 for 40 min, and then destained in a solution of 6.75% (v/v) glacial acetic acid and 9.45% (v/v) ethanol. Protein mass-band patterns were determined by comparison with a broad-range protein marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad).

2.4 Protein quantification and tryptic digestion of Cry proteins

The concentration in the supernatant of the solubilized proteins was determined by the Bradford assay (Bradford, 1976) using bovine serum albumin as a standard in order to perform a tryptic digestion of the Cry proteins sample. Preliminary tests were performed to establish the appropriate digestion protocol for crystal proteins, with urea or RapiGest SF (Waters Corp., Milford, MA) as the

denaturant, followed by one or two-steps of trypsin digestion (data not shown). The following optimal protocol was established. For protein denaturation, 10 μl of denaturing buffer (6 M urea and 100 mM Tris, pH 7.8) was added to previously evaporated crystal samples (protein content of approximately 30 μg). Cysteines were then reduced with 25 mM (final concentration) DTT for 30 min at 37 °C and alkylated with 70 mM (final concentration) iodoacetamide for 30 min in the dark. Unreacted iodoacetamide was neutralized by the addition of 6 μl of the reducing agent DTT and incubated for 30 min at room temperature. The samples were then diluted with 75 μl of 50 mM ammonium bicarbonate so that the final concentration of urea was below 1 M. Digestion was then performed at 37 °C overnight with trypsin (Gold Trypsin, Promega, Madison, WI), at an enzyme:protein weight ratio of 1:20. The reaction was stopped by adding 1 μl of concentrated formic acid (Sigma Aldrich, Saint Louis, MO). Two independent tryptic digestions were performed for each sample.

2.5 Preparation of artificial mixtures of Cry proteins

Two mixtures of Cry proteins were prepared in order to validate the MS-based Cry protein quantification method. In mixture 1, equal molar amounts of each Cry protein were combined (equivalent to a percentage composition of 33.3% each of Cry1Aa, Cry2Aa, and Cry6Aa). In mixture 2, Cry1Aa, Cry2Aa, and Cry6Aa were combined in a molar ratio of 13:4:13, respectively (equivalent to a percentage composition of 43.3% of Cry1Aa, 13.3% of Cry2Aa, and 43.3% of Cry6Aa). Each mixture was trypsinized in duplicate, as described in section 2.4. and then analyzed by LC-MS/MS-MRM.

2.6 Protein identification by LC-MS/MS and *in silico* digestion

Proteins were identified using a nano-LC system (Tempo MDLC, AB Sciex, Framingham, MA) coupled to a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP, AB Sciex). After pre-column desalting, the tryptic digests were separated on a C18 column (Thermo Scientific, Waltham, MA) at a flow rate of 300 nl/min, with a 90-min linear gradient from 5 to 35% acetonitrile in 0.1% formic acid. The mass spectrometer was interfaced with a nanospray source equipped with an uncoated fused silica emitter tip (20-μm inner diameter, 10 μm tip;

NewObjective, Woburn, MA) and was operated in the positive ion mode. The MS source parameters were as follows: capillary voltage, 2800 V; source temperature, 150 °C; declustering potential, 110 V; curtain and ion source gas (nitrogen), 20 psi; and collision gas (nitrogen) set to high. Analyses were performed using an information-dependent acquisition (IDA) method, as follows: single enhanced mass spectra (400–1400 *m*/*z*) were acquired and the eight most intense peaks were automatically chosen by the mass spectrometer and subjected to an enhanced product ion scan. Proteins were identified by using the search engine Mascot (version 2.3, Matrix Science, Boston, MA) to examine the deduced amino acid sequences of the Cry proteins predicted from the genome sequence. The database used for Mascot searches was built in-house. The following search parameters were used: one missed cleavage; carbamidomethylation of cysteines as a fixed modification; 0.5 Da peptide mass tolerance; and 0.3 Da fragment mass tolerance. Two separate LC- MS/MS analyses were performed for each digestion.

An *in silico* trypsin digestion of the predicted Cry protein sequences was performed using MS-Digest, a bioinformatics tool in the software package "Protein Prospector" of the University of California San Francisco (www.prospector.ucsf.edu/prospector/mshome.htm).

2.7 Synthesis of SIL peptides for protein quantification

SIL peptides, synthesized by a stepwise solid-phase peptide synthesis on an automated peptide synthesizer (Multipep, INTAVIS Bioanalytical Instruments, Köln, Germany), were obtained from the Proteomics Facility of the National Biotechnology Center (CNB, Madrid, Spain). Amino acid polymerization was performed using standard *N*-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry and PyBOP/*n*-methylmorpholine as coupling activation reagents. Fmoc-derivatized amino acid monomers were obtained from Merck (San Luis, MO). L-Lysine (^{13}C6; ^{15}N2)- and L-arginine (^{13}C6; ^{15}N4)- preloaded 2-chlorotrityl resins (Cambridge Isotope Laboratories, Tewksbury, MA) were used as a solid support. Once synthesized, the peptides were cleaved from the resin using a scavenger-containing trifluoroacetic acid-water cleavage solution and precipitated by the addition of cold ether. Crude peptides were purified by HPLC (Jasco PU-2089) equipped with a semi-preparative Kromasil C18 column (Teknokroma, Barcelona, Spain). Purity and

labelling efficiency were analyzed by mass spectrometry using a MALDI-TOF/TOF 4800 instrument (AB Sciex) and freeze-dried.

Prior to quantification, SIL peptides, isotopically labelled with either (^{13}C6,^{15}N2) lysine (+8 Da) or (^{13}C6, ^{15}N4) arginine (+10 Da), were reconstituted in 30% acetonitrile and 1% formic acid for a 5 nmol/μl stock solution. The stock solution was further diluted with 2% acetonitrile and 0.1% formic acid, as required.

2.8 Protein quantification by Multiple Reaction Monitoring (MRM)

The development of MRM methods and protein quantification were performed using Skyline (version 4.2.0.19009, MacCoss Lab Software, Seattle, WA). In MRM assays, the first quadrupole (Q1) acts as a *m/z* filter of the precursor ion of the different peptides, and after the fragmentation in the collision cell (Q2), predefined product ions are selected in the second mass filter (Q3). The precursor/product ion pair is called transition (Figure 1). Five transitions were selected for most peptides based on the intensity of y- or b- fragment ions in the MS/MS spectra obtained in the LC-MS/MS analysis described in section 2.6. MRM analyses were conducted using the nano-LC system (AB Sciex) coupled to the 4000QTRAP mass spectrometer (AB Sciex) with the chromatographic and source parameter settings as those described in section 2.6. MRM transitions for each peptide were recorded with a dwell time of 20 ms. Collision energies were automatically computed using the embedded rolling collision energy equations of the Skyline software. To confirm the identity of peptides, an MRM-initiated detection and sequencing (MIDAS) experiment was performed for each peptide. The mass spectrometer was instructed to switch from MRM to enhanced product ion scanning mode when an individual MRM transition signal exceeded 2000 counts. MS/MS data were analyzed using an in-house Mascot server (v 2.3). The data were compared against data deposited in the corresponding in-house database.

For stable isotope dilution, 20–2000 fmoles of SIL peptides (depending on the signal intensity of the endogenous peptide) were spiked into trypsin-digested Cry protein samples. To quantify the tryptic peptides in the Cry samples, the sum of the transition signal intensities of the endogenous peptides was calculated in reference to the sum of the transition intensities of the SIL peptides.

2.9 Purification of Bt crystals from Bt-based insecticides

The crystals of Bt-*kurstaki* ABTS-351 and Bt-*aizawai* ABTS-1857 were recovered directly from water dispersible granules of the commercial products DiPel® DF (Kenogard, Valent BioScience corporation, Barcelona, Spain; manufacturing batch 261-355-PG; manufactured in January 2016) and XenTari® GD (Kenogard; manufacturing batch 261-355-PG; manufacture in January 2016), respectively. Similarly, the crystals of Bt-*israelensis* AM65-52 and Bt-*tenebrionis* NB-176 were recovered directly from suspension concentrate formulations of the commercial products VectoBac® 12AS (Kenogard; manufacturing batch 276-006; manufactured in April 2017) and Novodor® (Kenogard; manufacturing batch 272-803-PG; manufactured in December 2016), respectively. In all cases, a sample of 1 g (solid formulations) or 1 ml (liquid formulations) was taken and washed six times in cold Milli-Q water by centrifugation at 15,000×*g* for 10 min. The final pellet was resuspended in 1 ml of 10 mM KCl and parasporal crystals were then purified as described in 2.2 section.

2.10 Total DNA extraction, genome sequencing and computational analysis

Bt strains ABTS-351, ABTS-1857, AM65-52, and NB-176 were directly isolated from the commercial products DiPel® DF, XenTari® GD, VectoBac® 12AS and Novodor®, respectively. Each bacterial strain was grown at 28 °C for 12 h in 5 ml of sterile CCY medium (Stewart et al., 1981). Total DNA (chromosomal and plasmid DNA) was extracted from vegetative cells following the protocol for DNA extraction from Gram-positive bacteria supplied in Wizard® genomic DNA purification kit (Promega, Madison, WI). Purified DNA samples were used for the preparation of DNA libraries and were sequenced using an Illumina NextSeq500 Sequencer (Genomics Research Hub Laboratory, Cardiff University, UK). The resulting reads were assembled using CLC Genomic Workbench 10.1.1 (Qiagen, Hilden, Germany) with the *de novo* assembly tool. Contigs were then analyzed using BLASTp (Altschul et al., 1990) against a custom insecticidal toxin database constructed using insecticidal protein sequences obtained from the GenBank of the National Center for Biotechnology Information (NCBI) and the BtToxin-scanner (Ye et al., 2012).

2.11 Determination of the relative proportion of Cry and Cyt proteins in Bt commercial products

Protein quality and band patterns were analyzed as described in section 2.3, and quantification and tryptic digestion of the purified crystals was performed (see section 2.4). Protein identification was achieved by LC-MS/MS analyses and *in silico* trypsin digestions were then performed using the amino acid sequences of the proteins identified by IDA analysis (see section 2.6) of Bt strains ABTS-351, ABTS-1857, AM65-52, and NB-176; SIL peptides were synthesized and prepared prior to quantification (see section 2.7); finally, MRM analyses were performed as described in section 2.8 to quantify the protein content of each commercial product.

3. RESULTS

3.1 Validation of the LC-MS/MS-MRM method by analysis of artificial Cry protein mixtures

For the initial validation of the LC-MS/MS-MRM technique, two protein mixtures containing Cry1Aa, Cry2Aa, and Cry6Aa were examined. The three proteins were identified in both Cry protein mixtures via proteotypic peptide detection (Figure 2 and Supplemental material, Table S1).

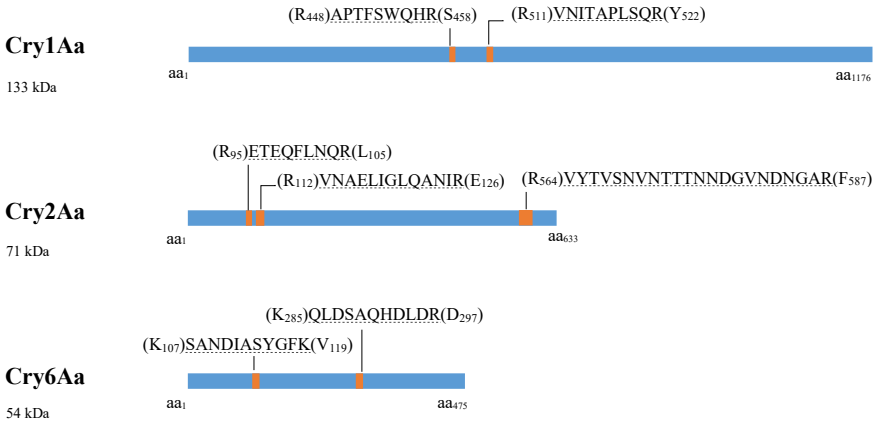

Figure 2. Schematic overview of the targeted proteotypic peptide in three Cry proteins present in known amounts in artificial mixtures used for LC-MS/MS-MRM method validation. These peptides were detected in the IDA analysis and checked against *in silico* digestion results of the artificial protein mixture. Two proteotypic peptides were used to identify Cry1Aa and Cry6Aa, and three to identify Cry2Aa. The location of the proteotypic peptides (orange bands) within full-length proteins (blue bars) are shown, with different letter corresponding to different amino acids and a number indicating the position of the amino acid in the protein. The residues in parentheses are the previous and subsequent amino acid in the protein sequence.

Table 1. Validation of LC-MS/MS-MRM method. Proteins were identified and quantified in two artificial mixtures of three Cry proteins

	Digestion	Cry protein	Proteotypic peptide	Peptide mean ± SD (fmol)	Protein mean ± SD (fmol)	Relative molar composition
Mixture 1	D1	Cry1Aa	APTFSWQHR	2511 ± 410	2226 ± 405	32%
			VNITAPLSQR	1942 ± 32		
		Cry2Aa	ETEQFLNQR	2558 ± 254	1666 ± 724	24%
			VNAELIGLQANIR	1407 ± 155		
			VYTVSNVNTTTNNDGVNDNGAR	1032 ± 28		
		Cry6Aa	QLDSAQHDLDR	3092 ± 51	3126 ± 128	44%
			SANDIASYGFK	3159 ± 204		
	D2	Cry1Aa	APTFSWQHR	2834 ± 103	2402 ± 511	35%
			VNITAPLSQR	1970 ± 157		
		Cry2Aa	ETEQFLNQR	2614 ± 458	1754 ± 772	26%
			VNAELIGLQANIR	1567 ± 39		
			VYTVSNVNTTTNNDGVNDNGAR	1081 ± 561		
		Cry6Aa	QLDSAQHDLDR	2218 ± 283	2654 ± 576	39%
			SANDIASYGFK	3090 ± 392		
Mixture 2	D1	Cry1Aa	APTFSWQHR	4510 ± 854	3630 ± 1172	43%
			VNITAPLSQR	2750 ± 543		
		Cry2Aa	ETEQFLNQR	714 ± 210	483 ± 211	6%
			VNAELIGLQANIR	430 ± 20		
			VYTVSNVNTTTNNDGVNDNGAR	304 ± 55		
		Cry6Aa	QLDSAQHDLDR	4258 ± 416	4380 ± 305	51%
			SANDIASYGFK	4503 ± 213		
	D2	Cry1Aa	APTFSWQHR	5906 ± 1484	4703 ± 1648	46%
			VNITAPLSQR	3501 ± 406		
		Cry2Aa	ETEQFLNQR	732 ± 72	591 ± 155	6%
			VNAELIGLQANIR	561 ± 84		
			VYTVSNVNTTTNNDGVNDNGAR	480 ± 203		
		Cry6Aa	QLDSAQHDLDR	4507 ± 518	4821 ± 486	48%
			SANDIASYGFK	5134 ± 217		

*Cry1Aa, Cry2Aa, and Cry6Aa were mixed in a molar ratio 1:1:1 for mixture 1 (33.3% of each Cry protein) and 13:4:13 for mixture 2 (equivalent to 43.3% of Cry1Aa, 13.3% of Cry2Aa, and 43.3% of Cry6Aa). The mixtures were digested with trypsin and proteins were identified and quantified, based on proteotypic peptide abundance and by comparing the signal intensities of the endogenous and the corresponding SIL peptide. Mean values represent averages of two independent analyses for each tryptic digestion (D1 and D2).

The MS/MS spectra and extracted ion chromatograms (Supplemental material, Figure S1 [A] Cry1Aa, [B] Cry2Aa, and [C] Cry6Aa) and the transitions selected for each target peptide (Supplemental material, Table S2) were determined. SIL peptides were synthesized and used for protein quantification of both artificial mixtures. In mixture 1, which contained equal amounts of each Cry protein, the relative abundance (in molarity) of Cry1Aa was determined at 32–35%, that of Cry2Aa at 24–26%, and that of Cry6Aa at 39–44% (Table 1). In mixture 2, containing Cry1Aa, Cry2Aa, and Cry6Aa in a molar ratio of 13:4:13, the measured relative abundances of the different proteins were also comparable with the expected relative abundances (in molarity) of 43, 13 and 43%, respectively, as follows: Cry1Aa, 43–46%; Cry2Aa, 6%; Cry6Aa, 48–51% (Table 1).

Therefore, using known mixtures of Cry1Aa, Cry2Aa, and Cry6Aa, three aspects of the procedure were validated: (i) all three types of Cry proteins were detected independently of their proportions in the mixture; (ii) the relative abundance determined by LC/MS-MS–based quantification was close to the true abundance of Cry proteins in both mixtures; and (iii) a high degree of reproducibility of the relative abundance values was observed in the analyses of different digestions of samples (Table 1). This indicated that the method was suitable for the quantification of Cry protein mixtures.

3.2 Gene content of the Bt strains in four commercial biopesticides and identification of the proteins present in the parasporal crystals

Prior to the proteomic analysis, it is necessary to determine which proteins could be present in the parasporal crystal according to the genes present in the genome of a given Bt strain. This information is necessary to perform the information-dependent acquisition (IDA) analysis as well as to select the proteotypic peptides that will be used to identify and quantify each crystal protein. Total genomic DNA sequencing revealed the different types of Cry and Cyt proteins encoded by the four strains of Bt present in the commercial products (Table 2). Once the potential composition of the crystals was known, IDA analyses were performed to identify which proteins encoded in the genome of each Bt strain were actually expressed and integrated into the parasporal crystal (Table 3, Supplemental Material Figure S3). In the DiPel® DF sample, peptides from five proteins were

detected, namely, Cry1Aa, Cry1Ab, Cry1Ac, Cry2Aa and Cry2Ab. Five proteins were detected in the parasporal crystal of XenTari® GD: Cry1Aa, Cry1Ab, Cry1Ca, Cry1Da and Cry2Ab. For VectoBac® 12AS, seven different proteins were identified: five Cry proteins (Cry4Aa, Cry4Ba, Cry11Aa, Cry60Aa, Cry60Ba) and two Cyt proteins (Cyt1Aa1, Cyt1Ca). In the Novodor® sample, all the Cry proteins predicted by genome analysis were detected: Cry3Aa, Cry23Aa and Cry37Aa. These results agree with the detection of putative insecticidal proteins by SDS-PAGE electrophoresis of spore-crystal mixtures and solubilized proteins (Supplemental material, Figure S2).

In the IDA analyses, although most proteins were identified by at least four peptides, Cry2Ab in DiPel® DF and Cyt1Ca in VectoBac® 12AS were each detected by a single peptide. Consequently, they were considered to be potential false positives, even though it could be that these proteins were present at concentrations that were below or at the detection limit. Similarly, three insecticidal proteins whose genes were identified by genome sequencing (Table 2), were not detected in the crystals from the commercial Bt insecticides. These were: Cry9Ea in XenTari® GD and Cry10Aa and Cyt2Ba in VectoBac® 12AS. We assume that the respective genes are either not expressed or that these proteins are present at concentrations below the detection threshold of the current method.

Table 2. Insecticidal protein genes identified in the genome of Bt strains isolated from four commercial products.

	DiPel® (ABTS-351)	XenTari® (ABTS-1857)	VectoBac® (AM65-52)	Novodor® (NB-176)
cry1Aa	+	+	-	-
cry1Ab	+	+	-	-
cry1Ac	+	-	-	-
cry1Ca	-	+	-	-
cry1Da	-	+	-	-
cry2Aa	+	-	-	-
cry2Ab	+	+	-	-
cry3Aa	-	-	-	+
cry4Aa	-	-	+	-
cry4Ba	-	-	+	-
cry9Ea	-	+	-	-
cry10Aa	-	-	+	-
cry11Aa	-	-	+	-
cry23Aa	-	-	-	+
cry37Aa	-	-	-	+
cry60Aa	-	-	+	-
cry60Ba	-	-	+	-
cyt1Aa	-	-	+	-
cyt1Ca	-	-	+	-
cyt2Ba	-	-	+	-

*The Bt strains ABTS-351, ABTS-1857, AM65-52, and NB-176 were isolated from the commercial products DiPel® DF, XenTari® GD, VectoBac® 12AS, and Novodor®, respectively. Genomes of these strains were sequenced using an Illumina NextSeq500 Sequencer and the resulting reads were assembled using CLC Genomic Workbench 10.1.1 with the *de novo* assembly tool. Contigs were then analyzed against a custom insecticidal toxin database constructed using insecticidal protein sequences. + indicates presence and – indicates absence

Table 3. Cry and Cyt proteins identified by IDA analysis of Bt strains present in four insecticidal products

Product (Bt strain)	Protein	Accession no.	Number of peptides used for protein identification
DiPel® DF	Cry1Aa	MK184461	6
(ABTS-351)	Cry1Ab	MK184462	7
	Cry1Ac	MK184463	9
	Cry2Aa	MK184464	11
	(Cry2Ab)*	MK184465	(1)
XenTari® GD	Cry1Aa	MK184475	4
(ABTS-1857)	Cry1Ab	MK184476	12
	Cry1Ca	MK184477	12
	Cry1Da	MK184478	4
	Cry2Ab	MK184479	5
VectoBac® 12AS	Cry4Aa	MK184469	3
(AM65-52)	Cry4Ba	MK184470	17
	Cry11Aa	MK184471	17
	Cry60Aa	MK184472	5
	Cry60Ba	MK184473	8
	Cyt1Aa	MK184474	2
	(Cyt1Ca)*	CAD30104	(1)
Novodor®	Cry3Aa	MK184466	16
(NB-176)	Cry23Aa	MK184467	7
	Cry37Aa	MK184468	4

*Proteins in parentheses were detected on the basis of the presence of a single peptide. They were therefore considered to be potential false positives or present at a low concentration, close to the limit of detection of this technique

3.3 Selection of proteotypic peptides and MRM parameters for improved protein quantification

Next, we proceeded to select the proteotypic peptides that were unique for each protein within each mixture and would provide a clear response in the instrument. Proteotypic peptides were identified by *in silico* trypsin digestion of the amino acid sequences of the proteins identified by IDA analysis (Supplemental material, Tables S3–S6). Cry proteins have a high degree of similarity, therefore the selection of target proteotypic peptides depends on the specific composition of each

Bt product. For example, the selected peptides for Cry1Aa in DiPel® DF were not necessarily proteotypic for Cry1Aa in XenTari® GD. Identifying proteins based on a specific list of Bt proteotypic peptides has the advantage of increasing the precision of ensuing identification and quantification.

MRM-initiated detection and sequencing (MIDAS) analyses (see Methods section 2.8*)* were performed to select the best MRM transition, in terms of sensitivity and selectivity, for each peptide. To increase sensitivity, the most intense *m/z* (mass/charge) ratio of each targeted peptide was selected at the first quadrupole, and in order to maximize selectivity, fragment ions with *m/z* ratios higher than those of the precursor ion were monitored. During the design of the MRM assays, several transitions from each peptide were tested, and those with the highest signal intensity and lowest level of interference signals were chosen (Supplemental material, Table S7–S10).

We selected the most suitable proteotypic peptides and MRM transitions for each crystal to enable highly-specific quantification of the Cry and Cyt proteins of interest. At least two proteotypic peptides were selected for each crystal protein in DiPel® DF, XenTari® GD, VectoBac® 12AS, and Novodor® (Supplemental Material, Figure S3[A], [B], [C] and [D], respectively). However, this was not always possible because of different limitations, especially those involving the high similarity between some of these proteins (for more details, see the Discussion section). Application of the developed LC-MS/MS-MRM method for the quantification of crystal proteins in Bt products Having established the proteins present in each sample, we next proceeded to determine the relative composition of the different Bt crystals. The relative molar abundance of each crystal protein in the four commercial Bt-based insecticides is shown in Figure 3 (and Supplemental material, Tables S11–S14). In all cases, the crystals contained a major protein that accounted for approximately 50% of all identified Cry and Cyt proteins. Parasporal crystals from DiPel® DF were found to be mainly composed of Cry2Aa, followed by Cry1Ab and Cry1Aa, and Cry1Ac as a minor component. Crystals from XenTari® GD mainly contained Cry1Ab and Cry1Aa, with Cry1Ca and Cry1Da as minor components. Quantification of Cry2Ab showed residual results and thus, not considered. The major component of VectoBac® 12AS crystals was Cyt1Aa, followed by Cry11Aa, Cry4Ba, and Cry60Ba, with a very low abundance of Cry60Aa and Cry4Aa. Finally,

Cry3Aa was the major protein in Novodor® crystals, followed by the Cry23Aa and Cry37Aa pair, which function as binary toxins (Figure 3). The results demonstrate that the LC-MS/MS-MRM method is suitable for the determination of the crystal protein content of Bt strains with very distinct protein compositions and toxicity spectra.

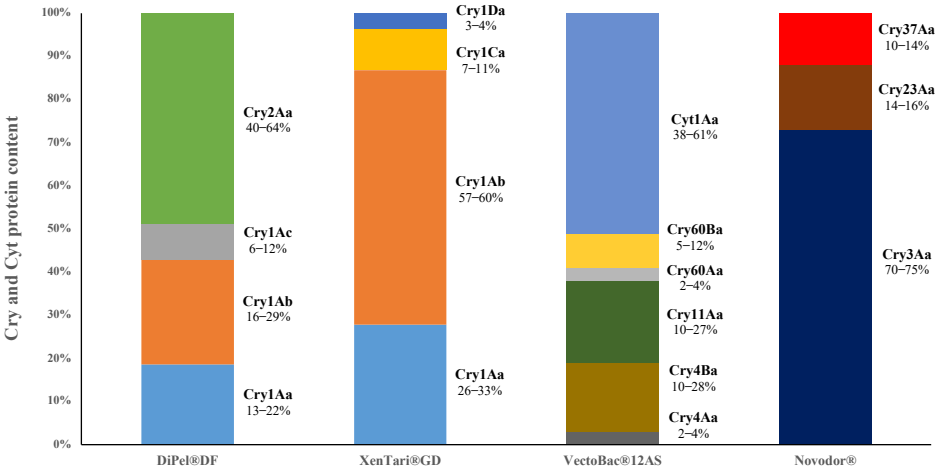

Figure 3. Relative molar composition of proteins in parasporal crystals of Bt-based insecticides. The composition is expressed as a range from two independent tryptic digestions with two technical replicates each.

4. DISCUSSION

In the current study, we developed an LC-MS/MS-MRM method to evaluate the protein composition of parasporal crystals purified directly from Bt-based commercial insecticides or from any Bt product or culture. The novel mass spectrometry method can be used not only to identify the components of the crystal, but also to determine their relative abundance, thus providing a method for qualitative and quantitative characterization of the active ingredients of insecticidal preparations based on Bt.

The set-up of an MRM experiment for the analysis of Bt Cry and Cyt proteins is complex and requires several intermediate steps. The major experimental hurdle to overcome is the complexity of the mixtures of Cry and Cyt proteins, which complicates the selection of suitable proteotypic peptides for the

different crystal proteins in the mixture. This selection can be challenging for the following reasons: (i) the small size of some proteins reduces the number of candidate peptides; (ii) there is a lack of spectral libraries for Bt; and (iii) certain proteins show high amino acid sequence similarity (Brun et al., 2009), which limits the number of candidate proteotypic peptides. Moreover, once the candidate proteotypic peptides are identified by *in silico* analysis, they have to be filtered to fulfill certain analytical criteria. These include sequence length (ideally 6–16 amino acids), chemical stability throughout the analytical process, and good detectability and MS response characteristics (Alves et al., 2011; Kamiie et al., 2008; Kuster et al., 2005). Finally, certain SIL peptides that are too long or with a strong hydrophobic character, can be difficult to synthesize and should be avoided (Hoofnagle et al., 2016). For some Bt proteins these factors make it difficult to select a minimum of two proteotypic peptides for every crystal protein detected, in order to provide highly selective and sensitive assays. Specifically, Cry1Ab and Cry2Aa in the DiPel® DF sample were quantified based on a single proteotypic peptide that was specific for each protein. However, given the novelty of the method developed in this study and the high reproducibility observed in the results obtained from different digestions of the samples, we are confident that the use of a single proteotypic peptide for the quantification of some proteins is a valid approach.

Careful choice of the MRM transitions is critical for the specificity of the assay, and the ability to detect and quantify target peptides (Picotti and Aebersold, 2012). Several transitions for each peptide were monitored in the current study (Supplemental material, Tables S7–S10), which resulted in improved discrimination of the different crystal toxins. Typically, monitoring three or four transitions per peptide provides a suitable balance between selectivity and throughput (Addona et al., 2009; Reiter et al., 2011). For this method, we monitored between 4 and 6 transitions per peptide, and in some cases, we were even able to select transitions for the double and triple-charged precursor ion. Overall, double-charged precursor ions are favoured by electrospray ionization, but in some cases, such as when the peptide sequence contains a histidine residue, triple-charged ions are also favoured (Willard and Kinter, 2001). Because of the lack of spectral libraries for Bt Cry proteins, we had to determine the precursor ion charge state as well as the product ions to be monitored based on the experimental data obtained. In addition, in order

to maximize selectivity and minimize background interference, several features were considered: (i) product ions with *m*/*z* values higher than those of the precursor ion were preferentially selected (Gallien et al., 2011), (ii) for a given MRM method, there were no peptides with the same *m/z* (even for those that differed in amino acid sequence) or retention time, (iii) runs without a spike of SIL peptides were performed previously to confirm the lack of interference in SIL transitions, (iv) the reference intensity dot product, which is the relative intensities of the transitions for endogenous and SIL peptides, had to be similar for each type of peptide. In this way, we ensured that maximum sensitivity and peptide discrimination was achieved.

Common Bt-based products for controlling pests of different orders (Dipel® DF, Xentari® GD, Vectobac® 12S and Novodor®) have been characterized in the current study using the novel validated method. The relative abundance of different Cry and Cyt proteins within the parasporal crystal of the Bt strain used in each product has been revealed for the first time.

DiPel® DF and XenTari® GD products are widely used in the control of lepidopteran pests. Although the former is effective against many leaf-feeding lepidopterans, it is less active against pests from the genus *Spodoptera* (Bravo et al., 2011). According to the manufacturer, the active ingredient of Dipel® DF, Bt strain ABTS-351, harbors the *cry1Aa, cry1Ab, cry1Ac* and *cry2* genes (not specified), and XenTari® GD Bt strain ABTS-1857 contains *cry1Aa, cry1Ab, cry1C and cry1D*. The difference in the gene content explains their spectrum of activity, however, the level of expression of these genes in each product is not given by the manufacturer (www.kenogard.com).

In the current study, we initially corroborated that the genes expressed in Dipel® DF were Cry1Aa, Cry1Ab, Cry1Ac and Cry2Aa. Next, we determined that the protein content in the crystal is distributed approximately evenly between Cry1A and Cry2A proteins (Figure 3). The broad spectrum of toxicity against lepidopterans attributed to Cry2A proteins, along with a balanced contribution of Cry1 proteins, explains the use of this Bt-based product against the recommended target species. In XenTari® GD, we confirmed the expression of Cry1Aa, Cry1Ab, Cry1C and Cry1D, and determined that crystals comprised up to 15% of Cry1C and Cry1D (Figure 3). Despite this small percentage, the occurrence of these two toxins is responsible for

the unique toxicity spectrum of this product, which has high toxicity to species in the genus *Spodoptera* that are major pests of horticultural and other crops (Hernández-Martínez et al., 2008; Porcar et al., 2000).

VectoBac® 12AS, which is based on a Bt serovar *israelensis*, is used for controlling medically-important vectors, such as mosquitoes (Dominic Amalraj et al., 2000). In the present study, we showed that Cry4Aa, Cry4Ba, Cry11Aa, Cry60Aa, Cry60Ba, Cyt1Aa and Cyt1Ca were expressed, but we found no evidence for the presence of Cry10Aa or Cyt2Ba in the commercial product. However, other studies using different techniques have reported small amounts of these proteins in the parasporal crystals of different strains of Bt-*israelensis* (Ben-Dov, 2014). Approximately 90% of the protein content of the crystals in VectoBac® 12AS was found to comprise Cry4Aa, Cry4Ba, Cry11Aa and Cyt1Aa (Figure 3), which are toxins with high larvicidal activity in dipterans when found in a mixture (Crickmore et al., 1995; Monnerat et al., 2014). The low concentration of Cry60Aa and Cry60Ba detected in the current study could suggest that minor crystal components may contribute to the toxicity of the parasporal crystal (Berry et al., 2002), by synergizing the potency of other Cry proteins in the product and may also contribute to the lack of reports of resistance to Bt serovar *israelensis* in dipteran populations subjected to long-term treatment with this type of insecticide (Becker et al., 2018; Ferré and Van Rie, 2002).

Novodor® is widely used for the control of coleopteran pests. We confirmed the expression of three Cry proteins (Figure 3). The major insecticidal protein found in Novodor® crystals was Cry3Aa, which is toxic to insects in the orders Coleoptera, Hemiptera, and Hymenoptera (Van Frankenhuyzen, 2009). Another identified component, Cry23A, is active against certain species of Coleoptera only in the presence of Cry37 (Donovan et al., 2000), which is believed to facilitate binding of the channel-forming toxins to midgut epithelial cells (de Maagd et al., 2003). The observation that the relative abundance of Cry37Aa in Novodor® crystals was similar to that of Cry23Aa supports the notion of a positive interaction between these two proteins.

Although the applicability of this LC-MS/MS-MRM method may be limited by the selection of proteotypic peptides and by the availability of the corresponding

SIL peptides, when proteins with a high homology are present in the same sample, we managed to analyze strains such as Dipel® DF and Xentari® GD that contained several isomorphic proteins in their crystals. Also, the need for genome sequencing of the bacterium no longer poses a limitation to the use of this method as genome sequencing technology is now routine in most laboratories across the world.

In conclusion, the method developed in the current study allows, for the first time, the analysis of novel Bt isolates as well as Bt strains recovered from commercial biopesticides targeted at a diversity of insect pests and vectors of medical importance. Determination of the composition of Bt parasporal crystals can provide direct economic and environmental benefits associated with the production of effective biological insecticides with minimal environmental impact (Hokkanen and Hajek, 2003; Jouzani et al., 2017). Application of the present method to quantify the effect of growth media and fermentation conditions on the production of each insecticidal crystal protein will allow unprecedented control over the insecticidal characteristics of products based on Bt, which is likely to increase the commercial competitiveness of Bt-based products. The novel method could find numerous applications in the development and innovation of Bt-based biological insecticides and provide precise information for Bt production processes and phytosanitary registration authorities responsible for the safety and efficacy of crop protection products based on this uniquely-valuable pathogen.

5. REFERENCES

Addona, T.A., Abbatiello, S.E., Schilling, B., Skates, S.J., Mani, D.R., Bunk, D.M., Spiegelman, C.H., Zimmerman, L.J., Ham, A.J.L., Keshishian, H., Hall, S.C., Allen, S., Blackman, R.K., Borchers, C.H., Buck, C., Cardasis, H.L., Cusack, M.P., Dodder, N.G., Gibson, B.W., Held, J.M., Hiltke, T., Jackson, A., Johansen, E.B., Kinsinger, C.R., Li, J., Mesri, M., Neubert, T.A., Niles, R.K., Pulsipher, T.C., Ransohoff, D., Rodriguez, H., Rudnick, P.A., Smith, D., Tabb, D.L., Tegeler, T.J., Variyath, A.M., Vega-Montoto, L.J., Wahlander, Å., Waldemarson, S., Wang, M., Whiteaker, J.R., Zhao, L., Anderson, N.L., Fisher, S.J., Liebler, D.C., Paulovich, A.G., Regnier, F.E., Tempst, P., Carr, S.A., 2009. Multi-site assessment of the precision and reproducibility of multiple reaction monitoring-based measurements of proteins in plasma. Nat. Biotechnol. 27, 633–641. https://doi.org/10.1038/nbt.1546

Altschul, S., Gish, W., Miller, W., Myers, E., 1990. Basic Local Alignment Search Tool. J. Mol. Biol.

Alves, G., Ogurtsov, A.Y., Yu, Y.K., 2011. Assigning statistical significance to proteotypic peptides via database searches. J. Proteomics 74, 199–211. https://doi.org/10.1016/j.jprot.2010.10.005

Becker, N., Ludwig, M., Su, T., 2018. Lack of resistance in *Aedes vexans* field populations after 36 years of *Bacillus thuringiensis* subsp. *israelensis* applications in the upper Rhine Valley, Germany. J. Am. Mosq. Control Assoc. 34, 154–157. https://doi.org/10.2987/17-6694.1

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

Bernardi, O., Sorgatto, R.J., Barbosa, A.D., Domingues, F.A., Dourado, P.M., Carvalho, R.A., Martinelli, S., Head, G.P., Omoto, C., 2014. Low susceptibility of *Spodoptera cosmioides, Spodoptera eridania* and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) to genetically-modified soybean expressing Cry1Ac protein. Crop Prot. 58, 33–40. https://doi.org/10.1016/j.cropro.2014.01.001

Berry, C., O'Neil, S., Ben-Dov, E., Jones, A.F., Murphy, L., Quail, M.A., Holden, M.T.G., Harris, D., Zaritsky, A., Parkhill, J., 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of *Bacillus thuringiensis* subsp. *israelensis*. Appl. Environ. Microbiol. 68, 5082–5095. https://doi.org/10.1128/AEM.68.10.5082-5095.2002

Bravo, A., 1997. Phylogenetic relationships of *Bacillus thuringiensis* δ-endotoxin family proteins and their functional domains. J. Bacteriol. 179, 2793–2801. https://doi.org/10.1128/jb.179.9.2793-2801.1997

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. https://doi.org/10.1016/j.toxicon.2006.11.022

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. https://doi.org/10.1016/j.ibmb.2011.02.006

Brun, V., Masselon, C., Garin, J., Dupuis, A., 2009. Isotope dilution strategies for absolute quantitative proteomics. J. Proteomics. https://doi.org/10.1016/j.jprot.2009.03.007

Crickmore, N., Bone, E.J., Williams, J.A., Ellar, D.J., 1995. Contribution of the individual components of the δ-endotoxin crystal to the mosquitocidal activity of *Bacillus thuringiensis* subsp. *israelensis*. FEMS Microbiol. Lett. 131, 249–254. https://doi.org/10.1016/0378-1097(95)00264-6

de Maagd, R.A., Bravo, A., Berry, C., Crickmore, N., Schnepf, H.E., 2003. Structure, diversity, and evolution of protein toxins from spore-forming entomopathogenic bacteria. Annu. Rev. Genet. 37, 409–433. https://doi.org/10.1146/annurev.genet.37.110801.143042

Dominic Amalraj, D., Sahu, S.S., Jambulingam, P., Boopathi Doss, P.S., Kalyanasundaram, M., Das, P.K., 2000. Efficacy of aqueous suspension and granular formulations of *Bacillus thuringiensis* (Vectobac) against mosquito vectors. Acta Trop. 75, 243–246. https://doi.org/10.1016/S0001-706X(00)00054-1

Donovan, W.P., Donovan, J.C., Slaney, A.C., 2000. *Bacillus thuringiensis* CryET33 and CryET34 compositions and uses therefor. United States Pat.

Dulmage, H.T., Boening, O.P., Rehnborg, C.S., Hansen, G.D., 1971. A proposed standardized bioassay for formulations of *Bacillus thuringiensis* based on the international unit. J. Invertebr. Pathol. 18, 240–245. https://doi.org/10.1016/0022-2011(71)90151-0

Ferré, J., Van Rie, J., 2002. Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. Annu. Rev. Entomol. 47, 501–533. https://doi.org/10.1146/annurev.ento.47.091201.145234

Floore, T.G., 2006. Mosquito larval control practices: past and present. J. Am. Mosq. Control Assoc. 22, 527–33. https://doi.org/10.2987/8756-971X(2006)22[527:MLCPPA]2.0.CO;2

Gallien, S., Duriez, E., Domon, B., 2011. Selected reaction monitoring applied to proteomics. J. Mass Spectrom. https://doi.org/10.1002/jms.1895

Hernández-Martínez, P., Ferré, J., Escriche, B., 2008. Susceptibility of *Spodoptera exigua* to 9 toxins from *Bacillus thuringiensis*. J. Invertebr. Pathol. 97, 245–250. https://doi.org/10.1016/J.JIP.2007.11.001

Hokkanen, H.M.T., Hajek, A., 2003. Environmental impacts of microbial insecticides: Need and methods for risk assessment. Kluwer Academic.

Holman, S.W., Sims, P.F.G., Eyers, C.E., 2012. The use of selected reaction monitoring in quantitative proteomics. Bioanalysis 4, 1763–1786. https://doi.org/10.4155/bio.12.126

Hoofnagle, A.N., Whiteaker, J.R., Carr, S.A., Kuhn, E., Liu, T., Massoni, S.A., Thomas, S.N., Reid Townsend, R., Zimmerman, L.J., Boja, E., Chen, J., Crimmins, D.L., Davies, S.R., Gao, Y., Hiltke, T.R., Ketchum, K.A., Kinsinger, C.R., Mesri, M., Meyer, M.R., Qian, W.J., Schoenherr, R.M., Scott, M.G., Shi, T., Whiteley, G.R., Wrobel, J.A., Wu, C., Ackermann, B.L., Aebersold, R., Barnidge, D.R., Bunk, D.M., Clarke, N., Fishman, J.B., Grant, R.P., Kusebauch, U., Kushnir, M.M., Lowenthal, M.S., Moritz, R.L., Neubert, H., Patterson, S.D., Rockwood, A.L., Rogers, J., Singh, R.J., Van Eyk, J.E., Wong, S.H., Zhang, S., Chan, D.W., Chen, X., Ellis, M.J., Liebler, D.C., Rodland, K.D., Rodriguez, H., Smith, R.D., Zhang, Z., Zhang, H., Paulovich, A.G., 2016. Recommendations for the generation, quantification, storage, and handling of peptides used for mass spectrometry-based assays. Clin. Chem. 62, 48–69. https://doi.org/10.1373/clinchem.2015.250563

Ibrahim, M.A., Griko, N., Junker, M., Bulla, L.A., 2010. *Bacillus thuringiensis* A genomics and proteomics perspective. Bioeng. Bugs 1, 31–50. https://doi.org/10.4161/bbug.1.1.10519

Jouzani, G.S., Valijanian, E., Sharafi, R., 2017. *Bacillus thuringiensis*: a successful insecticide with new environmental features and tidings. Appl. Microbiol. Biotechnol. https://doi.org/10.1007/s00253-017-8175-y

Kamiie, J., Ohtsuki, S., Iwase, R., Ohmine, K., Katsukura, Y., Yanai, K., Sekine, Y., Uchida, Y., Ito, S., Terasaki, T., 2008. Quantitative atlas of membrane transporter proteins: Development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. Pharm. Res. 25, 1469–1483. https://doi.org/10.1007/s11095-008-9532-4

Kati, H., Sezen, K., Demirbağ, Z., 2007. Characterization of a highly pathogenic *Bacillus thuringiensis* strain isolated from common cockchafer, *Melolontha melolontha*. Folia Microbiol. (Praha). 52, 146–152. https://doi.org/10.1007/BF02932153

Kuster, B., Schirle, M., Mallick, P., Aebersold, R., 2005. Scoring proteomes with proteotypic peptide probes. Nat. Rev. Mol. Cell Biol. https://doi.org/10.1038/nrm1683

Lange, V., Picotti, P., Domon, B., Aebersold, R., 2008. Selected reaction monitoring for quantitative proteomics: A tutorial. Mol. Syst. Biol. https://doi.org/10.1038/msb.2008.61

Li, J., Koni, P.A., Ellar, D.J., 1996. Structure of the mosquitocidal δ-endotoxin CytB from *Bacillus thuringiensis* sp. *kyushuensis* and implications for membrane pore formation. J. Mol. Biol. 257, 129–152. https://doi.org/10.1006/JMBI.1996.0152

Masson, L., Erlandson, M., Puzstai-Carey, M., Brousseau, R., Juárez-Pérez, V., Frutos, R., 1998. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 64, 4782–8.

Monnerat, R., Pereira, E., Teles, B., Martins, E., Praça, L., Queiroz, P., Soberon, M., Bravo, A., Ramos, F., Soares, C.M., 2014. Synergistic activity of *Bacillus thuringiensis* toxins against *Simulium spp.* larvae. J. Invertebr. Pathol. 121, 70–73. https://doi.org/10.1016/J.JIP.2014.07.003

Olson, S., 2015. An analysis of the biopesticide market now and where it is going. Outlooks Pest Manag. 26, 203–206. https://doi.org/10.1564/v26_oct_04

Palma, L., Muñoz, D., Berry, C., Murillo, J., Caballero, P., 2014. *Bacillus thuringiensis* toxins: An overview of their biocidal activity. Toxins (Basel). https://doi.org/10.3390/toxins6123296

Picotti, P., Aebersold, R., 2012. Selected reaction monitoring–based proteomics: workflows, potential, pitfalls and future directions. Nat. Methods 9, 555–566. https://doi.org/10.1038/nmeth.2015

Porcar, M., Juárez-Pérez, V., 2003. PCR-based identification of *Bacillus thuringiensis* pesticidal crystal genes. FEMS Microbiol. Rev. https://doi.org/10.1016/S0168-6445(02)00128-6

Porcar, M., Martinez, C., Caballero, P., 2000. Host range and gene contents of *Bacillus thuringiensis* strains toxic towards *Spodoptera exigua*. Entomol. Exp. Appl. 97, 339–346. https://doi.org/10.1023/A:1004140122226

Pusztai-Carey, M., Carey, P., 1994. Isolation, quantitation and purification of insecticidal proteins from *Bacillus thuringiensis* 13, 274. https://doi.org/10.1016/0734-9750(95)96017-f

Raymond, B., Federici, B.A., 2017. In defence of *Bacillus thuringiensis*, the safest and most successful microbial insecticide available to humanity—a response to EFSA. FEMS Microbiol. Ecol. 93. https://doi.org/10.1093/femsec/fix084

Reiter, L., Rinner, O., Picotti, P., Hüttenhain, R., Beck, M., Brusniak, M.Y., Hengartner, M.O., Aebersold, R., 2011. MProphet: Automated data processing and statistical validation for large-scale SRM experiments. Nat. Methods 8, 430–435. https://doi.org/10.1038/nmeth.1584

Siegel, J.P., 2001. The mammalian safety of *Bacillus thuringiensis*-based insecticides. J. Invertebr. Pathol. 77, 13–21. https://doi.org/10.1006/jipa.2000.5000

Stewart, G.S., Johnstone, K., Hagelberg, E., Ellar, D.J., 1981. Commitment of bacterial spores to germinate A measure of the trigger reaction. Biochem. J. 198, 101–106.

Thomas, W.E., Ellar, D.J., 1983. *Bacillus thuringiensis* var *israelensis* crystal delta-endotoxin: effects on insect and mammalian cells in vitro and in vivo. J. Cell Sci. 60, 181–97.

Van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J. Invertebr. Pathol. 101, 1–16. https://doi.org/10.1016/j.jip.2009.02.009

Willard, B.B., Kinter, M., 2001. Effects of the position of internal histidine residues on the collision-induced fragmentation of triply protonated tryptic peptides. J. Am. Soc. Mass Spectrom. 12, 1262–1271. https://doi.org/10.1016/S1044-0305(01)00312-9

Yamamoto, T., 1983. Identification of entomocidal toxins of *Bacillus thuringiensis* by high performance liquid chromatography. J. Gen. Microbiol. 129, 2595–2603. https://doi.org/10.1099/00221287-129-8-2595

Ye, W., Zhu, L., Liu, Y., Crickmore, N., Peng, D., Ruan, L., Sun, M., 2012. Mining new crystal protein genes from *Bacillus thuringiensis* on the Bbasis of mixed plasmid-enriched genome sequencing and a computational pipeline. Appl. Environ. Microbiol. 78, 4795–4801.

CHAPTER III

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Activity of the individual crystal proteins of *Bacillus thuringiensis* serovar *aizawai* strain ABTS-1857 for *Spodoptera* species: insecticidal effects of artificial mixtures of proteins

Abstract

Bacillus thuringiensis ser. *aizawai* strains are commonly used for the control of *Spodoptera* species. In this study the toxicity of the Bt strain ABTS-1857 (Xentari® GD) was estimated against second instars of *S. exigua* (LC_{50} = 7.8 ng/μl), *S. littoralis* (LC_{50} = 28.0 ng/μl) and *S. frugiperda* (LC_{50} = 120.2 ng/μl). The individual activity of each of the proteins present in the crystal confirmed the efficacy of Cry1Ca and Cry1Da against the tested species, which differed markedly in their susceptibility. Cry1Ca was highly toxic to *S. exigua* (LC_{50} = 3.1 ng/μl) and moderately toxic to *S. littoralis* (LC_{50} = 19.8 ng/μl), whereas Cry1Da was 1.8-fold more toxic to *S. frugiperda* (LC_{50} = 10.3 ng/μl) compared to *S. littoralis* (LC_{50} = 19.6 ng/μl). Artificial mixtures that contained four Cry1 proteins at a prevalence of 29% Cry1Aa, 58.5% Cry1Ab, 9% Cry1Ca and 3.5% Cry1Da did not differ significantly in their toxicity characteristics when compared to the natural mixture present in crystals of the wild-type strain in *S. exigua* (LC_{50} = 9.9 ng/μl) or *S. frugiperda* (LC_{50} = 188.1 ng/μl). Increasing amounts of Cry1Da, in combination with decreasing quantities of either Cry1Aa or Cry1Ab, had no significant effect in the toxicity towards *S. exigua* whereas mortality increased in *S. littoralis* and *S. frugiperda*. We conclude that the composition and prevalence of crystal proteins within the parasporal crystal of a Bt strain is crucial for its insecticidal activity.

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1. INTRODUCTION

Synthetic chemical insecticides have been the most widely used method of pest control over the last 70 years but there is currently growing concern related to their widespread use related to public health, their impact on pollinators and beneficial insect populations and the environment (Carson et al., 2002; Jarman and Ballschmiter, 2012). Insecticides based on entomopathogenic microorganisms have been proposed as an alternative to chemical insecticides for the control of agricultural and forest pests. Among the insecticides of microbial origin, those based on *Bacillus thuringiensis* have experienced the greatest commercial development and are now available in most regions of the world (Sanchis and Bourguet, 2008).

B. thuringiensis (Bt) is a spore forming Gram-positive bacterium that synthetizes a parasporal crystal during the sporulation phase of growth. The parasporal inclusion is formed by the aggregation of crystal toxins (Cry and Cyt), also known as delta-endotoxins, which exhibit insecticidal activity against a wide range of insect pests belonging to the orders Lepidoptera, Coleoptera or Diptera, among others (Schnepf et al., 1998). Since the cloning of the first *cry* gene (Schnepf and Whiteley, 1981), more than 700 insecticidal proteins have been produced and described from strains of Bt (http://www.btnomenclature.info/).

Cry toxins exhibit activity after solubilization and proteolytic activation in the midgut of the insect. Binding to specific receptors located in the brush border of epithelial cells enables toxins to form a lytic pore leading to cell disruption and subsequent insect death (Bravo et al., 2007; Vachon et al., 2012). These receptor interactions are responsible for the high specificity of Bt and a narrower spectrum of action compared to many synthetic insecticides. Despite the huge variety of characterized Bt strains only a few have been exploited commercially, mainly against lepidopteran pests.

The insecticidal activity of a particular Bt strain depends mainly on the proteins that make up the crystal, the relative proportion of each of the proteins and the interactions that occur among the component proteins. The relative proportion of toxins within the crystal is a further step in the characterization of a Bt strain that can provide crucial information on the predicted activity of the isolate prior to testing

in a bioassay. Currently, because of the difficulties faced in the identification and quantification of the different Cry and Cyt proteins in Bt crystals, commercial Bt-based bioinsecticides include no description of the composition of active ingredients and their characterization is based entirely on protein content and their potency against a reference insect species compared with an international Bt standard (Dulmage, 1981).

Recently, we have developed an analytical liquid chromatography-mass spectrometry (LC-MS/MS-MRM) based method to evaluate the protein composition of parasporal crystals purified directly from Bt-based insecticides or from any Bt product or culture. This method can be used not only to identify the components of the crystal, but also to determine their relative abundance, thus providing a method for qualitative and quantitative characterization of the active ingredients of insecticidal preparations based on Bt (Caballero et al., unpublished data). This method has been applied to identify and quantify the proteins that make up the crystal of some of the Bt strains that serve as an active ingredient in some of the most widely used Bt insecticides in the control of agricultural pests including the commercial product Xentari® GD (Kenogard) (Caballero et al., unpublished data). This product uses the Bt strain ABTS-1857 (ser. *aizawai*) as an active ingredient, which is recommended for the control of lepidopteran pests especially those belonging to the genus *Spodoptera* that are tolerant to other Bt-based insecticides.

A total of six *cry* genes were identified in the Bt strain ABTS-1857, but only four of them were expressed in sufficient quantity to be detected, including Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da, which were found in a relative molar ratio of 29:58.5:9:3.5, respectively (Caballero *et al.,* not published). Cry1Ca and Cry1Da are considered to be amongst the most toxic proteins for *Spodoptera exigua, Spodoptera littoralis* and *Spodoptera frugiperda* (BenFarhat-Touzri et al., 2019, 2016; Hernández-Martínez et al., 2008; Silva et al., 2015; Van Frankenhuyzen, 2009; Van Rie et al., 1989).

The aim of this study was to determine the toxicity of each of the individual crystal proteins against *S. exigua, S. littoralis* and *S. frugiperda*. and the influence of protein interactions in artificial mixtures comprising different proportions of each of the toxins on insect mortality.

2. MATERIAL AND METHODS

2.1. Insect rearing

Laboratory colonies of *S. exigua, S. littoralis* and *S. frugiperda* (Lepidoptera: Noctuidae) were maintained in a growth chamber at the Universidad Pública de Navarra under controlled conditions (25 ± 2 °C, 70 ± 5% humidity and 16:8 h light:dark photoperiod). Larvae were reared on a semi-synthetic diet (Greene et al., 1976). Adults had *ad libitum* access to 10% sugar solution. Newly moulted second instar larvae were selected for use in toxicity bioassays.

2.2. Bacterial strains, plasmids and culture media

The Bt strain ABTS-1857, a *B. thuringiensis* ser. *aizawai,* was isolated directly from the commercial product XenTari® GD (Kenogard; manufacturing batch 261-355-PG; manufactured in January 2016). Plasmid pJET (Fermentas) was used for cloning PCR fragments and sequencing. Plasmids pSTAB (Park et al., 1998) and pSTAB-RBS (a modified vector) were used as expression vectors. *E. coli* XL1-Blue cells were used as the cloning host. The acrystalliferous Bt strain BMB171 was used for toxin production and toxicity bioassays. BMB171 containing the empty pSTAB plasmid was used as negative control in bioassay experiments. Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract and 1% NaCl, pH 7) was used to grow both *B. thuringiensis* and *E. coli.* Ampicillin and erythromycin were used (100 µg/ml and 20 µg/ml, respectively) for positive clone selection.

2.3. Total DNA isolation, sequencing and computational analysis

Spores from the strain ABTS-1857 were inoculated on LB agar and incubated at 28 ºC overnight. A single colony was selected and grown at 28 °C for 12 h in 5 ml of sterile LB broth and total DNA was extracted from vegetative cells using the Wizard Genomic DNA Purification Kit (Promega) following the instructions for DNA isolation from Gram-positive bacteria. Purified total DNA was used as template for gene cloning.

2.4. PCR amplification of insecticidal genes

Specific oligonucleotide primer pairs were designed to amplify the open reading frames (ORF) of *cry1Aa, cry1Ab, cry1Ca* and *cry1Da* based on the

published sequence of each gene (GenBank AAA22353, X54939, M73251 and X54160, respectively). Primers included restriction sites for cloning (Table 1). DNA extracted from the strain ABTS-1857 was used as template in a 50 µl reaction mixture containing 10 µl of 5x HF reaction buffer, 1 µl of 100 mM dNTPs, 1 µl of each forward and reverse primer (10 µM), 0.5 U Phusion High-fidelity DNA polymerase (NEB) and 100 ng of total DNA. PCR cycling conditions were: 1 initial denaturation cycle at 98 °C for 30 s, 30 amplification cycles of 98 °C for 10 s, 55 °C for 1 min and 72 °C for 3 min and 30 s, followed by a final extension step at 72 °C for 10 min in a C1000 Touch thermal cycler (Bio-Rad). The PCR products were analyzed by electrophoresis on 1% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA, pH 8) at 100 V for 30 min.

Table 1. Primer sequences with restriction enzyme cleavage sites targeted at the *cry1Aa, cry1Ab, cry1Ca* and *cry1Da* genes of *B. thuringiensis* ser. *aizawai* strain ABTS-1857.

Primer and restriction enzyme*	Sequence of primer
F-Cry1A-S*all*	5'GGTCGACGGTATCTTAATAAAAGAGATGGAG3'
F-Cry1Ca-*Nco*I	5'CCCATGGAGGAAAATAATCAAAATCAATG3'
F-Cry1Da-S*all*	5'GGTCGACGGACTTTAGTAATTTAATAAAAAAAGGG3'
R-Cry1-P*ael*	5'GCATGCTTATT-CCTCCATAAGGAGTAATTCCAC3'
F-Seq-Cry1A	5'GATGAATTTTGTCTGGATGAA3'
R-Seq-Cry1A	5'GATAGCACTCATCAAAGGTAC3'
F-Seq-Cry1C	5'GGGGAGAACTTAACATCTAG3'
F-Seq-Cry1D	5'GCTCATACAACACTCTTCAC3'
R-Seq-Cry1C-D	5'GAAGTAAACAGGGCATTCAC3'

*Restriction enzyme cleavage sites are underlined. F: specific forward primer; R: specific reverse primer; Seq: specific intermediate primer.

2.5. Cloning and transformation of insecticidal genes

The PCR products corresponding to *cry1Aa, cry1Ab, cry1Ca* and *cry1Da* CDS were purified from the agarose gel with NucleoSpin Gel and PCR clean up kit (Macherey-Nagel Inc., Bethlehem, PA) and cloned bluntly into the pJET vector (CloneJET PCR Cloning Kit, Fermentas, Canada) to obtain the pJET-protoxin constructions (pJET-1Aa, pJET-1Ab, pJET-1Ca and p-JET-1Da). Transformation of *E. coli* XL1-Blue was performed following a standard protocol (Sambrook et al., 1989) for recombinant plasmid production containing each of the *cry* gene CDS.

Putative positive clones were checked by PCR in a 20 µl reaction mixture containing 2 µl of 10x NH4 reaction buffer, 1 µl 50 mM $MgCl_2$, 1 µl of 100 mM dNTPs, 1 µl of each forward and reverse primer (10 µM), and 0.2 µl of BioTaq Polymerse (Bioline). *E. coli* XL1-Blue cells harbouring the pJET constructions were cultured in 5 ml LB broth supplemented with 100 µg/mL ampicillin at 37 °C and with agitation at 200 rpm overnight. Recombinant plasmids were extracted with a NucleoSpin plasmid kit (Macherey-Nagel Inc., Bethlehem, PA) according to the manufacturer's protocol and verified by sequencing using an intermediate forward primer for each gene and the forward and reverse primers for the pJET vector (StabVida, Caparica, Portugal). Nucleotide sequences were aligned in SnapGene with those of toxins available in the GenBank database. Digestion of the pJET-protoxin plasmids were performed with the corresponding combination of restriction enzymes (Thermo Scientific). Subsequent purification of digestion products, previously resolved in an agarose gel, was performed using a NucleoSpin Gel and PCR clean up kit (Macherey-Nagel Inc., Bethlehem, PA). The pSTAB/pSTAB-RBS vector, previously digested with the corresponding restriction enzymes, was used as receptor for the inserts (pSTAB for *cry1Aa, cry1Ab* and *cry1Da;* pSTAB-RBS for *cry1Ca)* using the Rapid DNA ligation kit (Thermo Scientific) to obtain recombinant plasmids with the pSTAB/pSTAB-RBS-protoxin constructions. *E. coli* XL1-Blue cells were transformed with the ligation products as described before, and putative positive clones were checked by colony PCR prior to plasmid extraction. An acrystalliferous Bt strain, BMB171 (Li et al., 2000) was used as receptor in a transformation process performed as described previously (Cucarella et al., 2001). Putative positive clones were checked by colony PCR.

2.6. Insecticidal protein expression

The *B. thuringiensis* ser. *aizawai* strain ABTS-1857 and each of the BMB171-protoxin constructions were cultured in 50 ml CCY medium supplemented with 20 µg/ml erythromycin for the recombinant strains, in a rotary shaker set at 28 °C and 200 rpm for two-three days, until sporulation and lysis of 95% of the cells was observed. Inclusion bodies were observed by phase contrast microscopy at x1000 magnification. Spore and crystal mixtures were then centrifuged at 9000×*g* for 10 min at 4 °C. Pellets were washed three times in 1 M NaCl, six times in cold

distilled water and finally resuspended in 1.5 ml of 10 mM KCl. A volume (200 µl) was pelleted and then solubilized in 200 µl of 50 mM Na_2CO_3 (pH 11.3) and 10 mM dithiothreitol (DTT) solution by gentle agitation during 2 h at 37 ºC. Non-solubilized crystals were removed by centrifugation at 9000×*g* for 10 min at 4 °C. An aliquot (10 µl) was used for protein quantification by Bradford assay (Bradford, 1976) (Bio-Rad) using bovine serum albumin (BSA) as standard. For quality control, spore and crystal mixtures and solubilized proteins were resolved using a Criterion TGX™ 4-20% Precast Gel (BIO-RAD) at 50 mA for 1 h. The gels were stained with Coomassie brilliant blue R250 (Bio-Rad) for 40 min, and then distained in a solution of 10% (v/v) glacial acetic acid and 30% (v/v) ethanol. Protein mass-band patterns were determined by comparison with a broad-range protein marker (Precision Plus Protein™ Dual Colour Standards, Bio-Rad).

2.7. Protein toxicity bioassay

To determine the concentration-mortality response of insects inoculated with the wild-type Bt strain ABTS-1857, the individual Cry toxins and the protein mixtures, a total of six concentrations of solubilized crystals were prepared from the original solubilized protein stock to obtain final concentrations of 300, 100, 33.3, 11.1, 3.7, 1.4 ng/µl. Bioassays were performed using groups of 28 recently molted *S. exigua, S. littoralis* and *S. frugiperda* second instars using the droplet feeding method (Hughes and Wood, 1981). Toxicity experiments were replicated three times on different days using independent spore and crystal preparations. Combinations of two, three or four solubilized proteins were also prepared and tested in three independent replicates according to their relative abundance in the parasporal crystal of the strain ABTS-1857 (29% Cry1Aa, 58.5% Cry1Ab, 9% Cry1Ca and 3.5% Cry1Da), determined previously (Caballero et al., ms in revision). A total of 10 replicates using a single concentration (100 ng/µl) were performed to estimate percentage of mortality of the natural strain and the artificial mixture containing all four Cry1 proteins. Increasing amounts of the Cry1Da protein, ranging from 3.5 to 20 ng/µl, with corresponding reductions in Cry1Aa or Cry1Ab, were also tested for the three species, performing up to three independent bioassays. The strain BMB171 containing an empty pSTAB vector served as negative control. Larvae that

consumed inoculum suspension within a 10 min period were incubated individually on semi-synthetic diet at 25 ± 2 °C for 5 days and were then checked for mortality.

Concentration-mortality data were subjected to Probit analysis (Finney, 1971) using the POLO-PC software (LeOra Software, 1987). Mean lethal concentration (LC_{50}) values were considered to differ significantly if their 95% fiducial limits did not overlap. A paired t-test (SAS Institute, 1999) was used to compare the percentage of mortality obtained with the artificial mixture that contained the four Cry1 proteins with that obtained for the natural crystal when tested at 100 ng/μl against the three insect species.

3. RESULTS

3.1. Cloning and expression of single insecticidal genes

PCR amplification, with specific oligonucleotide primers for *cry1Aa, cry1Ab, cry1Ca* and *cry1Da* genes, resulted in amplicons of 3574, 3468, 3570 and 3497 bp, respectively. These sequences had 99% similarity with the corresponding sequences from GenBank (data not shown). In all cases, the recombinant strain BMB171 that individually expressed the *cry1Aa*, *cry1Ab*, *cry1Ca*, and *cry1Da* genes had a bipyramidal crystal located at the opposite end of the cell to that of the endospore. Protein profiles of the Bt wild-type isolate and the individual recombinant Cry toxins showed protein bands migrating at 130 kDa in all cases, which correspond to the expected size of Cry1 protoxins. A second band at 60-70 kDa appeared clearly in the wild-type isolate (Figure 1, lanes 1 and 2) and also in individual Cry1 crystal protein (Figure 1, lanes 5, 6 and 9-12) probably as the result of proteolysis. Single proteins showed a double band pattern (100-130 kDa), probably due to a partial proteolysis (Figure 1).

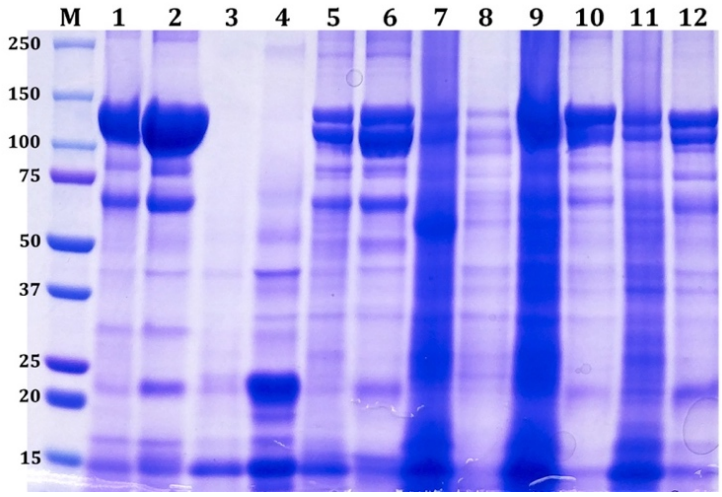


Figure 1. SDS-PAGE of *B. thuringiensis* ser. *aizawai* strain ABTS-1857 and its individual crystal proteins. M. Molecular weight marker; 1. ABTS-1857 spore/crystal mixture; 2. ABTS-1857 solubilized crystal proteins; 3. BMB171 acrystalliferous Bt strain spores; 4. Acrystalliferous Bt strain BMB171 after solubilization; 5. Cry1Aa spore/crystal mixture; 6. Cry1Aa solubilized crystal proteins; 7. Cry1Ab spore/crystal mixture; 8. Cry1Ab solubilized crystal proteins; 9. Cry1Ca spore/crystal mixture; 10. Cry1Ca solubilized crystal proteins; 11. Cry1Da spore/crystal mixture; 12. Cry1Da solubilized crystal proteins.

3.2. Toxicity of the Bt strain ABTS-1857 for *Spodoptera* species

The concentration-mortality results showed significant differences in the LC_{50} values of strain ABTS-1857 among the three insect species, which ranged from 7.8 to 120.2 ng/μl. This strain exhibited high toxicity towards *S. exigua* and *S. littoralis*, whereas *S. frugiperda* larvae were less susceptible (Table 2). In the control treatments larval mortality was less than 10% in all cases.

Table 2. Probit regression of concentration-mortality results for *S. exigua, S. littoralis and S. frugiperda* second instars treated with strain ABTS-1857. The median lethal concentration (LC_{50}) and relative potency values were calculated for each species.

Spodoptera	Regression		LC_{50} (ng/μl)	Relative Potency	F.L 95%		χ^2	df
	Slope ± SE	Intercept			Low	High		
exigua	1.0 ± 0.1	3.9	**7.8**	14.5	9.7	21.8	2.0	3
littoralis	2.2 ± 0.3	1.7	**28.0**	4.3	2.9	6.2	3.7	3
frugiperda	1.0 ± 0.1	2.9	**120.2**	1	-	-	0.5	3

F.L: 95% fiducial limits; χ^2: goodness-of-fit test; df: degrees freedom

3.3. Toxicity of the recombinant Cry1 proteins

The toxicity results of the individual Cry1Aa, Cry1Ab, Cry1Ca, and Cry1Da proteins were subjected to probit regression for *S. exigua*, *S. littoralis* and *S. frugiperda* second instars (Table 3). Crystal proteins Cry1Aa and Cry1Ab only showed little activity against *S. exigua* (data not shown), but the estimated LC_{50} value for all three species tested in this study consistently exceeded the highest concentration tested, 300 ng/μl. Cry1Ca was the most active protein for *S. exigua* larvae with an estimated LC_{50} of 3.1 ng/μl, and also showed moderate activity against *S. littoralis* larvae (LC_{50} = 19.8 ng/μl), but was not active for *S. frugiperda* larvae at the highest concentration of 300 ng/μl. Cry1Da showed low activity against *S. exigua* larvae and moderate toxicity to *S. littoralis* (LC_{50} = 19.6 ng/μl). This was the only active toxin against *S. frugiperda* with an estimated LC_{50} of 10.3 ng/μl (Table 3).

Table 3. Probit regression of concentration-mortality results for *S. exigua, S. littoralis and S. frugiperda* second instars treated with recombinant Cry1Aa, Cry1Ab, Cry1Ca, and Cry1Da proteins. Mean lethal concentration (LC_{50}) and relative potency values were calculated for each species. Missing values indicate that insect mortality did no-t exceed 50% at the highest concentration tested.

Species	Cry1	Regression line		LC_{50}	Relative	F.L 95%		χ^2
		Slope ± SE	Intercept	(ng/μl)	Potency	Low	High	
S. exigua	Aa	0.5 ± 0.2	3.7	**>300**	-	-	-	-
	Ab	1.5 ± 0.4	1.7	**>300**	-	-	-	-
	Ca	1.2 ± 0.1	4.4	**3.1**	27	9.7	75.6	13.3
	Da	0.5 ± 0.1	4.1	**83.0**	1	-	-	1.8
S. littoralis	Aa	14.1 ± 0.1	-25	**>300**	-	-	-	.
	Ab	13.6 ± 0.1	-24	**>300**	-	-	-	.
	Ca	1.5 ± 0.1	3.1	**19.8**	1	-	-	2.4
	Da	1.2 ± 0.1	4.4	**19.6**	1	0.8	1.34	1.9
S. frugiperda	Aa	-	-	**>300**	-	-	-	.
	Ab	-	-	**>300**	-	-	-	.
	Ca	-	-	**>300**	-	-	-	.
	Da	1.5 ± 0.2	4.6	**10.3**	1	-	-	0.6

SE: standard error; Int: intercept; F.L: fiducial limits; χ^2: goodness-of-fit with 3 degrees of freedom.

3.4. Toxicity of the different recombinant protein combinations

Different mixtures of recombinant proteins, in relative proportions similar to that found in the crystal produced by wild-type strain ABTS-1857, were tested for protein interactions. In *S. exigua*, recombinant protein mixtures containing two solubilized protoxins showed that those containing the Cry1Ca protein produced the highest mortalities (71-74%), whereas the lowest mortalities (<27%) were registered in mixtures that did not include Cry1Ca (Table 4).

Table 4. Percentage of mortality observed in *S. exigua, S. littoralis* and *S. frugiperda* second instars following inoculation with different mixtures of recombinant proteins.

Cry1 mixtures	Concentration (ng/μl)	Mortality ± SE (%)		
		S. exigua	*S. littoralis*	*S. frugiperda*
Aa + Ab	29 + 58.5	26 ± 9.4	5 ± 7.1	1.6 ± 2.6
Aa + Ca	29 + 9	71 ± 18.5	35 ± 18.6	5 ± 2.1
Ab + Ca	58.5 + 9	74 ± 11.5	45 ± 21.7	0 ± 0
Aa + Da	29 + 3.5	27 ± 18.2	46 ± 18.2	30 ± 5.0
Ab + Da	58.5 + 3.5	7 ± 1.8	47 ± 15.0	33 ± 4.2
Ca + Da	9 + 3.5	71 ± 14.5	71 ± 18.7	33 ± 12.0
Aa + Ab + Ca	29 + 58.5 + 9	80 ± 9.0	57 ± 22.4	7 ± 5.7
Aa + Ab + Da	29 + 58.5 + 3.5	33 ± 10.5	54 ± 19.8	21 ± 6.2
Aa + Ca + Da	29 + 9 + 3.5	62 ± 18.4	77 ± 13.1	31 ± 6.0
Ab + Ca + Da	58.5 + 9 + 3.5	86 ± 9.1	81 ± 10.3	33 ± 10.0
Aa + Ab + Ca + Da	29 + 58.5 + 9 + 3.5	85 ± 10.6	76 ± 16.5	38 ± 9.7

Similar results were obtained when mixtures that included three recombinant proteins were used, where Cry1Ca appeared to be the determinant toxic component for mortality in *S. exigua* larvae, with 62-86% mortality (Table 4). The absence of Cry1Ca showed a negative impact in mortality (33%). Finally, the artificial combination that reflects the natural mixture occurring in the parasporal crystal of the strain ABTS-1857 resulted in 85% mortality, similar to the 93% mortality observed when 100 ng/μl of the solubilized crystal proteins of the wild-type isolate were used to inoculate *S. exigua* larvae ($p = 0.31$).

In *S. littoralis* the mixture of recombinant proteins Cry1Aa + Cry1Ab resulted in larval mortalities of 5% at the tested concentration (Table 4). However, mixtures of two proteins containing Cry1Ca or Cry1Da resulted in moderate mortality (35-47%), while the Cry1Ca + Cry1Da mixture produced the highest mortality (71%) (Table 4). The same trend was observed in combinations of three proteins although, in general, higher percentages of mortality (54-81%) were observed. No statistical differences were observed when *S. littoralis* larvae were treated with the natural mixture or an artificial combination of the four expressed proteins, scoring 76% mortality when compared to the solubilized wildtype crystal (85%) ($p = 0.30$). In *S. frugiperda* no mortality was registered for the Cry1Aa + Cry1Ab combination. Only protein combinations of either two or three toxins that included Cry1Da resulted in mortality ranging from 21 to 33% (Table 4). Similar mortality for the artificial mixture (38%) was obtained when compared to the natural solubilized crystal (40%) ($p = 0.24$).

3.5. Toxicity of the natural and the predicted artificial protein mixture

The LC_{50} for the artificial mixture containing the four proteins at their predicted abundance was estimated for *S. exigua* and *S. frugiperda* second instars and compared to that obtained for the natural mixture (Table 2). For *S. exigua* the LC_{50} value for the artificial mixture was 9.9 ng/μl and was 188.1 ng/μl for *S. frugiperda* compared to 7.8 and 120.2 ng/μl for the natural mixture, respectively (Table 5).

Table 5. Mean lethal concentration (LC_{50}) and relative potency (R. P.) values for the natural and artificial mixture of proteins expressed in the Bt strain ABTS-1857 at the estimated relative abundance for *S. exigua* and *S. frugiperda* second instars.

Species	Mixture	Regression		LC_{50} (ng/μl)	Relative Potency	95% F.L.		χ^2
		Slope ± SE	Int.			Low	High	
S. exigua	Natural	1.1 ± 0.1	4.0	**7.8**	1.3	0.8	2.1	1.6
	Artificial	0.9 ± 0.1	4.2	**9.9**	1	-	-	2.5
S. frugiperda	Natural	1.0 ± 0.1	3.0	**120.2**	1.6	0.6	3.8	0.7
	Artificial	1.0 ± 0.2	2.7	**188.1**	1	-	-	1.4

F.L: fiducial limits; χ^2 goodness of fit test with 3 degrees of freedom.

3.6. Toxicity effects of increased amounts of Cry1Da

Due to the low proportion of Cry1Da in the parasporal crystal, different combinations containing increasing amounts of this toxin with corresponding reductions in Cry1Aa and Cry1Ab, were tested against second instar larvae of the three *Spodoptera* species (Table 6). For *S. exigua* no enhanced activity was observed, with mortality that varied from 92% for the original mixture to 88-92% at the highest dose (mixture containing 20 ng/μl of Cry1Da). For *S. littoralis* mortality increased from 76% to 86-87% in mixtures containing 10 ng/μl of Cry1Da and to 96-99% when the mixture contained 20 ng/μl of this toxin. Finally, a marked increase was detected for *S. frugiperda* when larvae were inoculated with mixtures containing 10 ng/μl (52-53% mortality) or 20 ng/μl (82-83% mortality) of Cry1Da (Table 6).

Table 6. Percentages of mortality of *S. exigua, S. littoralis* and *S. frugiperda* second instars inoculated with artificial mixtures containing increased amounts of recombinant Cry1Da (total Cry protein concentration of 100 ng/μl).

Cry1 protein mixture	Mortality ± SE (%)		
(Aa + Ab + Ca + **Da**)	*S. exigua*	*S. littoralis*	*S. frugiperda*
29 + 58.5 + 9 + **3.5**	92 ± 7.5	76 ± 3.7	35 ± 5.0
22.5 + 58.5 + 9 + **10**	86 ± 4.9	87 ± 1.5	53 ± 3.0
29 + 52 + 9 + **10**	84 ± 7.5	86 ± 7.0	52 ± 6.5
12.5 + 58.5 + 9 + **20**	92 ± 4.0	96 ± 5.5	83 ± 4.9
29 + 42 + 9 + **20**	88 ± 12	99 ± 1.3	82 ± 6.5

4. DISCUSSION

In this study, the toxicity of the Bt strain ABTS-1857 and of each of the four proteins that make up its crystal (Cry1Aa, Cry1Ab, Cry1Ca, and Cry1Da), was determined in *S. exigua*, *S. littoralis* and *S. frugiperda* larvae. The contribution of each of the expressed proteins to the toxicity of different artificial mixtures that included two, three, or four proteins was also elucidated. *S. exigua* larvae, was the most susceptible species for solubilized crystals from the Bt strain ABTS-1857, followed by *S. littoralis* and *S. frugiperda* as the least susceptible species, in line with the results of published previously (Hernández-Martínez et al., 2010; Horikoshi et al., 2019). The composition of the parasporal crystal of the strain ABTS-1857, was determined by proteomic analysis by which Cry1Aa, Cry1Ab, Cry1Ca, and

Cry1Da were quantified in relative proportions of 0.29, 0.585, 0.09, and 0.035, respectively (Caballero et al., unpublished data).

Since the toxicity of a Bt isolate is due to the combination of different Cry proteins we first attempted to determine the concentration-mortality data of each of the individual Cry proteins in order to identify the protein or proteins that constitute the main mortality factor for each of the three species of *Spodoptera.* Cry1Aa and Cry1Ab, despite being the proteins with major representation in the crystal (29 and 58,5%, respectively), showed low or no activity against the tested insects, as consistent with the results of previous studies (Xue et al., 2005; Yinghua et al., 2017). Nevertheless, the two remaining proteins (Cry1Ca and Cry1Da), which represented just 12.5% of total toxin produced by the strain ABTS-1857, were both toxic to all three *Spodoptera* species, as reported previously (BenFarhat-Touzri et al., 2019; Hernández-Martínez et al., 2008; Jakka et al., 2014; Valicente et al., 2010; Van Frankenhuyzen, 2009). Toxicity of the Bt isolate can be attributed to the presence of both toxins, which are considered among the most toxic insecticidal proteins against *Spodoptera* species (Van Rie et al., 1989). Proteins with high insecticidal activity, albeit at a low abundance in the crystal, have been also reported by others (Masson et al., 1998). Rather than low expression per se, small amounts of these proteins might be required in order to function synergistically with other toxins (BenFarhat-Touzri et al., 2018; Chang et al., 2001).

The three *Spodoptera* species tested in our study were not all equally susceptible to both proteins. Whether *S. exigua* showed to be very susceptible to Cry1Ca, *S. littoralis* was 3.3-fold more tolerant and *S. frugiperda* was resistant to this toxin in our study. In contrast, Cry1Da had low toxicity against the beet armyworm, but was highly toxic to the other species of *Spodoptera*. Determining the most toxic protein for each species can provide useful information for the development of future insecticidal products or engineering of transgenic plants (Christov et al., 2005; Lin et al., 2003).

Proteins with no activity when inoculated individually may be very active when combined in a determined proportion (Ben-Dov, 2014; Monnerat et al., 2014). Consequently, we tested mixtures of 2 - 4 proteins to identify the most toxic combinations. Mixtures that included Cry1Ca where the most active against *S.*

exigua larvae (62-86% mortality) and *S. littoralis* (35-81% mortality), whereas mixtures containing Cry1Da appeared to be the most toxic to *S. littoralis* (46-81%) and *S. frugiperda* (21-33% mortality). A previous study reported toxicity of Cry1Ca against *S. frugiperda* (Soares Figueiredo et al., 2019). Lastly, a combination of the four proteins in the proportions in which they are found in the wild-type strain did not differ significantly in toxicity compared to the wild-type crystal. Furthermore, the toxicity of the artificial mixture was estimated for *S. exigua* and *S. frugiperda* larvae and no significant differences were detected for either of these species. The low proportion of Cry1Da, despite its high toxicity towards some *Spodoptera* species, suggests that this toxin was only required in small amounts in order to exert toxic activity (Chang et al., 2001; Masson et al., 1998). However, when Cry1Da was supplemented in the natural mixture, enhanced toxicity was detected in *S. littoralis* (BenFarhat-Touzri et al., 2018). We examined the effect of increasing the amount of Cry1Da with corresponding reductions in Cry1Aa or Cry1Ab. No effect was detected in *S. exigua,* probably due to the low performance of the toxin. However, increased mortality was recorded in *S. littoralis* and *S. frugiperda* when the concentration of Cry1Da was present at 10 or 20 ng/μl, whereas no differences were detected when the substituted proteins were Cry1Aa or Cry1Ab.

In conclusion, experimental evidence on the role of each protein alone and in mixtures in the insecticidal activity of the wild-type strain was elucidated. We also corroborated previous findings on the relative proportions of Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da in Bt ser. *aizawai* strain ABTS-1857 in terms of insect mortality when testing artificial mixtures containing the four solubilized crystal proteins compared to the toxicity of the natural solubilized crystal.

5. REFERENCES

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

BenFarhat-Touzri, D., Driss, F., Jemli, S., Tounsi, S., 2018. Molecular characterization of Cry1D-133 toxin from *Bacillus thuringiensis* strain HD133 and its toxicity against *Spodoptera littoralis*. Int. J. Biol. Macromol. 112, 1–6. https://doi.org/10.1016/J.IJBIOMAC.2018.01.081

BenFarhat-Touzri, D., Driss, F., Tounsi, S., 2016. A promising HD133-like strain of *Bacillus thuringiensis* with dual efficiency to the two Lepidopteran pests: *Spodoptera littoralis* (Noctuidae) and *Ephestia küehniella* (Pyralidae). Toxicon 118, 112–120.

https://doi.org/10.1016/J.TOXICON.2016.04.045

BenFarhat-Touzri, D., Jemli, S., Driss, F., Tounsi, S., 2019. Molecular and structural characterization of a novel Cry1D toxin from *Bacillus thuringiensis* with high toxicity to *Spodoptera littoralis* (Lepidoptera: Noctuidae). Int. J. Biol. Macromol. 126, 969–976. https://doi.org/10.1016/J.IJBIOMAC.2018.12.175

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Bravo, A., Gill, S.S., Soberón, M., 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. Toxicon. https://doi.org/10.1016/j.toxicon.2006.11.022

Carson, R., Wilson, E.O., Lear, L.J., 2002. Silent Spring. Houghton Mifflin.

Chang, L., Grant, R., Aronson, A., 2001. Regulation of the packaging of *Bacillus thuringiensis* δ-endotoxins into inclusions. Appl. Environ. Microbiol. 67, 5032–5036. https://doi.org/10.1128/AEM.67.11.5032

Christov, N.K., Imaishi, H., Ohkawa, H., 2005. Green-tissue-specific expression of a reconstructed Cry1C gene encoding the active fragment of *Bacillus thuringiensis* δ-endotoxin in Hhploid tobacco plants conferring resistance to S*podoptera litura*. Biosci. Biotechnol. Biochem. 63, 1433–1444. https://doi.org/10.1271/bbb.63.1433

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penadés, J.., 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. J. Bacteriol. 183, 2888–2896.

Dulmage, H.T., 1981. Insecticidal activity of isolated of *Bacillus thuringiensis* and their potential for pest control. Microb. Control pests plant Dis. 1970–1980, Ed.H.D Burges, Academic Press, London.

Finney, D., 1971. Probit analysis. 3rd Ed. Cambridge Univ. Press 333.

Greene, G.L., Leppla, N.C., Dickerson, W.A., 1976. Velvetbean caterpillar: A rearing procedure and artificial medium. J. Econ. Entomol. 69, 487–488. https://doi.org/10.1093/jee/69.4.487

Hernández-Martínez, P., Ferré, J., Escriche, B., 2008. Susceptibility of *Spodoptera exigua* to 9 toxins from *Bacillus thuringiensis*. J. Invertebr. Pathol. 97, 245–250. https://doi.org/10.1016/J.JIP.2007.11.001

Hernández-Martínez, P., Navarro-Cerrillo, G., Caccia, S., de Maagd, R.A., Moar, W.J., Ferré, J., Escriche, B., Herrero, S., 2010. Constitutive activation of the midgut response to *Bacillus thuringiensis* in Bt-resistant *Spodoptera exigua*. PLoS One 5, e12795. https://doi.org/10.1371/journal.pone.0012795

Horikoshi, R.J., Bernardi, O., Amaral, F.S. de A. e A., Miraldo, L.L., Durigan, M.R., Bernardi, D., Silva, S.S., Omoto, C., 2019. Lack of relevant cross-resistance to Bt insecticide XenTari in strains of *Spodoptera frugiperda* (J. E. Smith) resistant to Bt maize. J. Invertebr. Pathol. 161, 1–6. https://doi.org/10.1016/J.JIP.2018.12.008

Hughes, P.R., Wood, H.A., 1981. A synchronous peroral technique for the bioassay of insect viruses. J. Invertebr. Pathol. 37, 154–159. https://doi.org/10.1016/0022-2011(81)90069-0

Jakka, S.R.K., Knight, V.R., Jurat-Fuentes, J.L., 2014. *Spodoptera frugiperda* (J.E. Smith) with field-evolved resistance to Bt maize are susceptible to Bt pesticides. J. Invertebr. Pathol. 122, 52–4. https://doi.org/10.1016/j.jip.2014.08.009

Jarman, W.M., Ballschmiter, K., 2012. From coal to DDT: The history of the development of the pesticide DDT from synthetic dyes till Silent Spring. Endeavour. https://doi.org/10.1016/j.endeavour.2012.10.003

LeOra Software, 1987. LeOra Software (1987) POLO PC, a User Guide to Probit or Logit Analysis. Le Ora Software, Berkelay, CA, USA.

Li, L., Yang, C., Liu, Z., Li, F., Yu, Z., 2000. Screening of acrystalliferous mutants from *Bacillus thuringiensis* and their transformation properties. Wei Sheng Wu Xue Bao 40, 85–90.

Lin, C.H., Chen, Y.Y., Tzeng, C.C., Tsay, H.S., Chen, L.J., 2003. Expression of a *Bacillus thuringiensis* cry1C gene in plastid confers high insecticidal efficacy against tobacco cutworm - a *Spodoptera* insect. Bot. Bull. Acad. Sin. 44, 199–210.

Masson, L., Erlandson, M., Puzstai-Carey, M., Brousseau, R., Juárez-Pérez, V., Frutos, R., 1998. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 64, 4782–8.

Monnerat, R., Pereira, E., Teles, B., Martins, E., Praça, L., Queiroz, P., Soberon, M., Bravo, A., Ramos, F., Soares, C.M., 2014. Synergistic activity of *Bacillus thuringiensis* toxins against *Simulium spp.* larvae. J. Invertebr. Pathol. 121, 70–73. https://doi.org/10.1016/J.JIP.2014.07.003

Park, H.W., Ge, B., Bauer, L.S., Federici, B.A., 1998. Optimization of Cry3A yields in *Bacillus thuringiensis* by use of sporulation-dependent promoters in combination with the STAB-SD mRNA sequence. Appl. Environ. Microbiol. 64, 3932–8.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular cloning: a laboratory manual., Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press.

Sanchis, V., Bourguet, D., 2008. *Bacillus thuringiensis*: applications in agriculture and insect resistance management. A review. Agron. Sustain. Dev. 28, 11–20. https://doi.org/10.1051/agro:2007054

SAS Institute, 1999. SAS/STAT users guide, release 8.2.

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806. https://doi.org/1092-2172

Schnepf, H.E., Whiteley, H.R., 1981. Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in Escherichia coli. Proc. Natl. Acad. Sci. U. S. A. 78, 2893–7. https://doi.org/10.1073/PNAS.78.5.2893

Silva, M.C., Siqueira, H.A.A., Silva, L.M., Marques, E.J., Barros, R., 2015. Cry proteins from *Bacillus thuringiensis* active against diamondback moth and fall armyworm. Neotrop. Entomol. 44, 392–401. https://doi.org/10.1007/s13744-015-0302-9

Soares Figueiredo, C., Nunes Lemes, A.R., Sebastião, I., Desidério, J.A., 2019. Synergism of the *Bacillus thuringiensis* Cry1, Cry2, and Vip3 proteins in *Spodoptera frugiperda* control. Appl.

Biochem. Biotechnol. 1–12. https://doi.org/10.1007/s12010-019-02952-z

Vachon, V., Laprade, R., Schwartz, J.-L., 2012. Current models of the mode of action of *Bacillus thuringiensis* insecticidal crystal proteins: A critical review. J. Invertebr. Pathol. 111, 1–12. https://doi.org/10.1016/J.JIP.2012.05.001

Valicente, F.H., de Toledo Picoli, E.A., de Vasconcelos, M.J.V., Carneiro, N.P., Carneiro, A.A., Guimarães, C.T., Lana, U.G., 2010. Molecular characterization and distribution of *Bacillus thuringiensis* cry1 genes from Brazilian strains effective against the fall armyworm, *Spodoptera frugiperda*. Biol. Control 53, 360–366. https://doi.org/10.1016/j.biocontrol.2010.02.003

Van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J. Invertebr. Pathol. 101, 1–16. https://doi.org/10.1016/j.jip.2009.02.009

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. 186, 239–47.

Xue, J.L., Cai, Q.X., Zheng, D.S., Yuan, Z.M., 2005. The synergistic activity between Cry1Aa and Cry1C from *Bacillus thuringiensis* against *Spodoptera exigua* and *Helicoverpa armigera*. Lett. Appl. Microbiol. 40, 460–465. https://doi.org/10.1111/j.1472-765X.2005.01712.x

Yinghua, S., Yan, D., Jin, C., Jiaxi, W., Jianwu, W., 2017. Responses of the cutworm *Spodoptera litura* (Lepidoptera: Noctuidae) to two Bt corn hybrids expressing Cry1Ab. Sci. Rep. 7, 41577. https://doi.org/10.1038/srep41577

CHAPTER IV

CHAPTER IV

General discussion

Bacillus thuringiensis-based products have been used worldwide as an alternative to chemical pesticides for pest control during the last decades. Recently, many chemical compounds have been banned by the authorities due to their negative effect on the ecosystem, including the killing of beneficial insects and pollinators, and the persistence of high levels of toxic residues on the food and water. This increase the risk for human health and the conservation of the environment affecting the survival of different animal and plant species (Carson et al., 2002; Fishel, 2016; Jarman and Ballschmiter, 2012; WHO, 1990). The mode of action of conventional chemical pesticides is not specific to target insects since they are nervous toxins and the nervous system is one of the most widespread life systems in the animal kingdom (Das, 2013). The repeated and systematic use of the same active ingredients has caused a high pressure of selection upon the insect population leading to the selection of resistant biotypes, which may show cross-resistance events to different compounds, generating an alert upon companies of the sector (Tabashnik, 2008). An increasing need for alternatives to chemical pesticides has raised in the last decades, and microbial agents, among others, have proven effective, specific and secure in their use (Chandler et al., 2011).

The entomopathogenic bacterium *B. thuringiensis* (Bt) was at first considered to be effective only for the control of lepidopteran species in the field. However, the discovery of new Bt isolates and novel insecticidal genes active against dipteran and coleopteran species (Goldberg and Margalit, 1977; Krieg et al., 1983) expanded the host spectrum and *B. thuringiensis* turned out as the most used microbe for the development of biological solutions due to: 1) the huge variety of reported insecticidal genes which now cover the entire spectrum of hosts causing economic losses in crops and 2) because it is a facultative pathogen that can be produced in large fermenters at a very competitive cost. The insecticidal genes from Bt have been engineered to express one or more insecticidal proteins in order to obtain resistant plants to one or more insect pests (de Maagd et al., 1999; Zhao et

al., 2003). The active ingredient of Bt-based sprayable products is comprised by the spore and the parasporal crystal of a natural strain, which is responsible for the insecticidal potency and the host spectrum activity (Schnepf et al., 1998). The parasporal crystal is made up of proteins commonly known as delta-endotoxins (Cry and Cyt) which aggregate during the stationary phase of growth. Generally, Bt strains have been classified according to their flagellar antigen (H serotyping), which have led to Bt serovars subdivided in serotypes (Lecadet et al., 1999); however, this classification has no relation with the insecticidal activity of the strain. It was believed that strains belonging to the same serovar would harbour a similar arsenal of *cry* genes, such as *cry1Ca* and *cry1Da* in ser. *aizawai* strains, but this was dismissed. The genomic sequence of a Bt isolate provides essential information on the content of insecticidal genes. This information, although necessary, is not sufficient by itself to predict the potential target pests. Therefore, a very useful complementary information to anticipate the insecticide activity of the crystal produced by a Bt strain would consist in the knowledge of the proteins that are part of the crystal and the relative proportion each of them. Routine laboratory methods, including acrylamide gels (SDS-PAGE), are used to correlate the protein band patterns with the gene expression. However, this technique is not always reliable since many proteins that are very similar in their physicochemical characteristics and have the same electrophoretic mobility exhibit very different insecticidal properties. Thus, a key factor is the gene expression and their level of expression (Martinez et al., 2004; Porcar et al., 2000). An infallible tool to determine the toxicity is an *in vivo* bioassay using a described method according to the feeding behaviour of the target species (Navon and Ascher, 2000). But bioassays are a tough and time-consuming task where different factors, such as bacteria culture, preparation of dilutions, and in occasions the researcher's subjectivity in mortality registration, may alter the results.

The composition of chemical pesticides is reflected in the label of the products and described in detail providing the exact amount of each compound in the formulation. Regarding this information, appliers can identify which are the active molecules that give the product its insecticidal power (www.syngenta-us.com/insecticides/) (Alewu B, 2018). However, and in contrast to traditional pesticides, Bt-based products only provide information related to the strain that is used as active ingredient, according to its H serotyping, and its potency, defined by

International Units (IU), which express the efficacy of an isolate compared to a defined Bt standard strain against a model insect (Dulmage, 1981). But the serovar of the Bt strains does not provide the users information referred to the insecticidal components responsible for the insecticidal activity, making the selection of the most suitable product a hard task for farmers. On the other hand, the IU only reflect the efficacy of the Bt strain compared to a single Bt standard strain against a certain insect pest. This value does not permit the users to predict the toxicity of the product against other insect species, but the reference used to estimate the IU. Companies usually provide appliers with commercial information about the products and indicate the gene content of the used Bt strains and very few venture to estimate the protein abundance present in the formulation, and when provided do not show how the prediction was made.

The identification and quantification of the insecticidal components that are present in the synthetized parasporal crystal would allow to predict the insecticidal activity of a strain and fully characterize a Bt isolate unravelling the mystery within the crystal. Several attempts using different techniques have been made in order to quantify the relative abundance of δ-endotoxins of Bt strains (Masson et al., 1998; Pusztai-Carey, M. & Carey, 1994; Yamamoto, 1983), but these approaches have not been successful when proteins with similar amino acid sequences are present in the same crystal. The main objective of this thesis was to develop a method that elucidated the presence and the relative molar proportion of each protein that make up the crystal of natural Bt strains (**Chapter II**). A mass spectrometry method (LC-MS/MS-MRM) using isotopically-labelled proteotypic peptides (SIL) was developed to accomplish this task and efficiently used to determine the parasporal composition of four Bt-based products that are most widely used today worldwide. The method was set up using two mixtures containing three Cry proteins (1Aa, 2Aa and 6Aa) that contained equal molar amounts of each of the toxins (1:1:1), and molar ratios of 43:13:43, respectively. The proteomic analysis output revealed a 32-35% of Cry1Aa, 24-26% of Cry2Aa and a 39-44% of Cry6Aa for the first mixture, and a 43-46% of Cry1Aa, 6% of Cry2Aa and a 48-51% of Cry6Aa, for the second mixture. The obtained results were very consistent with the input proportions, certifying the accuracy and reproducibility of the method.

The validated proteomic method was applied to the parasporal inclusions of the Bt strains used as active ingredients in DiPel® DF, XenTari® GD, VectoBac® 12S and Novodor®. One of the hardest tasks was to select proteotypic peptides for each protein in the mixture, due to the high similarity between the amino acidic sequences of some toxins, mainly those sharing up to 95% identity (Brun et al., 2009; Crickmore et al., 2018), or due to difficulties, in terms of hydrophobicity, in the synthesis of some of them. The genomic sequencing revealed the presence of 5 genes in the total DNA extracted from the Bt ser. *kurstaki* strain ABTS-351 (DiPel®). We corroborated the presence of 4 proteins in the crystal as indicated by the manufacturer (www.kenogard.com), but also quantified their molar rates (13-22% Cry1Aa, 16-29% Cry1Ab, 6-12% Cry1Ac and 40-64% Cry2Aa). This composition is consistent with the target species of this product, which turns to be wide due mainly to the presence of several Cry1A proteins that make up half of the crystal and a major Cry2 protein. These proteins are highly toxic to several leaf-feeding lepidopteran species such as, *Helicoverpa armigera, Heliothis virescens, Mamestra brassicae, Trichoplusia ni* or *Lymantria dispar*, excluding the genus *Spodoptera,* among others (Avilla et al., 2005; Bravo et al., 2011; Lenin et al., 2001; Li et al., 2006). A total of 6 genes were identified in the Bt ser. *aizawai* strain ABTS-1857 (XenTari®) but only 4 were expressed taking part in the parasporal crystal. The major protein was Cry1Ab (57-60%) followed by Cry1Aa (26-33%). Surprisingly the responsible toxins for the insecticidal activity towards *Spodoptera* species only represent 10-15% of the parasporal crystal (7-11% Cry1Ca and 3-4% Cry1Da) (Van Frankenhuyzen, 2009), which suggests a high toxicity effect of these two proteins. The genome of the Bt ser. *israelensis* strain AM65-52 (VectoBac®) harboured up to 8 insecticidal genes but only 6 were detected according to the developed LC-MS/MS-MRM method. Cyt1Aa was the major component of the parasporal crystal (38-61%) despite its low toxicity towards some dipteran species when administered alone (Ben-Dov, 2014). Almost half of the inclusion body was comprised by Cry4Ba (10-28%), Cry11Aa (10-27%) and Cry60Ba (5-12%), with a small contribution of Cry4Aa (2-4%) and Cry60Aa (2-4%). The insecticidal activity of the strain is mainly attributed to the Cry4 and Cry11 proteins, but the Cyt1Aa plays a crucial role in delaying the selection of resistant biotypes (Monnerat et al., 2014; Wirth et al., 2005). The role of Cry60 proteins has not been yet elucidated, but their low proportion could suggest their contribution to toxicity via synergism effects or by

avoiding resistance. In the Bt ser. *tenebrionis* strain NB-74 (Novodor®) all the identified genes were expressed and the parasporal crystal was comprised by Cry3Aa, Cry23Aa and Cry37Aa proteins, 70-75%, 14-16%, 10-14% at molar ratios, respectively. The former protein is well studied and show a high toxicity effect against species of the orders Coleoptera, Hemiptera, and Hymenoptera (Kurt et al., 2005; López-Pazos et al., 2009; Porcar et al., 2010; Van Frankenhuyzen, 2009). The latter proteins act as binary toxins, so both of them are needed in order to exert toxicity against some coleopteran species. It is believed that Cry37 facilitate the binding of the Cry23 which forms a toxic channel in midgut epithelial cells (Donovan et al., 2000). The estimated amount of each of the two components of this binary toxin was very similar, which is an indirect confirmation of the efficacy of the developed LC-MS/MS-MRM method.

The composition of the parasporal crystal gives a very valuable information that allow researchers to predict the insecticidal potency as well as the host range of a certain Bt strain. However, in each insect species the interactions between the toxins that make up the crystal have a direct impact in mortality ratios. In this thesis we deeply studied the Bt ser. *aizawai* strain ABTS-1857 (**Chapter III**), used in the commercial product XenTari® and the role of each individual protein expressed in the parasporal crystal. First, we tested the solubilized natural strain against three species of the genus *Spodoptera,* including *S. exigua, S. littoralis* and *S. frugiperda,* in order to verify its activity as reported by other studies (Smith and Terry, 2001). The most susceptible insect was *S. exigua* with an estimated LC_{50} of 7.8 ng/μl followed by *S. littoralis.* which was 3.3-fold more tolerant, showing a LC_{50} of 28 ng/μl. *S. frugiperda* larvae showed a much higher LC_{50}, scoring 120.2 ng/μl to kill 50% of the insect population. According to these results we could confirm the toxicity of the Bt strain ABTS-1857 towards the tested *Spodoptera* species but remarked that susceptibility is dependent on species. This event could be due to the different activity of individual toxins, so we estimated their toxic effect against each pest under the same laboratory conditions. The major proteins that comprised the parasporal crystal, Cry1Aa and Cry1Ab, did not perform well when administered to second instar larvae (LC_{50} > 300 ng/μl) as previously described (Hernández-Martínez et al., 2008; Yinghua et al., 2017), although some authors have indicated a toxicity of Cry1Ab towards *S. exigua,* we did not obtain mortalities over 30% at the

maximum concentration (Hernández-Martínez et al., 2008; MacIntosh et al., 1990; Moar et al., 1990). According to several studies, Cry1Ca and Cry1Da are the main responsible proteins for the toxicity against the cotton leafworm and the armyworms (Lee et al., 2001; Porcar et al., 2000; Van Rie et al., 1989). *S. exigua* turned to be very susceptible to the former protein (LC_{50} = 3.1 ng/μl) but not to the latter protein (LC_{50} = 83 ng/μl) (Luo et al., 1999). Cry1Ca and Cry1Da showed to be equally active against *S. littoralis* larvae, with estimated LC_{50} values of 19.8 ng/μl and 19.6 ng/μl, respectively. We observed that *S. frugiperda* was only susceptible to Cry1Da with a calculated LC_{50} = 10.3 ng/μl, as previously described. However, some authors also report a positive effect of Cry1C against the fall armyworm whether by exerting a weak toxicity or by forming cation channels in the brush border membrane of the larvae (Lorence et al., 1995; Luo et al., 1999). According to these resulted we concluded that i) Cry1Ca was the best protein for the control of the beet armyworm, ii) Cry1Ca and Cry1Da caused the same mortal effect on the cotton leafworm, and iii) Cry1Da was the only protein, within the crystal of the Bt strain ABTS-1857, toxic for the fall armyworm. It is important to notice that the obtained results in a laboratory are subjected to the populations used and maintained in it, meaning that the same crystal protein dilutions may perform different against other insect populations maintained elsewhere or natural populations. Some reports have concluded different susceptibilities of *S. exigua* populations when treated with the Cry1Ca protein, supporting this idea (Hernández-Martínez et al., 2008). Field trials are then mandatory when registering a Bt-based product, preferably at the locations where the biopesticide will be sprayed out.

Proteins in a mixture may interact having an important impact in the mortality results. The mode of action of Cry proteins in lepidopterans has been widely studied and interactions with specific receptors located in the brush border of epithelial cells of the midgut seems to be a crucial step for their high specificity (Gómez et al., 2007; Soberón et al., 2016). However, some crystal toxins may share binding sites in some insect populations which leads to variations in susceptibility or the appearance of cross-resistance events (Hernández and Ferré, 2005). In this work we studied the effect of different artificial mixtures containing two, three or four Cry1 proteins according to their concentration in 100 ng/μl of solubilized Cry1 crystal proteins (29 -Aa, 58.5 -Ab, 9 -Ca and 3.5 ng/μl -Da) against the three *Spodoptera*

species. For *S. exigua* all combinations that contained Cry1Ca performed much better than those without it, scoring mortalities from 62-86%. These results are consistent with the high toxicity observed when inoculated individually. We corroborated the absence of synergism between Cry1Aa and Cry1Ab, which were not toxic when inoculated individually, as suggested by other authors (Moar et al., 1990). Nevertheless, we could not detect the described synergistic effect when larvae were treated simultaneously with Cry1Aa and Cry1C (Xue et al., 2005) at the tested concentrations. For *S. littoralis* we observed that Cry1Ca and Cry1Da gave the same activity when combined with the rest of the proteins in mixtures of two or three toxins, and the best results were obtained when administered together, reaching 71 ± 18.7% mortality with the Cry1Ca + Cry1Da mixture, and 81± 10.3% with the Cry1Ab + Cry1Ca + Cry1Da mixture. For *S. frugiperda* only combinations that contained Cry1Da showed insecticidal activity towards second instar larvae as expected, reaching up to around 33% mortality in the best cases (Cry1Ab + Cry1Da; Cry1Ca + Cry1Da or Cry1Ab + Cry1Ca + Cry1Da). An artificial combination that contained the four proteins in the estimated concentration of 29, 58.5, 9 and 3.5 ng/μl for each protein (Cry1-Aa, -Ab, -Ca and -Da, respectively), was tested and compared to the natural mixture found in the wildtype strain ABTS-1857. No significant differences were detected between the artificial and the natural mixture ($p>0.05$) when treating the three *Spodoptera* species. Furthermore, the LC_{50} of the artificial mixture was estimated for *S. exigua* (9.9 ng/μl) and *S. frugiperda* larvae (188.1 ng/μl) and the potency of the insecticidal protein combination was compared to that obtained for the parasporal crystal of the wildtype strain. No significant differences were obtained for neither of the two species. This data supports the hypothesis of the determined proportions for the four Cry1 proteins in the parasporal crystal of the strain ABTS-1857.

According to some researchers Cry1D is only required in small amounts in order to exert toxicity when in presence of other toxins, including Cry1C (Chang et al., 2001; Masson et al., 1998). However, this hypothesis was discarded when increasing amounts of Cry1D, along with the natural strain HD-133, resulted in higher mortality rates for *S. littoralis* larvae (BenFarhat-Touzri et al., 2018). In our research we introduced some changes in the molar ratios of the four proteins, and increased the proportion of Cry1Da, from 3.5% to 20%, combined with decreased

amounts of either Cry1Aa or Cry1Ab. We demonstrated that increasing amounts of Cry1Da resulted in higher toxicity for *S. littoralis* and specially for *S. frugiperda,* reaching up to 96-100% and 83-88% mortality, respectively, at higher molar ratios of Cry1Da. No apparent differences were detected when the increments were made in substitution of Cry1Aa or Cry1Ab.

The use of *B. thuringiensis* as microbial control agent presents many advantages compared to chemical pesticides, including the high specificity of its insecticidal crystal proteins and the security for non-target organisms. A huge number of genes have been described, meaning an enormous reservoir of insecticidal weapons to combat different pests belonging to different orders. We have demonstrated that the development of a method to determine the qualitative and quantitative abundance of crystal proteins is of great interest to fully characterize Bt isolates and enable the prediction of their potency and host range activity.

REFERENCES

Alewu B, N.C., 2018. Pesticides and human health. Intech open 2, 64. https://doi.org/10.5772/32009

Avilla, C., Vargas-Osuna, E., González-Cabrera, J., Ferré, J., González-Zamora, J.E., 2005. Toxicity of several delta-endotoxins of *Bacillus thuringiensis* against *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Spain. J. Invertebr. Pathol. 90, 51–4. https://doi.org/10.1016/j.jip.2005.04.003

Ben-Dov, E., 2014. *Bacillus thuringiensis* subsp. *israelensis* and its dipteran-specific toxins. Toxins (Basel). 6, 1222–1243. https://doi.org/10.3390/toxins6041222

BenFarhat-Touzri, D., Driss, F., Jemli, S., Tounsi, S., 2018. Molecular characterization of Cry1D-133 toxin from *Bacillus thuringiensis* strain HD133 and its toxicity against *Spodoptera littoralis*. Int. J. Biol. Macromol. 112, 1–6. https://doi.org/10.1016/J.IJBIOMAC.2018.01.081

Bravo, A., Likitvivatanavong, S., Gill, S.S., Soberón, M., 2011. *Bacillus thuringiensis*: A story of a successful bioinsecticide. Insect Biochem. Mol. Biol. https://doi.org/10.1016/j.ibmb.2011.02.006

Brun, V., Masselon, C., Garin, J., Dupuis, A., 2009. Isotope dilution strategies for absolute quantitative proteomics. J. Proteomics. https://doi.org/10.1016/j.jprot.2009.03.007

Carson, R., Wilson, E.O., Lear, L.J., 2002. Silent Spring. Houghton Mifflin.

Chandler, D., Bailey, A.S., Mark Tatchell, G., Davidson, G., Greaves, J., Grant, W.P., 2011. The development, regulation and use of biopesticides for integrated pest management. Philos. Trans. R. Soc. B Biol. Sci. 366, 1987–1998. https://doi.org/10.1098/rstb.2010.0390

Chang, L., Grant, R., Aronson, A., 2001. Regulation of the packaging of *Bacillus thuringiensis* δ-endotoxins into inclusions. Appl. Environ. Microbiol. 67, 5032–5036. https://doi.org/10.1128/AEM.67.11.5032

Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., Schnepf, E., Sun, M., Zeigler, D.R., 2018. *Bacillus thuringiensis* toxin nomenclature [WWW Document]. URL http://www.btnomenclature.info/

Das, S.K., 2013. Mode of action of pesticides and the novel trends a critical review. Int. Res. J. Agric. Soil Sci. 3. https://doi.org/10.14303/irjas.2013.127

de Maagd, R.., Bosch, D., Stiekema, W., 1999. Toxin-mediated insect resistance in plants. Trends Plant Sci. 4, 9–13.

Donovan, W.P., Donovan, J.C., Slaney, A.C., 2000. *Bacillus thuringiensis* CryET33 and CryET34 compositions and uses therefor. United States Pat.

Dulmage, H.T., 1981. Insecticidal activity of isolated of *Bacillus thuringiensis* and their potential for pest control. Microb. Control pests plant Dis. 1970–1980, Ed.H.D Burges, Academic Press, London.

Fishel, F.M., 2016. Pest management and pesticides : A historical early uses of pesticides and other insect-vectored diseases. IFAS extension-UF 2–6.

Goldberg, L.J., Margalit, J., 1977. A bacterial spore demonstrating rapid larvicidal activity against *Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegypti* and *Culex pipiens* . Mosq. News 37, 355–358.

Gómez, I., Pardo-López, L., Muñoz-Garay, C., Fernandez, L.E., Pérez, C., Sánchez, J., Soberón, M., Bravo, A., 2007. Role of receptor interaction in the mode of action of insecticidal Cry and Cyt toxins produced by *Bacillus thuringiensis*. Peptides 28, 169–173. https://doi.org/10.1016/j.peptides.2006.06.013

Hernández-Martínez, P., Ferré, J., Escriche, B., 2008. Susceptibility of *Spodoptera exigua* to 9 toxins from *Bacillus thuringiensis*. J. Invertebr. Pathol. 97, 245–250. https://doi.org/10.1016/J.JIP.2007.11.001

Hernández, C.S., Ferré, J., 2005. Common receptor for *Bacillus thuringiensis* toxins Cry1Ac, Cry1Fa, and Cry1Ja in *Helicoverpa armigera, Helicoverpa zea*, and *Spodoptera exigua*. Appl. Environ. Microbiol. 71, 5627–5629. https://doi.org/10.1128/AEM.71.9.5627-5629.2005

Jarman, W.M., Ballschmiter, K., 2012. From coal to DDT: The history of the development of the pesticide DDT from synthetic dyes till Silent Spring. Endeavour. https://doi.org/10.1016/j.endeavour.2012.10.003

Krieg, A., Huger, A.M., Langenbruch, G.A., Schnetter, W., 1983. *Bacillus thuringiensis* var. *tenebrionis*, a new pathotype effective against larvae of Coleoptera. J. Appl. Entomol. 96, 500–508.

Kurt, A., Özkan, M., Sezen, K., Demirbağ, Z., Özcengiz, G., 2005. Cry3Aa11: A new Cry3Aa δ-endotoxin from a local isolate of *Bacillus thuringiensis*. Biotechnol. Lett. 27, 1117–1121. https://doi.org/10.1007/s10529-005-8460-1

Lecadet, M.-M., Frachon, E., Dumanoir, V.C., Ripouteau, H., Hamon, S., Laurent, P., Thiery, I., 1999. Updating the H-antigen classification of *Bacillus thuringiensis*. J. Appl. Microbiol. 86, 660–672. https://doi.org/10.1046/j.1365-2672.1999.00710.x

Lee, I.H., Je, Y.H., Chang, J.H., Roh, J.Y., Oh, H.W., Lee, S.G., Shin, S.C., Boo, K.S., 2001. Isolation and characterization of a *Bacillus thuringiensis* ssp. *kurstaki* strain toxic to *Spodoptera exigua* and *Culex pipiens*. Curr. Microbiol. 43, 284–287. https://doi.org/10.1007/s002840010302

Lenin, K., Mariam, M.A., Udayasuriyan, V., 2001. Expression of a cry2Aa gene in an acrystalliferous *Bacillus thuringiensis* strain and toxicity of Cry2Aa against *Helicoverpa armigera*. World J. Microbiol. Biotechnol. 17, 273–278. https://doi.org/10.1023/A:1016674417728

Li, Y.-X., Greenberg, S.M., Liu, T.-X., 2006. Effects of Bt cotton expressing Cry1Ac and Cry2Ab and non-Bt cotton on behavior, survival and development of *Trichoplusia ni* (Lepidoptera: Noctuidae). Crop Prot. 25, 940–948. https://doi.org/10.1016/J.CROPRO.2005.12.007

López-Pazos, S.A., Cortázar Gómez, J.E., Cerón Salamanca, J.A., 2009. Cry1B and Cry3A are active against *Hypothenemus hampei* Ferrari (Coleoptera: Scolytidae). J. Invertebr. Pathol. 101, 242–245. https://doi.org/10.1016/j.jip.2009.05.011

Lorence, A., Darszon, A., Díaz, C., Liévano, A., Quintero, R., Bravo, A., 1995. δ-Endotoxins induce cation channels in *Spodoptera frugiperda* brush border membranes in suspension and in planar lipid bilayers. FEBS Lett. 360, 217–222. https://doi.org/10.1016/0014-5793(95)00092-N

Luo, K., Banks, D., Adang, M.J., 1999. Toxicity, binding, and permeability analyses of four Bacillus thuringiensis Cry1 δ-endotoxins using brush border membrane vesicles of Spodoptera exigua and Spodoptera frugiperda. Appl. Environ. Microbiol. 65, 457–464.

MacIntosh, S.C., Stone, T.B., Sims, S.R., Hunst, P.L., Greenplate, J.T., Marrone, P.G., Perlak, F.J., Fischhoff, D.A., Fuchs, R.L., 1990. Specificity and efficacy of purified *Bacillus thuringiensis* proteins against agronomically important insects. J. Invertebr. Pathol. 56, 258–266. https://doi.org/10.1016/0022-2011(90)90109-J

Martinez, C., Porcar, M., Lopez, A., de Escudero, I.R., Perez-Llarena, F.J., Caballero, P., 2004. Characterization of a *Bacillus thuringiensis* strain with a broad spectrum of activity against lepidopteran insects. Entomol. Exp. Appl. 111, 71–77. https://doi.org/10.1111/j.0013-8703.2004.00156.x

Masson, L., Erlandson, M., Puzstai-Carey, M., Brousseau, R., Juárez-Pérez, V., Frutos, R., 1998. A holistic approach for determining the entomopathogenic potential of *Bacillus thuringiensis* strains. Appl. Environ. Microbiol. 64, 4782–8.

Moar, W.J., Masson, L., Brousseau, R., Trumble, J.T., 1990. Toxicity to Spodoptera exigua and Trichoplusia ni of individual P1 protoxins and sporulated cultures of Bacillus thuringiensis subsp. kurstaki HD-1 and NRD-12. Appl. Environ. Microbiol. 56, 2480–3.

Monnerat, R., Pereira, E., Teles, B., Martins, E., Praça, L., Queiroz, P., Soberon, M., Bravo, A., Ramos, F., Soares, C.M., 2014. Synergistic activity of *Bacillus thuringiensis* toxins against *Simulium spp.* larvae. J. Invertebr. Pathol. 121, 70–73. https://doi.org/10.1016/J.JIP.2014.07.003

Navon, A., Ascher, K.R.S., 2000. Bioassays of entomopathogenic microbes and nematodes. CABI Pub.

Porcar, M., García-Robles, I., Domnguez-Escrib, L., Latorre, A., 2010. Effects of *Bacillus thuringiensis* Cry1Ab and Cry3Aa endotoxins on predatory coleoptera tested through artificial diet-incorporation bioassays. Bull. Entomol. Res. 100, 297–302. https://doi.org/10.1017/S0007485309990290

Porcar, M., Martinez, C., Caballero, P., 2000. Host range and gene contents of *Bacillus thuringiensis* strains toxic towards *Spodoptera exigua*. Entomol. Exp. Appl. 97, 339–346. https://doi.org/10.1023/A:1004140122226

Pusztai-Carey, M. & Carey, P.R., 1994. Isolation, quantitation and purification of insecticidal proteins from Bacillus thuringiensis 13, 274. https://doi.org/10.1016/0734-9750(95)96017-f

Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., Zeigler, D.R., Dean, D.H., 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62, 775–806. https://doi.org/1092-2172

Smith, A., Terry, A., 2001. *Bacillus thuringiensis* containing compositions. US006264942B1.

Soberón, M., Monnerat, R., Bravo, A., 2016. Mode of action of Cry toxins from *Bacillus thuringiensis* and resistance mechanisms. https://doi.org/10.1007/978-94-007-6725-6

Tabashnik, B.E., 2008. Delaying insect resistance to transgenic crops. Proc. Natl. Acad. Sci. U. S. A. 105, 19029–30. https://doi.org/10.1073/pnas.0810763106

Van Frankenhuyzen, K., 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. J. Invertebr. Pathol. 101, 1–16. https://doi.org/10.1016/j.jip.2009.02.009

Van Rie, J., Jansens, S., Höfte, H., Degheele, D., Van Mellaert, H., 1989. Specificity of *Bacillus thuringiensis* delta-endotoxins. Importance of specific receptors on the brush border membrane of the mid-gut of target insects. Eur. J. Biochem. 186, 239–47.

WHO, 1990. World Health Organization & United Nations Environment Programme. (1990). Public health impact of pesticides used in agriculture. World Health Organization [WWW Document].

Wirth, M.C., Park, H.W., Walton, W.E., Federici, B.A., 2005. Cyt1A of *Bacillus thuringiensis* delays evolution of resistance to Cry11A in the mosquito *Culex quinquefasciatus*. Appl. Environ. Microbiol. 71, 185–189. https://doi.org/10.1128/AEM.71.1.185-189.2005

Xue, J.L., Cai, Q.X., Zheng, D.S., Yuan, Z.M., 2005. The synergistic activity between Cry1Aa and Cry1C from *Bacillus thuringiensis* against *Spodoptera exigua* and *Helicoverpa armigera*. Lett. Appl. Microbiol. 40, 460–465. https://doi.org/10.1111/j.1472-765X.2005.01712.x

Yamamoto, T., 1983. Identification of entomocidal toxins of *Bacillus thuringiensis* by high performance liquid chromatography. J. Gen. Microbiol. 129, 2595–2603. https://doi.org/10.1099/00221287-129-8-2595

Yinghua, S., Yan, D., Jin, C., Jiaxi, W., Jianwu, W., 2017. Responses of the cutworm *Spodoptera litura* (Lepidoptera: Noctuidae) to two Bt corn hybrids expressing Cry1Ab. Sci. Rep. 7, 41577. https://doi.org/10.1038/srep41577

Zhao, J.Z., Cao, J., Li, Y., Collins, H.L., Roush, R.T., Earle, E.D., Shelton, A.M., 2003. Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat. Biotechnol. 21, 1493–1497. https://doi.org/10.1038/nbt907

CONCLUSIONES

CONCLUSIONES

1. Se ha puesto a punto un método rápido, fiable y preciso que permite determinar la composición cualitativa y cuantitativa del cristal de las cepas de *Bacillus thuringiensis.* La aplicación eficiente del método requiere disponer de información sobre la secuencia completa del genoma y de las proteínas que presumiblemente forman parte del cristal. Los péptidos proteotípicos de cada proteína presente en cristal se identifican mediante cromatografía líquida acoplada a un espectrómetro de masas (LC-MS/MS) en combinación con una monitorización de múltiples reacciones (MRM). La síntesis de dichos péptidos, marcados isotópicamente (SIL), permite determinar la proporción molar relativa de cada proteína en el cristal.

2. Para validar el método, se emplearon las proteínas Cry1Aa, Cry2Aa y Cry6Aa y se determinó su proporción relativa en dos mezclas artificiales donde sus respectivas concentraciones molares fueron (1:1:1) y (13:1:13). La fiabilidad y precisión de los resultados obtenidos permitieron verificar la utilidad del método para el propósito que ha sido desarrollado.

3. El método puesto a punto se empleó con éxito para determinar la composición cualitativa y cuantitativa del cristal de las cepas Bt que constituyen el ingrediente activo de cuatro de los productos comerciales más utilizados en el control de plagas de lepidópteros (DiPel® DF y XenTari® GD), dípteros (VectoBac® 12AS) y coleópteros (Novodor®).

4. Se confirmó la toxicidad del producto comercial XenTari® GD para las tres especies del género *Spodoptera* (*S. exigua*, *S. littoralis* y *S. frugiperda*) evaluadas. No obstante, el valor estimado de la CL_{50} para la mezcla de esporas y cristales producida por la cepa ABTS-1857 fue significativamente diferente para larvas L_2 de *S. exigua* (7.8 ng/μl), *S. littoralis* (28.0 ng/μl) y *S.frugiperda* (120.2 ng/μl).

5. Las proteínas individuales (Cry1Aa, Cry1Ab, Cry1Ca y Cry1Da) que componen el cristal de la cepa ABTS-1857, mostraron una toxicidad especifica para cada

una de las tres especies de *Spodoptera*. La toxicidad de Cry1Aa y Cry1Ab fue muy baja para las tres especies de *Spodoptera* (CL_{50} > 300 ng/μl). Cry1Ca fue la más tóxica para larvas de *S. exigua* (CL_{50}= 3.1 ng/μl) seguida por Cry1Da (CL_{50}= 83 ng/μl). Las larvas de *S. littoralis* fueron igualmente susceptibles a Cry1Ca (19.8 ng/μl) y Cry1Da (19.6 ng/μl), mientras que las larvas de *S. frugiperda* sólo fueron susceptibles a Cry1Da (CL_{50}= 10.3 ng/μl).

6. Se analizaron distintas combinaciones de dos, tres o cuatro proteínas del cristal de la cepa ABTS-1857, para determinar posibles interacciones entre ellas. Las combinaciones que incluían Cry1Ca fueron las que presentaron una mayor actividad (62-86%) para larvas L_2 de *S. exigua.* Las larvas L_2 de *S. littoralis* fueron más susceptibles a las mezclas que incluían Cry1Ca y Cry1Da (71-81%). Todas las combinaciones que incluían la proteína Cry1Da presentaron los porcentajes de mortalidad más elevados (21-33%) en larvas L_2 de *S. frugiperda*.

7. Se demostró experimentalmente que los valores de la CL_{50} estimados para la mezcla de espora y cristales de la cepa silvestre ABTS-1857 en larvas L_2 de *S. exigua* y *S. frugiperda* no difirieron estadísticamente de los valores obtenidos para una mezcla artificial de las cuatro proteínas recombinantes simulando la composición natural del cristal.

8. La actividad insecticida de mezclas artificiales con crecientes concentraciones de Cry1Da, en detrimento de Cry1Aa o Cry1Ab, resultó en un notable incremento de la mortalidad larvaria en *S. littoralis* (10-20%) y en *S. frugiperda* (15-50%). Este efecto se atribuyó a la susceptibilidad de ambas especies a Cry1Da. En cambio, dicho efecto no se observó en las larvas de *S. exigua,* debido a la baja toxicidad de Cry1Da para esta especie.

9. Los resultados de esta tesis doctoral son de gran aplicación en la caracterización de productos basados en Bt y proporciona información sobre el posible rango de huéspedes de insecticidas si se conoce la toxicidad de los componentes del cristal. Adicionalmente, permite un preciso y fiable control de calidad de los distintos lotes de Bt producidos a nivel industrial y el control de calidad del producto final.

CONCLUSIONS

CONCLUSIONS

1. A fast, reliable and precise method has been developed to determine the qualitative and quantitative crystal composition of *B. thuringiensis* strains. For an efficient application of the method the information related to the complete sequence of the genome and the proteins that are presumably present in the crystal are required. The proteotypic peptides of each protein that make up the crystal are identified by liquid chromatography coupled to a mass spectrum(LC-MS/MS) in combination with multiple monitored reactions (MRM). The synthesis of isotopically labelled peptides (SIL), allow the determination of the relative molar proportion of each protein in the crystal.

2. To validate the method, the Cry1Aa, Cry2Aa and Cry6Aa proteins were used and their relative proportion was determined in two artificial mixtures with (1: 1: 1) and (13: 1: 13) molar concentrations, respectively. The reliability and precision of the obtained results allowed us to verify the usefulness of the method for the purpose to which has been developed

3. The developed method was successfully applied to determine the qualitative and quantitative composition of the crystal of the Bt strains that constitute the active ingredient of four of the most used commercial products for the control of lepidopteran (DiPel® DF and XenTari® GD), dipteran (VectoBac® 12AS) and coleopteran pests (Novodor®).

4. The toxicity of the commercial product XenTari® GD against three species of the genus *Spodoptera* (*S. exigua, S. littoralis* and *S. frugiperda*) was confirmed. However, the estimated value of the LC_{50} for the mixture of spores and crystals produced by the strain ABTS-1857 was significantly different for *S. exigua* (7.8 ng/µl), *S. littoralis* (28.0 ng/µl) and *S. frugiperda* (120.2 ng/µl) second instar.

5. The individual proteins (Cry1Aa, Cry1Ab, Cry1Ca and Cry1Da) that make up the crystal of the strain ABTS-1857, showed a specific toxicity for each of the three *Spodoptera* species. Cry1Aa and Cry1Ab showed a very low toxicity for the three species of *Spodoptera* (LC_{50} > 300 ng/µl). Cry1Ca was the most toxic

protein for of *S. exigua* larvae (LC_{50} = 3.1 ng/μl) followed by Cry1Da (LC_{50} = 83 ng/μl). *S. littoralis* second instar were equally susceptible to Cry1Ca (19.8 ng/μl) and Cry1Da (19.6 ng/μl), whereas *S. frugiperda* was only susceptible to the Cry1Da protein (LC_{50} = 10.3 ng/μl).

6. Different combinations containing two, three or four crystal proteins of the strain ABTS-1857 were analyzed to determine possible interactions between them. The combinations that included Cry1Ca showed the highest activity (62-86%) for *S. exigua* larvae. *S. littoralis* second instar were more susceptible to mixtures that contained the Cry1Ca and Cry1Da proteins (71-81%). All combinations that included the Cry1Da protein showed the highest mortality percentages (21-33%) in *S. frugiperda*.

7. The estimated LC_{50} values for the mixture of spores and crystals of the wild strain ABTS-1857 in *S. exigua* and *S. frugiperda* second instar were not statistically different from the obtained values for an artificial mixture that contained the four recombinant proteins simulating the natural composition of the crystal.

8. The insecticidal activity of artificial mixtures with increased concentrations of Cry1Da to the detriment of Cry1Aa or Cry1Ab, resulted in a notable increase in larval mortality in *S. littoralis* (10-20%) and *S. frugiperda* (15-50%). This effect was attributed to the fact that both species are quite susceptible to the Cry1Da protein. In contrast, this effect was not observed in *S. exigua* larvae, due to the low toxicity of Cry1Da for this species.

9. The results obtained in this doctoral thesis are of great application in the characterization of Bt-based products and provide information on the possible range of insecticide hosts if the toxicity of the crystal components is known. Additionally, it allows a precise and reliable quality control of different batches in the industrial production of Bt and the quality control of the final product.

LIST OF PUBLICATIONS

Caballero J., Williams T., 2019. Identification and quantification of Cry and Cyt proteins of *Bacillus thuringiensis.* European Patent Application Number: EP19382073.5.

Caballero J., Jiménez-Moreno N., Orera I., Williams T., Fernández A.B., Villanueva M., Ferré J., Caballero P.* & Ancín-Azpilicueta C., 2019.Unravelling the composition of insecticidal crystal proteins in *Bacillus thuringiensis* by genome sequencing and mass spectrometry. Applied and Environmental Microbiology, to be submitted.

Caballero J., Villanueva M., Jiménez-Moreno N., Ancín-Azpilicueta C., Caballero P.*, 2019. Activity of the individual crystal proteins of *Bacillus thuringiensis* serovar *aizawai* strain ABTS-1857 for *Spodoptera* species: insecticidal effect of artificial mixture proteins. Toxins, to be submitted.

SUPPLEMENTAL MATERIAL

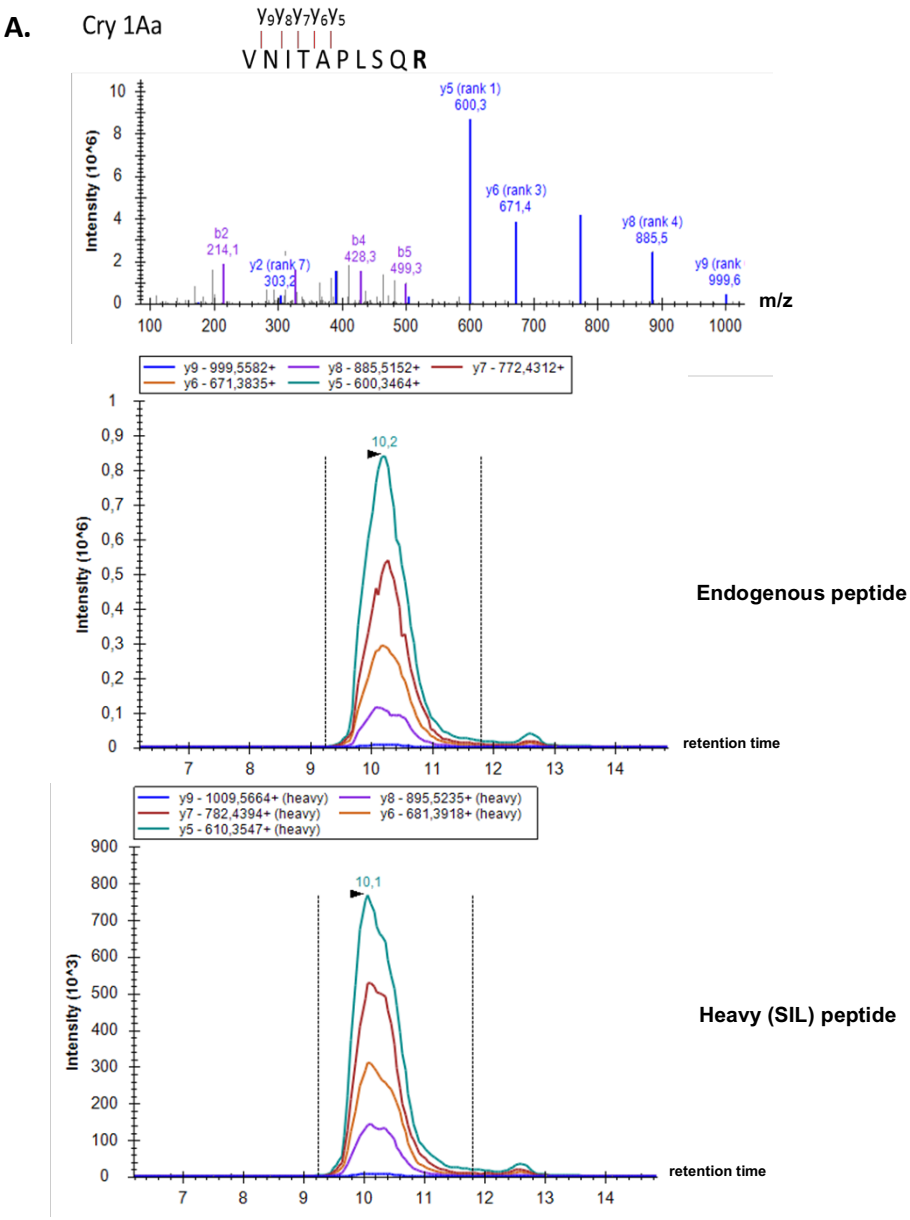


Figure S1. Example MS/MS spectra and extracted ion chromatograms of targeted proteotypic peptides in protein mixtures analysed by LC-MS/MS-MRM. MS/MS spectrum (y-ions and b-ions) of a targeted proteotypic peptide, and extracted ion chromatograms showing the five monitored transitions for the endogenous peptide (left) and the SIL (heavy)-labelled peptide (right) of a targeted peptide of Cry1Aa (A), Cry2Aa (B), and Cry6Aa (C) present in mixtures comprising known amounts of these three proteins. Data in (A), (B), and (C) are from the dataset shown in Table A1.

B.

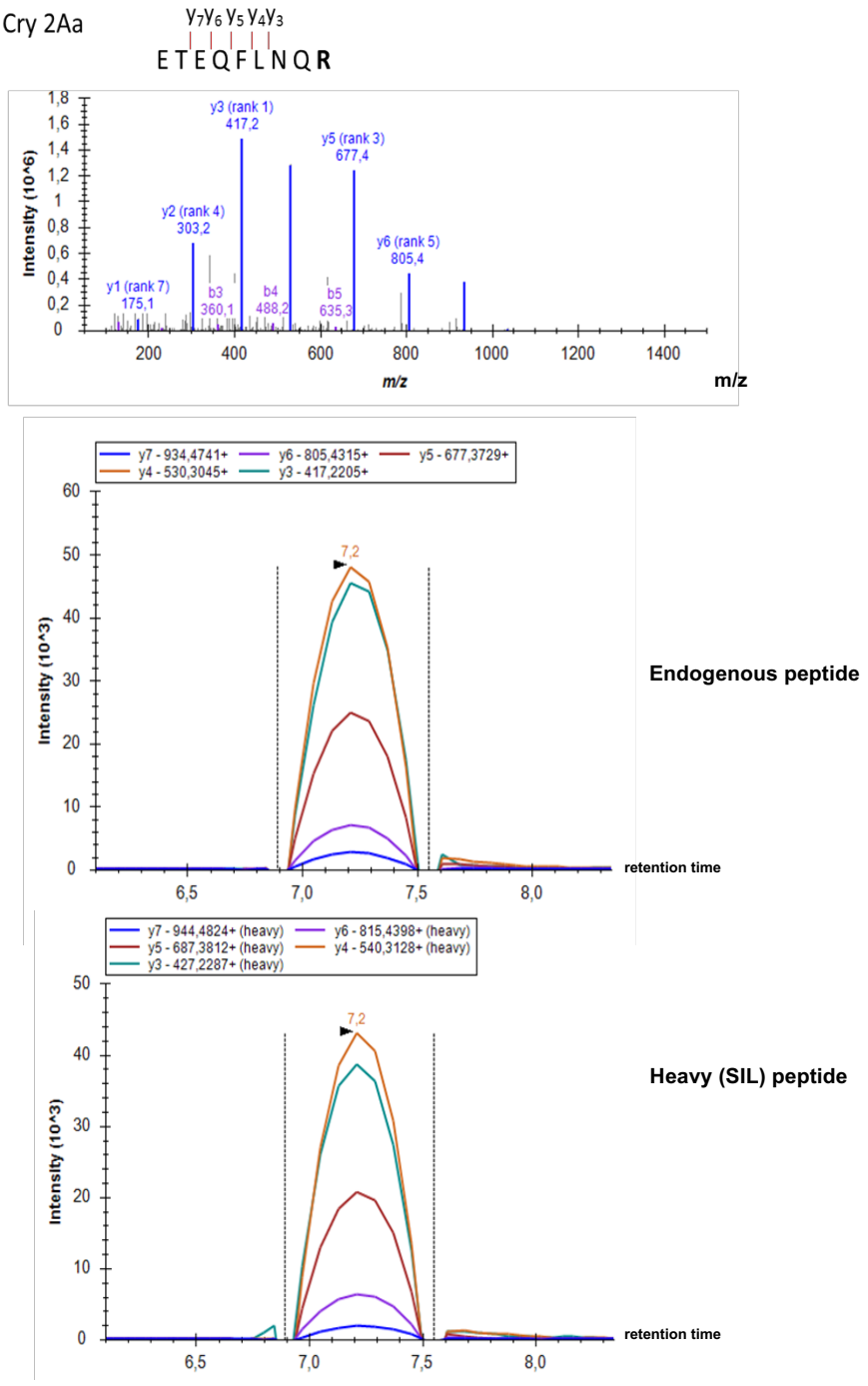


Figure S1. Continued

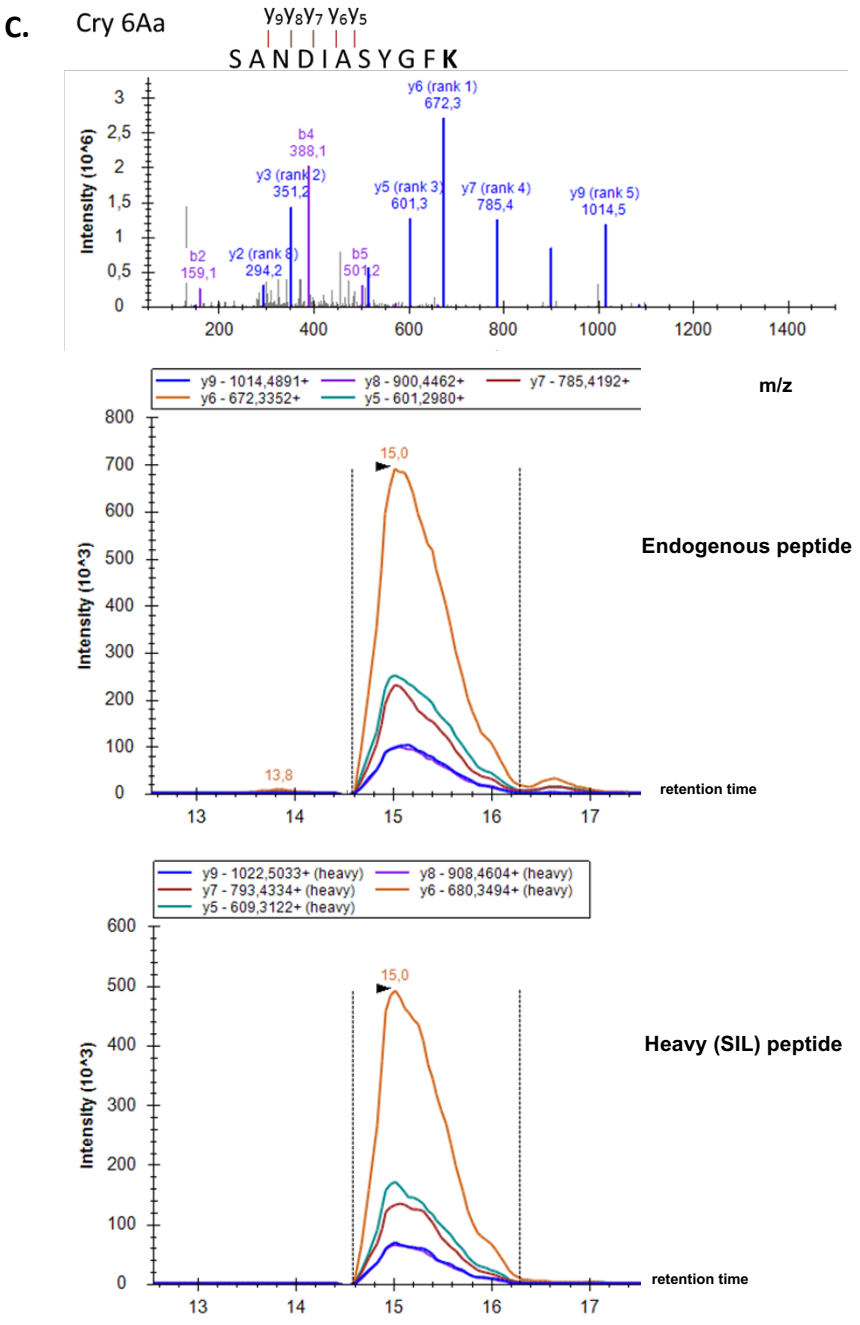


Figure S1. Continued.

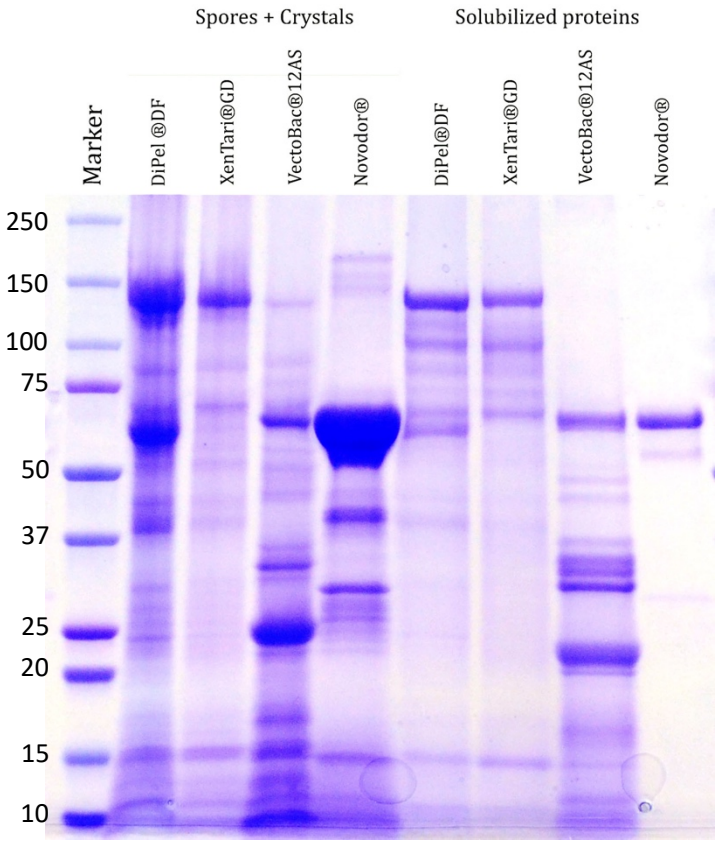


Figure S2. SDS-PAGE analysis of spore and crystal preparations, and solubilized proteins from four Bt strains grown from insecticide products. Bt spores were isolated directly from the commercial products and grown in CCY medium until lysis of sporulated cells. Mixtures of spores and crystals were sedimented by centrifugation and washed in distilled water. A total amount ranging from 5-10 µg of sample were loaded per lane. Different major protein bands were detected in the spore and crystal samples. DiPel® DF revealed the presence of two major bands at 130 kDa and 70 kDa, which may correspond to Cry1 and Cry2 proteins, respectively, while a protein band of 130 kDa was detected in the XenTari® GD sample, which is consistent with the presence of Cry1 protein. In VectoBac® 12S the three observed protein bands of 130 would correspond to Cry4 and Cry10, while the protein bands of 70 and 25 kDa would correspond to Cry11, and Cry60 and Cyt1 proteins, respectively. In the Novodor® sample a major band at 70kDa was resolved, in agreement with the size of Cry3 proteins. Cry23 and Cry37 in this product would be represented by the bands at 30 and 15 kDa, respectively. Three independent biological replicates were evaluated.

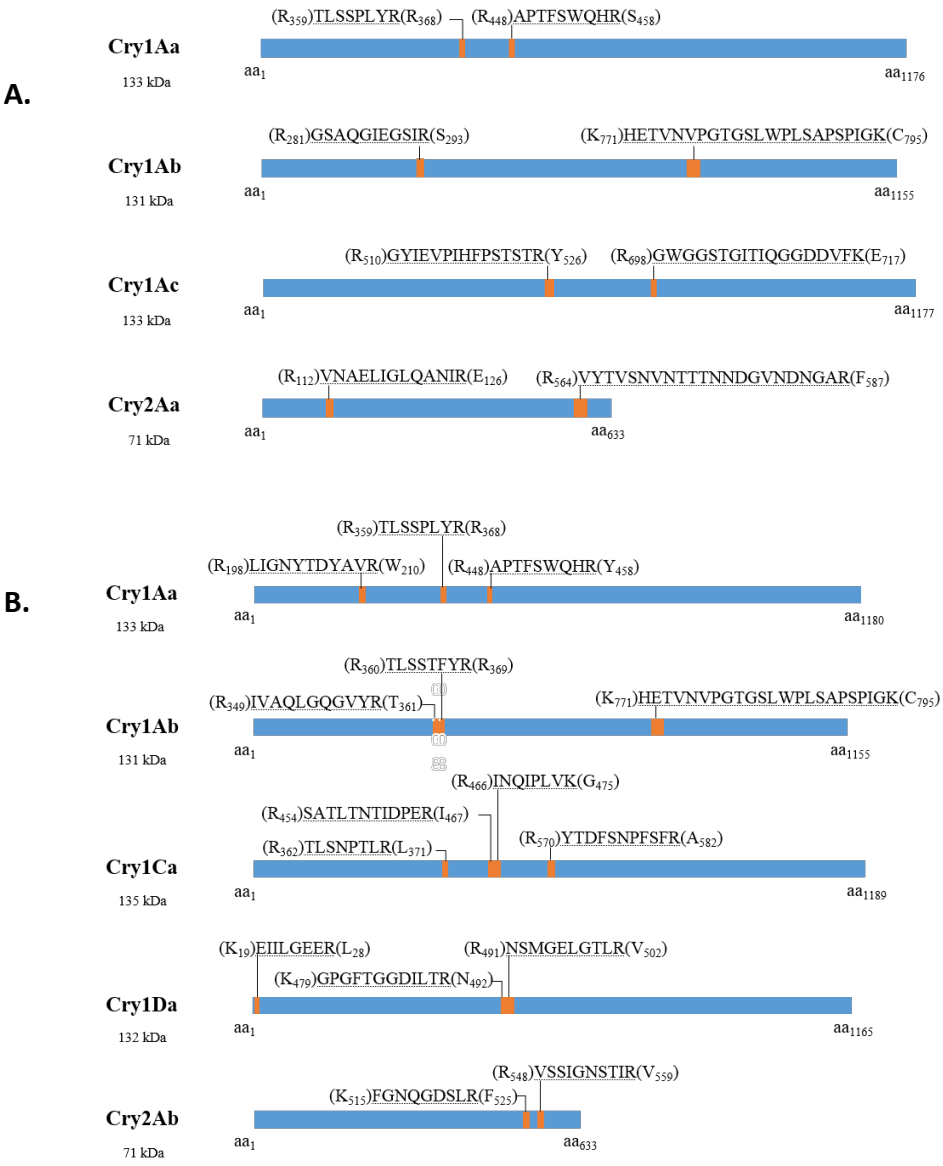
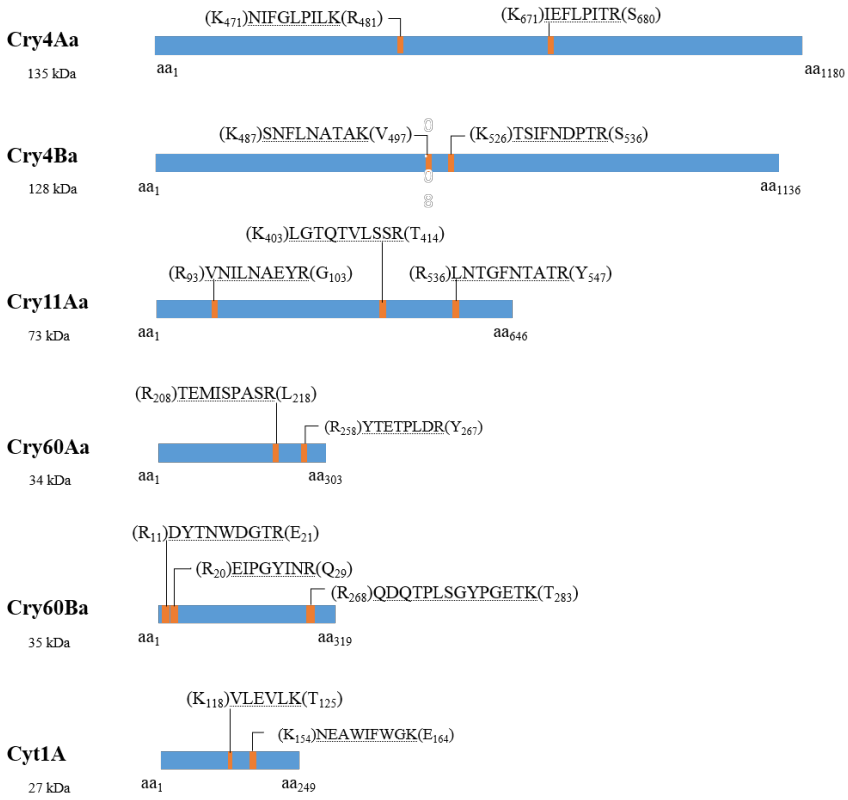


Figure S3. Proteins and proteotypic peptides selected for Dipel® DF (A), XenTari® GD (B), VectoBac® 12AS (C) and Novodor® (D) crystals. These peptides were detected in the IDA analysis and checked against the *in silico* digestion results. The location of the proteotypic peptides (orange bands) within full-length proteins (blue bars) are shown. Lower cases with numbers represent the initial and last amino acid within the sequence; and upper cases represent the amino acid sequence of each of the proteotypic peptides in each protein, indicating their position with numbers within the whole protein with different letters corresponding to different amino acids and the number indicating the position of the amino acid in the protein.

C.



D.

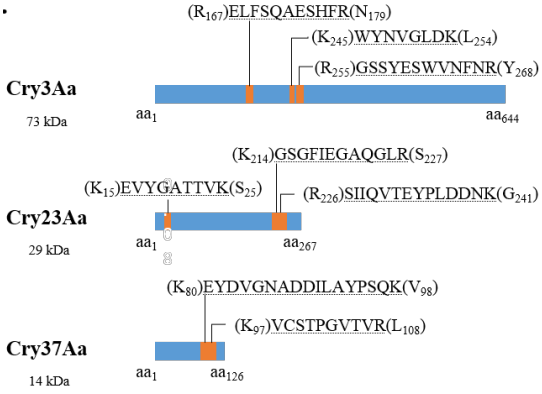


Figure S3. Continued.

Table S1. Proteotypic peptides in Cry protein mixtures. The protein mixtures contained Cry1Aa, Cry2Aa, and Cry6Aa proteins. Cry proteins were subjected to *in silico* tryptic digestion. The target peptides used to quantify the Cry proteins are shown in bold. Residues in parentheses are the previous and subsequent amino acid in the protein sequence.

Protein	Proteotypic peptide sequence (N to C)	Position in protein
Cry 1Aa	(R)VCPGR(G)	1022-1026
	(R)GMAQR(I)	282–286
	(R)VTAYK(E)	1032–1036
	(K)IDESK(L)	737–741
	(R)VHSIR(E)	935–939
	(R)LSDER(N)	676–680
	(R)GYILR(V)	1027–1031
	(R)AGFSHR(L)	425–430
	(K)CGEPNR(C)	794–799
	(R)NQAISR(L)	94–99
	(K)SYTDGR(R)	1113–1118
	(R)YNQFR(R)	229–233
	(K)QELSEK(V)	664–669
	(R)IEEFAR(N)	88–93
	(R)IEQNIR(Q)	287–292
	(R)YNDLTR(L)	193–198
	(K)TQDGHAR(L)	852–858
	(R)TVSQLTR(E)	259–265
	(R)VWGPDSR(D)	218–224
	(R)DVSVFGQR(W)	174–181
	(R)TLSSPLYR(R)	360–367
	(R)ENPCEFNR(G)	1120–1127
	(R)WYNTGLER(V)	210–217
	(R)TSPGQISTLR(V)	502–511
	(K)GPGFTGGDILR(R)	490–500
	(R)**VNITAPLSQR**(Y)	512–521
	(R)NLLQDPNFR(G)	681–689
	(R)**APTFSWQHR**(S)	449–457
	(K)STNLGSGTSVVK(G)	478–489
	(R)WGFDAATINSR(Y)	182–192
	(K)ELEYFPETDK(V)	1143–1152
	(R)LIGNYTDYAVR(W)	199–209
	(K)LEWETNIVYK(E)	892–901
	(R)IFTAFSLYDAR(N)	964–974
	(R)DYTPLPVGYVTK(E)	1131–1142
	(R)EWEADPTNPALR(E)	116–127
	(K)GHVDVEEQNNQR(S)	993–1004
	(R)GSTDITIQGGDDVFK(E)	701–715
	(K)NGDFNNGLSCWNVK(G)	979–992
	(K)AVNELFTSSNQIGLK(T)	622–636
	(R)GYIEDSQDLEIYLIR(Y)	752–766
	(R)CAPHLEWNPDLDCSCR(D)	800–815
	(R)EIYTNPVLENFDGSFR(G)	266–281
	(R)LEGLSNLYQIYAESFR(E)	100–115
	(R)LSHVTMLSQAAGAVYTLR(A)	431–448
	(R)SVLVLPEWEAEVSQEVR(V)	1005–1021
	(R)GTVDSLDVIPPQDNSVPPR(A)	406–424
	(R)GYNEAPSVPADYASVYEEK(S)	1094–1112

	(R)LGNLEFLEEKPLVGEALAR(V)	859–877
	(R)IEFVPAEVTFEAEYDLER(A)	601–618
	(R)ELTLTVLDIVALFSNYDSR(R)	235–253
	(R)SAEFNNIIPSSQITQIPLTK(S)	458–477
	(R)QPHLMDILNSITIYTDVHR(G)	293–311
	(K)EGYGEGCVTIHEIENNTDELK(F)	1037–1057
	(K)HETVNVPGTGSLWPLSAQSPIGK(C)	771–793
	(K)ENYVTLLGTFDECYPTYLYQK(I)	716–736
	(R)EAYLPELSVIPGVNAAIFEELEGR(I)	940–963
	(K)VWIEIGETEGTFIVDSVELLLMEE(-)	1153–1176
	(K)TDVTDYHIDQVSNLVECLSDEFCLDEK(Q)	637–663
	(-)MDNNPNINECIPYNCLSNPEVEVLGGER(I)	1–28
	(K)ESVDALFVNSQYDQLQADTNIAMIHAADK(R)	905–933
	(K)CAHHSHHFSLDIDVGCTDLNEDLGVWVIFK(I)	820–849
	(R)TVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR(I)	567–600
	(R)IILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYR(Q)	369–403
	(K)FSNCVEEEIYPNNTVTCNDYTVNQEEYGGAYTSR(N)	1058–1091
Cry 2Aa	(K)GLNTR(L)	233–237
	(R)VTINGR(V)	559–564
	(K)VGSLIGK(R)	65–71
	(R)CGAFSAR(G)	406–412
	(R)TFISEK(F)	510–515
	(K)SLDTIQK(E)	30–36
	(R)LNTDTLAR(V)	105–112
	(K)EWMEWK(R)	37–42
	(K)FGNQGDSLR(F)	516–524
	(-)MNNVLNSGR(T)	1–9
	(R)VSSIGNSTIR(V)	549–558
	(R)SWLDSGTDR(E)	376–384
	(R)FEQSNTTAR(Y)	525–533
	(R)AYLVSVHNR(K)	462–470
	(R)LHDMLEFR(T)	238–245
	(R)NISGVPLVIR(N)	425–434
	(R)**ETEQFLNQR**(L)	96–104
	(R)NIESPSGTPGGAR(A)	449–461
	(R)GNGNSYNLYLR(V)	538–548
	(R)**VNAELIGLQANIR**(E)	113–125
	(R)GNSNYFPDYFIR(N)	413–424
	(R)NEDLTRPLHYNQIR(N)	435–448
	(R)DYSNYCINTYQSAFK(G)	218–232
	(R)DVILNADEWGISAATLR(T)	190–206
	(R)TDHSLYVAPVVGTVSSFLLK(K)	44–63
	(R)TYMFLNVFEYVSIWSLFK(Y)	246–263
	(R)TTICDAYNVVAHDPFSFEHK(S)	10–29
	(R)**VYTVSNVNTTTNNDGVNDNGAR**(F)	565–586
	(R)EGVATSTNWQTESFQTTLSLR(C)	385–405
	(R)LSITFPNIGGLPGSTTTHSLNSAR(V)	316–339
	(R)ILSELWGIIFPSGSTNLMQDILR(E)	73–95
	(R)LPQFQIQGYQLLLLPLFAQAANLHLSFIR(D)	161–189
	(R)VNYSGGVSSGLIGATNLNHNFNCSTVLPPLSTPFVR(S)	340–375
Cry 6Aa	(K)LNSNK(K)	20–24
	(K)TTLPR(H)	7–11
	(K)TTLER(T)	234–238
	(K)DGYFK(K)	128–132
	(K)ELLEK(V)	224–228

(R)CGILIK(E)	162–167
(-)MIIDSK(T)	1–6
(K)VAGDPSIK(K)	119–126
(K)AEQDLEK(K)	242–248
(K)QYEEAAK(N)	171–177
(K)NQIDEIK(K)	278–284
(R)NSNLEYK(C)	444–450
(R)HSLIHTIK(L)	12–19
(K)NLYPLIIK(S)	100–107
(K)LEGVINIQK(R)	194–202
(K)TSAEWWNK(N)	92–99
(K)**SANDIASYGFK**(V)	108–118
(K**)QLDSAQHDLDR**(D)	286–296
(R)DFTLNAYSTNSR(Q)	379–390
(K)NIVTSLDQFLHGDQK(K)	178–192
(K)YGPGDMTNGNQFIISK(Q)	26–41
(K)LQGIWATIGAQIENLR(T)	328–343
(K)EVQTALNQAHGESSPAHK(E)	206–223
(K)LQDELDNIVDNNSDDDAIAK(A)	134–153
(K)IIGMLNSINTDIDNLYSQGQEAIK(V)	300–323
(K)QEWATIGAYIQTGLGLPVNEQQLR(T)	42–65
(R)THVNLSQDISIPSDFSQLYDVYCSDK(T)	66–91
(K)VEYSFLLGPLLGFVVYEILENTAVQHIK(N)	250–277
(K)CPENNFMIYWYNNSDWYNNSDWYNN(-)	451–475
(R)TTSLQEVQDSDDADEIQIELEDASDAWLVVAQEAR(D)	344–378

Table S2. MRM parameters for the detection and quantification of proteotypic peptides used for the quantification of Cry proteins in defined laboratory mixtures of Cry1Aa, Cry2Aa, and Cry6Aa proteins. The parameters were obtained after the analysis of two mixtures with different molar ratios of the proteins (from 13% to 43%). Two independent tryptic digestions of each protein mixture and two separate analyses for each digested sample were performed

Protein	Peptide sequence (N to C)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*) (*z*=+1)	Fragment ion
Cry1Aa	APTFSWQHR	565.3 (*z*=+2)	28.0	961.5	y7
				860.4	y6
				713.3	y5
				626.3	y4
				440.2	y3
		570.3 (*z*=+2)	28.0	971.5	y7
				870.4	y6
				723.4	y5
				636.3	y4
				450.2	y3
	VNITAPLSQR	549.8 (*z*=+2)	27.1	999.6	y9
				885.5	y8
				772.4	y7
				671.4	y6
				600.3	y5
		554.8 (*z*=+2)	27.1	1009.6	y9
				895.5	y8
				782.4	y7
				681.4	y6
				610.4	y5
Cry2Aa	ETEQFLNQR	582.8 (*z*=+2)	29.0	934.5	y7
				805.4	y6
				677.4	y5
				530.3	y4
				417.2	y3
		587.8 (*z*=+2)	29.0	944.5	y7
				815.4	y6
				687.4	y5
				540.3	y4
				427.2	y3
	VNAELIGLQANIR	705.9 (*z*=+2)	36.0	884.5	y8
				771.4	y7
				714.4	y6
				601.3	y5
				473.3	y4
		710.9 (*z*=+2)	36.0	894.5	y8
				781.5	y7
				724.4	y6
				611.4	y5
				483.3	y4
	VYTVSNVNTTTNNDGVNDNGAR	1163.0 (*z*=+2)	62.0	646.3	y6
				532.2	y5

				417.2	y4
				303.2	y3
				701.3	b3
		1168.0 (z=+2)	62.0	656.3	y6
				542.3	y5
				427.2	y4
				313.2	y3
				364.2	b3
		775.7 (z=+3)	31.1	646.3	y6
				532.2	y5
				417.2	y4
				303.2	y3
				364.2	b3
		779.0 (z=+3)	31.1	656.2	y6
				542.3	y5
				427.2	y4
				313.2	y3
				364.2	b3
Cry6Aa	SANDIASYGFK	586.8 (z=+2)	29.2	1014.5	y9
				900.4	y8
				785.4	y7
				672.3	y6
				601.3	y5
		590.8 (z=+2)	29.2	1022.5	y9
				908.5	y8
				793.4	y7
				680.3	y6
				609.3	y5
	QLDSAQHDLDR	649.3 (z=+2)	32.7	941.4	y8
				783.4	y6
				655.3	y5
				518.3	y4
				403.2	y3
		654.3 (z=+2)	32.7	951.5	y8
				793.4	y6
				665.3	y5
				528.3	y4
				413.2	y3
		433.2 (z=+3)	20.5	854.4	y7
				783.4	y6
				655.3	y5
				518.3	y4
				403.2	y3
		436.5 (z=+3)	20.5	864.4	y7
				793.4	y6
				665.3	y5
				528.3	y4
				413.2	y3

Table S3. Proteotypic peptides of Cry proteins predicted by *in silico* tryptic digestion of DiPel® DF crystals. The target peptides used to quantify the Cry proteins are shown in bold. Residues in parentheses are the previous and subsequent amino acids in the protein sequence

Protein	Proteotypic peptide (N to C)	Position in protein
Cry1Aa	(R)GMAQR(I)	282–286
	(R)AGFSHR(L)	425–430
	(R)IEQNIR(Q)	287–292
	(R)**TLSSPLYR**(R)	360–367
	(R)**APTFSWQHR**(S)	449–457
	(R)LSHVTMLSQAAGAVYTLR(A)	431–448
	(R)GTVDSLDVIPPQDNSVPPR(A)	406–424
	(R)ELTLTVLDIVALFSNYDSR(R)	235–253
	(R)QPHLMDILNSITIYTDVHR(G)	293–311
	(R)IILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYR(Q)	369–403
Cry1Ab	(R)TYPIR(T)	254–258
	(K)AYTDGR(R)	1092–1097
	(R)DNPCESNR(G)	1099–1106
	(R)TLSSTLYR(R)	361–368
	(R)**GSAQGIEGSIR**(S)	282–292
	(R)SGFSNSSVSIIR(A)	438–449
	(R)LIGNYTDHAVR(W)	199–209
	(K)GHVDVEEQNNHR(S)	968–979
	(R)GYGDYTPLPAGYVTK(E)	1107–1121
	(R)LQADTNIAMIHAADK(R)	894–908
	(K)ESVDALFVNSQYDR(L)	880–893
	(R)ELTLTVLDIVSLFPNYDSR(T)	235–253
	(K)**HETVNVPGTGSLWPLSAPSPIGK**(C)	772–794
	(R)GYDGAYESNSSVPADYASAYEEK(A)	1069–1091
	(K)FSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSR(N)	1033–1066
Cry1Ac	(R)GSAQGIER(S)	282–289
	(R)TLSSTFYR(R)	361–368
	(R)NLLQDSNFK(D)	682–690
	(R)SGSSSSVSIIR(A)	438–448
	(R)LNSSGNNIQNR(G)	500–510
	(R)NFSGTAGVIIDR(F)	590–601
	(K)AVNALFTSTNQLGLK(T)	623–637
	(R)**GYIEVPIHFPSTSTR**(Y)	511–525
	(R)**GWGGSTGITIQGGDDVFK**(E)	699–716
	(R)SAEFNNIIASDSITQIPAVK(G)	458–477
	(R)FEFIPVTATLEAEYNLER(A)	602–619
	(R)ELTLTVLDIVALFPNYDSR(R)	235–253
	(K)GNFLFNGSVISGPGFTGGDLVR(L)	478–499
	(K)ENYVTLSGTFDECYPTYLYQK(I)	717–737
	(K)TNVTDYHIDQVSNLVTYLSDEFCLDEK(R)	638–664
Cry2Aa	(K)VGSLIGK(R)	65–71
	(R)CGAFSAR(G)	406–412
	(K)SLDTIQK(E)	30–36
	(K)EWMEWK(R)	37–42
	(-)MNNVLNSGR(T)	1–9
	(R)SWLDSGTDR(E)	376–384
	(R)FEQSNTTAR(Y)	525–533

(R)AYLVSVHNR(K)	462–470
(R)NISGVPLVIR(N)	425–434
(R)ETEQFLNQR(L)	96–104
(R)NIESPSGTPGGAR(A)	449–461
(R)**VNAELIGLQANIR**(E)	113–125
(R)NEDLTRPLHYNQIR(N)	435–448
(R)DYSNYCINTYQTAFR(G)	218–232
(R)TDHSLYVAPVVGTVSSFLLK(K)	44–63
(R)TTICDAYNVVAHDPFSFEHK(S)	10–29
(R)**VYTVSNVNTTTNNDGVNDNGAR**(F)	565–586
(R)EGVATSTNWQTESFQTTLSLR(C)	385–405
(R)LSITFPNIGGLPGSTTTHSLNSAR(V)	316–339
(R)ILSELWGIIFPSGSTNLMQDILR(E)	73–95
(R)VNYSGGVSSGLIGATNLNHNFNCSTVLPPLSTPFVR(S)	340–375

Table S4. Proteotypic peptides of Cry proteins predicted by *in silico* tryptic digestion of XenTari® GD crystals. The target peptides used to quantify the Cry proteins are shown in bold. Residues in parentheses are the previous and subsequent amino acids in the protein sequence

Protein	Proteotypic peptide (N to C)	Position in protein
Cry1Aa	(R)GMAQR(I)	282–286
	(R)AGFSHR(L)	425–430
	(R)IEQNIR(Q)	287–292
	(R)**TLSSPLYR**(R)	360–367
	(R)**APTFSWQHR**(S)	449–457
	(R)**LIGNYTDYAVR**(W)	199–209
	(R)LSHVTMLSQAAGAVYTLR(A)	431–448
	(R)GTVDSLDVIPPQDNSVPPR(A)	406–424
	(R)ELTLTVLDIVALFSNYDSR(R)	235–253
	(R)SAEFNNIIPSSQVTQIPLTK(S)	458–477
	(R)QPHLMDILNSITIYTDVHR(G)	293–311
	(K)HETVNVPGTGSLWPLSAQSPIGK(C)	771–793
	(K)VWIEIGETEGTFIVDSVELLLMEEVDA(-)	1157–1183
	(R)TVGFTTPFNFPNGSSVFTLSAHVFNSGNEVYIDR(I)	567–600
	(R)IILGSGPNNQELFVLDGTEFSFASLTTNLPSTIYR(Q)	369–403
	(K)FSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSR(N)	1058–1091
Cry1Ab	(R)TYPIR(T)	254–258
	(R)QGFSHR(L)	424–429
	(R)**TLSSTLYR**(R)	361–368
	(R)LSHVSMFR(S)	430–437
	(R)ALAQGIEGSIR(S)	282–292
	(R)APMFSWIHR(S)	450–458
	(R)**IVAQLGQGVYR**(T)	350–360
	(R)SGFSNSSVSIIR(A)	438–449
	(R)LIGNYTDHAVR(W)	199–209
	(K)GHVDVEEQNNQR(S)	968–979
	(K)SGTVDSLDEIPPQNNNVPPR(Q)	404–423
	(R)ELTLTVLDIVSLFPNYDSR(T)	235–253
	(R)SPHLMDILNSITIYTDAHR(G)	293–311
	(R)SAEFNNIIPSSQITQIPLTK(S)	459–478
	(K)**HETVNVPGTGSLWPLSAPSPIGK**(C)	772–794
	(R)TVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYIDR(I)	568–601
	(R)RPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYR(K)	369–402
Cry1Ca	(R)YASSR(D)	525–529
	(K)SYTDGR(R)	1126–1131
	(R)IAEFAR(N)	87–92
	(R)DIPSFR(I)	143–148
	(R)EGYSHR(L)	423–428
	(R)GLNNLPK(S)	213–219
	(R)**TLSNPTLR**(L)	363–370
	(R)DSVIFGER(W)	173–180
	(R)**INQIPLVK**(G)	467–474
	(R)ILDGLLER(D)	135–142
	(R)EANQEPPR(S)	345–352
	(R)NFYWGGHR(V)	319–326
	(R)LCHATFVQR(S)	429–437

	(R)SFTFNGPVFR(T)	353–362
	(K)LQLETNIVYK(E)	901–910
	(K)TMEIGENLTSR(T)	557–567
	(K)DLEYFPETDK(V)	1156–1165
	(R)YPIQPVGQLTR(E)	254–264
	(R)**SATLTNTIDPER**(I)	455–466
	(R)IFTAYSLYDAR(N)	973–983
	(R)**YTDFSNPFSFR**(A)	571–581
	(K)STYQDWITYNR(L)	220–230
	(K)EWEEDPNNPATR(T)	115–126
	(K)AVNALFTSSNQIGLK(T)	631–645
	(R)LQVDTNIAMIHAADK(R)	928–942
	(R)LLQQPWPAPPFNLR(G)	371–384
	(R)WGLTTINVNENYNR(L)	181–194
	(R)VISSLIGGGNITSPIYGR(E)	327–344
	(R)HIDEYADHCANTYNR(G)	198–212
	(R)GVEGVEFSTPTNSFTYR(G)	385–401
	(R)SGTPFLTTGVVFSWTHR(S)	438–454
	(R)VWGGTSVITGPGFTGGDILR(R)	478–497
	(R)GTVDSLTELPPEDNSVPPR(E)	404–422
	(K)IEIILADATFEAESDLER(A)	610–627
	(R)DLTLTVLDIAAFFPNYDNR(R)	234–252
	(R)NTFGDFVSLQVNINSPITQR(Y)	499–518
	(R)VIVLTGAASTGVGGQVSVNMPLQK(T)	533–556
	(K)EGYGEGCVTIHEIEDNTDELK(F)	1046–1066
	(R)NAAIANLEGLGNNFNIYVEAFK(E)	93–114
	(K)HEIVNVPGTGSLWPLSAQSPIGK(C)	780–802
	(K)ENYVTLPGTVDECYPTYLYQK(I)	725–745
	(R)ISGFEVPLLSVYAQAANLHLAILR(D)	149–172
	(R)NPHLFDILNNLTIFTDWFSVGR(N)	297–318
	(R)NQGYDEAYGNNPSVPADYASVYEEK(S)	1101–1125
	(R)ANPDIIGISEQPLFGAGSISSGELYIDK(I)	582–609
	(K)TDVTDYHIDQVSNLVDCLSDEFCLDEK(Q)	646–672
	(-)MEENNQNQCIPYNCLSNPEEVLLDGER(I)	1–27
	(K)CAHHSHHFTLDIDVGCTDLNEDLGVWVIFK(I)	829–858
	(R)EVYTDPLINFNPQLQSVAQLPTFNVMESSAIR(N)	265–296
	(K)FSNCVEEEVYPNNTVTCNNYTGTQEEYEGTYTSR(N)	1067–1100
Cry1Da	(R)ISGPR(I)	432–436
	(R)SGTFR(Y)	525–529
	(K)SYTDR(R)	1102–1106
	(R)VTFTGR(L)	502–507
	(R)SPLYGR(E)	338–343
	(R)YASVANR(S)	518–524
	(R)AFSDWEK(D)	112–118
	(K)DPTNPALR(E)	119–126
	(R)DVSVFGER(W)	173–180
	(K)**EIILGEER**(L)	20–27
	(R)TGTTTNLIR(S)	329–337
	(R)ITQIPWVK(A)	460–467
	(R)LEGLSNLYK(V)	99–107
	(R)LPQSYYIR(F)	508–515
	(R)**NSMGELGTLR**(V)	492–501
	(R)LCHATFLER(I)	423–431
	(K)AHTLASGASVIK(G)	468–479
	(K)TMDAGEPLTSR(S)	543–553

	(K)**GPGFTGGDILTR**(N)	480–491
	(R)SASPTNEVSPSR(I)	448–459
	(R)IAGTVFSWTHR(S)	437–447
	(R)WGYDTATINNR(Y)	181–191
	(R)FLSDWIVYNR(F)	221–230
	(R)TYPIQTATQLTR(E)	253–264
	(R)ETLQLETTIVYK(E)	875–886
	(K)GHVEVEEQNNHR(S)	978–989
	(R)YSQPPSYGISFPK(T)	530–542
	(R)YAYWGGHLVNSFR(T)	316–328
	(K)VVNALFTSTNQLGLK(T)	607–621
	(R)SFAHTTLFTPITFSR(A)	554–568
	(R)CAPHLEWNPDLHCSCR(D)	785–800
	(R)AQEEFDLYIQSGVYIDR(I)	569–585
	(R)IQFNDMNSALITAIPLFR(V)	131–148
	(R)IEFIPVTATFEAEYDLER(A)	586–603
	(R)EGNTERPVTITASPSVPIFR(T)	344–363
	(-)MEINNQNQCVPYNCLSNPK(E)	1–19
	(R)QLTISVLDIVAFFPNYDIR(T)	234–252
	(R)SPHLVDFLNSFTIYTDSLAR(Y)	296–315
	(R)GYDEAYGNNPSVPADYASVYEEK(S)	1079–1101
	(K)ENYVTLPGTFDECYPTYLYQK(I)	701–721
	(R)EAYLPELSVIPGVNAAIFEELEER(I)	925–948
	(R)VQNYEVALLSVYVQAANLHLSILR(D)	149–172
	(K)SGPIDSFSELPPQDASVSPAIGYSHR(L)	397–422
	(R)YSDLTSLIHVYTNHCVDTYNQGLR(R)	192–215
	(R)TLSYITGLDNSNPVAGIEGVEFQNTISR(S)	364–391
	(K)TDVTDYHIDQVSNLVACLSDEFCLDEK(R)	622–648
	(K)HEIVNVPGTGSLWPLSVENQIGPCGEPNR(C)	756–784
	(R)EVYLDLPFINENLSPAASYPTFSAAESAIIR(S)	265–295
	(K)FNNCVEEEVYPNNTVTCINYTATQEEYEGTYTSR(N)	1043–1076
Cry2Ab	(K)GLNTR(L)	233–237
	(R)NEDLR(R)	435–439
	(R)VTINGR(V)	559–564
	(K)VGSLVGK(R)	65–71
	(K)FLNQR(L)	100–104
	(R)SGAFTAR(G)	406–412
	(R)TFISEK(F)	510–515
	(R)ILSELR(N)	73–78
	(K)SLDTVQK(E)	30–36
	(K)EWTEWK(K)	37–42
	(R)LNTDTLAR(V)	105–112
	(-)MNSVLNSGR(T)	1–9
	(K)**FGNQGDSLR**(F)	516–524
	(R)SWLDSGSDR(E)	376–384
	(R)**VSSIGNSTIR**(V)	549–558
	(R)NISGVPLVVR(N)	425–434
	(R)LHDMLEFR(T)	238–245
	(R)AYMVSVHNR(K)	462–470
	(R)FEQNNTTAR(Y)	525–533
	(R)NIASPSGTPGGAR(A)	449–461
	(R)RPLHYNEIR(N)	440–448
	(R)QVDNFLNPNR(N)	130–139
	(R)GNGNSYNLYLR(V)	538–548
	(R)GNSNYFPDYFIR(N)	413–424

(R)DYSNYCINTYQSAFK(G)	218–232
(R)DVILNADEWGISAATLR(T)	190–206
(R)VNAELTGLQANVEEFNR(Q)	113–129
(R)NLIFPSGSTNLMQDILR(E)	79–95
(K)NNHSLYLDPIVGTVASFLLK(K)	44–63
(R)TTICDAYNVAAHDPFSFQHK(S)	10–29
(R)TYMFLNVFEYVSIWSLFK(Y)	246–263
(R)VYTATNVNTTTNNDGVNDNGAR(F)	565–586
(R)NAVPLSITSSVNTMQQLFLNR(L)	140–160
(R)EGVATVTNWQTESFETTLGLR(S)	385–405
(R)LSNTFPNIVGLPGSTTTHALLAAR(V)	316–339
(R)LPQFQMQGYQLLLLPLFAQAANLHLSFIR(D)	161–189
(R)VNYSGGISSGDIGASPFNQNFNCSTFLPPLLTPFVR(S)	340–375

Table S5. Proteotypic peptides of Cry proteins predicted by *in silico* tryptic digestion of VectoBac® 12AS. The target peptides used to quantify the Cry proteins are shown in bold. Residues in parentheses are the previous and subsequent amino acids in the protein sequence

Protein	Proteotypic peptide (N to C)	Position in protein
Cry4Aa	(K)GLNLIK(T)	271–276
	(K)FEAYLK(N)	227–232
	(K)ANSLGTASK(V)	546–554
	(-)MNPYQNK(N)	1–7
	(R)YPIENSPK(Q)	29–36
	(K)SLSIPATYK(T)	506–514
	(K)**IEFLPITR**(S)	672–679
	(K)**NIFGLPILK**(R)	472–480
	(R)YASNGSANTR(A)	592–601
	(K)LNISNNYTR(Y)	20–28
	(K)QLLQSTNYK(D)	37–45
	(K)MDFFITNGTR(L)	443–452
	(K)YLNDYNNISK(M)	433–442
	(K)YPIGVQSELTR(E)	318–328
	(K)NEYETLNASQK(K)	8–18
	(K)SSVFGNHNVTDK(L)	398–409
	(K)EIASTYISNANK(I)	128–139
	(R)SDVYTNTTVLIDK(I)	659–671
	(K)DFQYLEFSNEVK(F)	634–645
	(K)FAPNQNISLVFNR(S)	646–658
	(R)SFNVISTYHNHLK(T)	144–156
	(K)ELTAGSGQITYDVNK(N)	457–471
	(K)VVQGPGHTGGDLIDFK(D)	555–570
	(K)AIEDYTNYCVTTYK(K)	256–269
	(K)NTIYTHLTTQIPAVK(A)	531–545
	(K)YYDFQYQEDSLTR(R)	343–355
	(R)EIYQVLNFEESPYK(Y)	329–342
	(K)TWENNPNPQNTQDVR(T)	157–171
	(K)TQVYTFAWTHSSVDPK(N)	515–530
	(K)ITCQHSNFQQSYFIR(I)	575–589
	(K)LETVQQIINTFYANPIK(N)	690–706
	(R)RPHLFTWLDSLNFYEK(A)	356–371
	(K)MTTAVLDLVALFPNYDVGK(Y)	299–317
	(K)SLGLATNIYIFLLNVISLDNK(Y)	412–432
	(K)TTPDSNLDGNINWNTYNTYR(T)	277–296
	(R)QFDYLEPLPTAIDYYPVLTK(A)	236–255
	(R)ENQGNPTLFPTYDNYSHILSFIK(S)	483–505
	(R)AVINLSIPGVAELGMALNPTFSGTDYTNLK(Y)	602–631
	(K)AQTTPNNFFTSHYNMFHYTLDNISQK(S)	372–397
	(K)NTLQSELTDYDIDQAANLVECISEELYPK(E)	707–735
Cry4Ba	(K)MTVVK(D)	116–120
	(K)IDNTK(L)	277–281
	(R)YPADK(I)	272–276
	(R)SYGLR(I)	536–540
	(R)TDANAK(M)	110–115
	(K)NTNYK(D)	17–21
	(K)TEFTR(E)	285–289

	(K)VAGAILK(F)	51–57
	(K)GLDVLR(N)	231–236
	(R)FLSANK(I)	337–342
	(K)FNTWK(R)	130–134
	(K)MDFYK(I)	396–400
	(K)YEEFR(Y)	585–589
	(R)MEIQCK(T)	521–526
	(K)**SNFLNATAK**(V)	488–496
	(R)VSFAWTHK(I)	460–467
	(R)EPNNQSYR(T)	136–143
	(K)**TSIFNDPTR**(S)	527–535
	(K)SIAALEAALTR(D)	303–313
	(K)DYLDQYTTK(F)	121–129
	(R)DVHLFTWLK(R)	314–322
	(K)DPFDAIVPMR(L)	592–601
	(K)TDVIDYNSNR(V)	450–459
	(K)TSITDTSSPSNR(V)	381–392
	(R)EVVNALFTNDAK(D)	651–662
	(R)TAVITQFNLTSAK(L)	144–156
	(R)EIYTALVESPSSK(S)	290–302
	(K)SNGQWITFNDYK(R)	239–250
	(R)DGLINAQEWSLAR(S)	191–203
	(K)EYIAHSITWYNK(G)	219–230
	(R)VDFWTNTIYQDLR(F)	324–336
	(R)SAGDQLYNTMVQYTK(E)	204–218
	(-)MNSGYPLANDLQGSMK(N)	1–16
	(K)GPGHTGGDLVALTSNGTLSGR(M)	500–520
	(K)IDGTLASYNSNITPTPEGLR(T)	401–420
	(R)YAANSPIVLNVSYVLQGVSR(G)	543–562
	(R)EMTIQVLDILALFASYDPR(R)	252–270
	(K)IVDPNNQIYTDAITQVPAVK(S)	468–487
	(R)GTTISTESTFSRPNNIIPTDLK(Y)	563–584
	(R)VWNDFMTNTGNLIDQTVTAYVR(T)	88–109
	(R)IEIIPITQSVLDETENQNLESER(E)	628–650
	(R)LSSNQLITIAIQPLNMTSNNQVIIDR(I)	602–627
	(K)DWLAMCENNQQYGVNPAAINSSSVSTALK(V)	22–50
	(K)FVNPPAGTVLTVLSAVLPILWPTNTPTPER(V)	58–87
	(K)DALNIGTTDYDIDQAANLVECISEELYPK(E)	663–691
	(R)TTFFGFSTNENTPNQPTVNDYTHILSYIK(T)	421–449
	(R)ETAVYFSNLVGYELLLLPIYAQVANFNLLLIR(D)	159–190
Cry11Aa	(K)AAFSK(V)	56–60
	(R)LTTVK(F)	286–290
	(R)YEGLK(L)	244–248
	(K)LSQDR(V)	89–93
	(R)LPAGIR(V)	559–564
	(K)LFNQK(V)	190–194
	(K)DFYSK(K)	476–480
	(K)VLDYR(T)	195–199
	(K)DGLTFR(N)	220–225
	(R)AISACPR(G)	431–437
	(K)NADINVK(F)	382–388
	(R)TFISNEAK(Y)	525–532
	(K)QPGFTPATAK(G)	117–126
	(R)MYTEEFGR(L)	205–212
	(K)LLMGEVNQR(L)	277–285

	(K)**LGTQTVLSSR**(T)	404–413
	(R)**VNILNAEYR**(G)	94–102
	(R)**LNTGFNTATR**(Y)	537–546
	(R)VQSQNSGNNR(M)	565–574
	(R)TEVETLINQK(L)	79–88
	(K)GYFLNLSGAIIQR(L)	127–139
	(K)ESAFTTQINPLLK(-)	634–646
	(R)GIIEVSDVFDAYIK(Q)	103–116
	(K)VLSLIFPGSQPATMEK(V)	61–76
	(R)SFLEDTPDQATDGSIK(F)	506–521
	(K)FNYSFTNEPADIPAR(E)	291–305
	(R)TQTFYQNPNNEPIAPR(D)	348–363
	(K)FTQWFQSTLYGWNIK(L)	389–403
	(R)GVSLAYNHDLTTLTYNR(I)	438–454
	(R)TGTIPPNYLAYDGYYIR(A)	414–430
	(R)DIINQILTAPAPADLFFK(N)	364–381
	(R)GVHPIYDPSSGLTGWIGNGR(T)	310–329
	(R)TNNFNFADNNGNEIMEVR(T)	330–347
	(R)NMCNLYVFPFAEAWSLMR(Y)	226–243
	(K)DGILAGSAWGFTQADVDSFIK(L)	169–189
	(R)IEYDSPTTENIIVGFAPDNTK(D)	455–475
	(K)SHYLSETNDSYVIPALQFAEVSDR(S)	482–505
	(K)LQSSLSLWDYVGVSIPVNYNEWGGLVYK(L)	249–276
	(R)LPQFEVQTYEGVSIALFTQMCTLHLTLLK(D)	140–168
Cry60Aa	(K)IGGAIK(V)	97–102
	(R)YSSPGK(T)	267–272
	(R)GIVKPVR(L)	294–300
	(K)GLYAFIR(Y)	252–258
	(R)**TEMISPASR**(L)	209–217
	(R)**YTETPLDR**(Y)	259–266
	(-)MEITDIVLK(I)	1–9
	(R)SSQTQLHTIK(F)	69–78
	(K)TWDSNLIHLR(D)	273–282
	(R)VPMILNSNLIGK(R)	175–186
	(R)DGQILNVYDNR(G)	283–293
	(K)MESVTNTTVHGFK(I)	84–96
	(R)LANQSWPGKPIVFK(S)	218–231
	(K)AIYEYELNDTVTIPETK(V)	33–49
	(K)SGGSNGSLNLSGFGYSDLYK(G)	232–251
	(K)VFQTTPIPIASALTITENR(S)	50–68
	(R)YYDDYANMFFSYIFQSK(T)	188–204
	(K)IYDFIEWDYVTNQDGIPYTLFDK(A)	10–32
	(K)TWEITENVSVASHTSLTSQLIIMQADIR(V)	147–174
Cry60Ba	(K)IGGGIK(S)	99–104
	(K)IPYCDR(S)	313–318
	(K)TWYSDK(V)	283–288
	(R)**EIPGYINR**(Q)	21–28
	(R)VTSTLIIMK(T)	164–172
	(R)**DYTNWDGTR**(E)	12–20
	(R)VPMELTTNLR(G)	177–186
	(K)VVETTTHTTTK(G)	85–95
	(-)MTITNIELAIR(D)	1–11
	(R)**QDQTPLSGYPGETK**(T)	269–282
	(K)LWEITDNITVPPHSR(V)	149–163
	(R)IVTLPSNADVNMSQTAK(I)	296–312

	(R)QVIDGPNIYDYVISDSVAVPK(T)	29–49
	(R)GTNSSGEGSFPTSNGLFSYTTSAR(G)	187–210
	(K)TVIFNVNPTPYTGPNIISENNTDVNQNK(R)	50–77
	(K)FPVGELGFEQTLELPLTGEYNSSSTTGNTCANEK(L)	115–148
Cyt1Aa	(K)VSAVK(E)	199–203
	(R)VITLR(V)	26–30
	(R)FSMPK(G)	79–83
	(K)VNPWK(T)	14–18
	(K)**VLEVLK**(T)	119–124
	(K)TPQSTAR(V)	19–25
	(K)**NEAWIFWGK**(E)	155–163
	(-)MENLNHCPLEDIK(V)	1–13
	(K)FAQPLVSSSQYPIADLTSAINGTL(-)	226–249
	(K)EQVLFFTIQDSASYNVNIQSLK(F)	204–225
	(K)TVLGVALSGSVIDQLTAAVTNTFTNLNTQK(N)	125–154
	(K)GLEIANTITPMGAVVSYVDQNVTQTNNQVSVMINK(V)	84–118
	(K)ETANQTNYTYNVLFAIQNAQTGGVMYCVPVGFEIK(V)	164–198

Table S6. Proteotypic peptides of Cry proteins in Novodor® crystals obtained by *in silico* tryptic digestion. The target peptides used to quantify the Cry proteins are shown in bold. Residues in parentheses are the previous and subsequent amino acids in the protein sequence

Protein	Proteotypic peptide (N to C)	Position in protein
Cry3Aa	(K)DVIQK(G)	64–68
	(K)TELTR(D)	297–301
	(K)VYIDK(I)	633–637
	(K)NPVSSR(N)	153–158
	(K)IADYAK(N)	121–126
	(-)MNPNNR(S)	1–6
	(R)NPHSQGR(I)	159–165
	(R)IQFHTR(F)	340–345
	(R)SEHDTIK(T)	7–13
	(K)IEFIPVN(-)	638–644
	(K)ITQLPLVK(A)	509–516
	(K)**WYNVGLDK**(L)	246–253
	(K)EDIAEFYK(R)	223–230
	(K)LQSGASVVAGPR(F)	520–531
	(R)GTIPVLTWTHK(S)	486–496
	(K)SVDFFNMIDSK(K)	497–507
	(R)KPHLFDYLHR(I)	330–339
	(K)LTQEYTDHCVK(W)	235–245
	(R)**ELFSQAESHFR**(N)	168–178
	(R)**GSSYESWVNFNR**(Y)	256–267
	(R)DVLTDPIVGVNNLR(G)	302–315
	(K)AFMEQVEALMDQK(I)	108–120
	(K)SSEPVQNLEFNGEK(V)	385–398
	(K)DAQIYGEEWGYEK(E)	210–222
	(R)GYGTTFSNIENYIR(K)	316–329
	(R)MTADNNTEALDSSTTK(D)	48–63
	(R)AVANTNLAVWPSAVYSGVTK(V)	402–421
	(K)GYSHQLNYVMCFLMQGSR(G)	468–485
	(R)EMTLTVLDLIALFPLYDVR(L)	271–289
	(K)VEFSQYNDQTDEASTQTYDSK(R)	422–442
	(R)NVGAVSWDSIDQLPPETTDEPLEK(G)	444–467
	(K)ALAELQGLQNNVEDYVSALSSWQK(N)	129–152
	(R)FTGGDIIQCTENGSAATIYVTPDVSYSQK(Y)	532–560
	(R)IHYASTSQITFTLSLDGAPFNQYYFDK(T)	565–591
	(K)TTENNEVPTNHVQYPLAETPNPTLEDLNYK(E)	14–43
	(R)NSMPSFAISGYEVLFLTTYAQAANTHLFLLK(D)	179–209
	(K)GDTLTYNSFNLASFSTPFELSGNNLQIGVTGLSAGDK(V)	596–632
Cry23Aa	(K)NSNIK(F)	262–266
	(K)VTIPPK(T)	140–145
	(K)AGTSISTK(Q)	91–98
	(K)GDGIAHFK(G)	207–214
	(K)STYDPSFK(V)	25–32
	(K)**EVYGATTVK**(S)	16–24
	(K)**GSGFIEGAQGLR**(S)	215–226
	(R)**SIIQVTEYPLDDNK**(G)	227–240
	(-)MGIINIQDEINNYMK(E)	1–15
	(R)STPITYLINGSLAPNVTLK(N)	243–261

	(K)VFNESVTPQFTEIPTEPVNNQLTTK(R)	33–57
	(R)DGALIAAVYVSVADLADYNPNLNLTNK(G)	180–206
	(R)VDNTGSYPVESTVSFTWTETHTETSAVTEGVK(A)	59–90
	(K)TYVEAAYIIQNGTYNVPVNVECDMSGTLFCR(G)	146–176
Cry37Aa	(K)STPER(S)	49–53
	(K)YSLTPA(-)	121–126
	(R)LDGDEK(G)	108–113
	(K)QDDWGK(S)	43–48
	(K)GSYVTIK(Y)	114–120
	(R)STYTQTIK(I)	54–61
	(K)**VCSTPGVTVR**(L)	98–107
	(K)AYLTNPDHDFEIWK(Q)	29–42
	(K)**EYDVGNADDILAYPSQK**(V)	81–97
	(K)ISSDTGSPINQMCFYGDVK(E)	62–80
	(-)MTVYNATFTINFYNEGEWGGPEPYGYIK(A)	1–28

Table S7. MRM parameters for the detection and quantification of proteotypic peptides used for the quantification of Cry proteins in DiPel® DF parasporal crystals. The parameters were obtained after two LC-MS/MS-MRM analyses of two independent tryptic digestions of DiPel® DF purified and solubilized crystals

Protein	Peptide sequence (N to C)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*) (*z* = +1)	Fragment ion
Cry1Aa	APTFSWQHR	565.3 (*z* = +2)	28	961.5	y7
				860.4	y6
				713.3	y5
				626.3	y4
				270.1	b3
		570.3 (*z* = +2)	28	971.5	y7
				870.4	y6
				723.4	y5
				636.3	y4
				270.1	b3
	TLSSPLYR	468.8 (*z* = +2)	22.5	835.5	y7
				722.4	y6
				635.4	y5
				548.3	y4
				451.3	y3
		473.8 (*z* = +2)	22.5	845.5	y7
				732.4	y6
				645.4	y5
				558.3	y4
				461.3	y3
Cry1Ab	HETVNVPGTGSLWPLSAP SPIGK	782.1 (*z* = +3)	31.3	966.6	y10
				756.4	y8
				669.4	y7
				598.4	y6
				414.3	y4
				680.3	b6
		784.8 (*z* = +3)	31.3	974.6	y10
				764.4	y8
				677.4	y7
				606.4	y6
				422.3	y4
				680.3	b6
	GSAQGIEGSIR	537.8 (*z* = +2)	26.4	731.4	y7
				674.4	y6
				561.3	y5
				432.3	y4
				375.2	y3
		542.8 (*z* = +2)	26.4	741.4	y7
				684.4	y6
				571.3	y5
				442.3	y4
				385.2	y3
Cry1Ac	GYIEVPIHFPSTSTR	852.4 (*z* = +2)	44.3	1142.6	y10
				932.5	y8

				648.3	y6
				334.2	b3
				463.2	b4
		857.4	44.3	1152.6	y10
		(*z* = +2)		942.5	y8
				658.3	y6
				334.2	b3
				463.2	b4
		568.6	24.7	1142.6	y10
		(*z* = +3)		932.5	y8
				795.4	y7
				648.3	y6
				464.2	y4
		572.0	24.7	1152.6	y10
		(*z* = +3)		942.5	y8
				805.4	y7
				658.3	y6
				474.3	y4
	GWGGSTGITIQGGDDVFK	897.9	46.9	737.3	y7
		(*z* = +2)		680.3	y6
				623.3	y5
				508.3	y4
				393.2	y3
		901.9	46.9	745.4	y7
		(*z* = +2)		688.3	y6
				631.3	y5
				516.3	y4
				401.3	y3
		599.0	25.6	737.3	y7
		(*z* = +3)		680.3	y6
				623.3	y5
				508.3	y4
				393.2	y3
		601.6	25.6	745.4	y7
		(*z* = +3)		688.3	y6
				631.3	y5
				516.3	y4
				401.3	y3
Cry2Aa	VNAELIGLQANIR	705.9	36	884.5	y8
		(*z* = +2)		771.4	y7
				714.4	y6
				601.3	y5
				473.3	y4
		710.9	36	894.5	y8
		(*z* = +2)		781.5	y7
				724.4	y6
				611.3	y5
				483.3	y4
		470.9	21.7	771.4	y7
		(*z* = +3)		714.4	y6
				601.3	y5
				473.3	y4
				402.2	y3

	474.3 (*z* = +3)	21.7	781.5 724.4 611.3 483.3 412.3	y7 y6 y5 y4 y3
VYTVSNVNTTTNNDGVND NGAR	1163.0 (*z* = +2)	62	646.3 532.2 417.2 303.2 364.2	y6 y5 y4 y3 b3
	1168.0 (*z* = +2)	62	656.3 542.3 427.2 313.2 364.2	y6 y5 y4 y3 b3
	775.7 (*z* = +3)	31.1	802.4 745.4 646.3 532.2 417.2	y8 y7 y6 y5 y4
	779.0 (*z* = +3)	31.1	812.4 755.4 656.3 542.3 427.2	y8 y7 y6 y5 y4

Table S8. MRM parameters for the detection and quantification of proteotypic peptides used for the quantification of Cry proteins in XenTari® GD parasporal crystals. The parameters were obtained after two LC-MS/MS-MRM analyses of two independent tryptic digestions of Xentari® GD purified and solubilized crystals

Protein	Peptide sequence (N to C)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*) (*z* = +1)	Fragment ion
Cry1Aa	TLSSPLYR	468.8 (*z* = +2)	22.5	722.4	y6
				635.4	y5
				548.3	y4
				451.3	y3
		473.8 (*z* = +2)	22.5	732.4	y6
				645.4	y5
				558.3	y4
				461.3	y3
	LIGNYTDYAVR	642.8 (*z* = +2)	32.4	887.4	y7
				724.4	y6
				623.3	y5
				508.3	y4
				345.2	y3
		647.8 (*z* = +2)	32.4	897.4	y7
				734.4	y6
				633.3	y5
				518.3	y4
				355.2	y3
	APTFSWQHR	565.3 (*z* = +2)	28	961.5	y7
				860.4	y6
				713.3	y5
				626.3	y4
				440.2	y3
				270.1	b3
		570.3 (*z* = +2)	28	971.5	y7
				870.4	y6
				723.3	y5
				636.3	y4
				450.2	y3
				270.1	b3
Cry1Ab	TLSSTLYR	470.8 (*z* = +2)	22.6	839.5	y7
				726.4	y6
				639.3	y5
				552.3	y4
				451.3	y3
		475.8 (*z* = +2)	22.6	849.5	y7
				736.4	y6
				649.3	y5
				562.3	y4
				461.3	y3
	IVAQLGQGVYR	602.3 (*z* = +2)	30.1	792.4	y7
				679.4	y6
				622.3	y5

				494.3	y4
				437.3	y3
		607.3	30.1	802.4	y7
		(*z* = +2)		689.4	y6
				632.3	y5
				504.3	y4
				447.3	y3
		401.9	19.5	792.4	y7
		(*z* = +3)		679.4	y6
				622.3	y5
				494.3	y4
				437.3	y3
		405.2	19.5	802.4	y7
		(*z* = +3)		689.4	y6
				632.3	y5
				504.3	y4
				447.3	y3
	HETVNVPGTGSLWPLSAP SPIGK	782.1	31.3	966.6	y10
		(*z* = +3)		756.4	y8
				669.4	y7
				598.4	y6
				414.3	y4
				680.3	b6
		784.8	31.3	974.6	y10
		(*z* = +3)		764.4	y8
				677.4	y7
				606.4	y6
				422.3	y4
				680.3	b6
Cry1Ca	YTDFSNPFSFR	690.8	35.1	1116.5	y9
		(*z* = +2)		854.4	y7
				767.4	y6
				653.3	y5
		695.8	35.1	1126.5	y9
		(*z* = +2)		864.4	y7
				777.4	y6
				663.3	y5
	SATLTNTIDPER	659.3	33.3	945.5	y8
		(*z* = +2)		844.4	y7
				516.2	y4
				401.2	y3
		664.3	33.3	955.5	y8
		(*z* = +2)		854.4	y7
				526.2	y4
				411.2	y3
	INQIPLVK	462.8	22.1	811.5	y7
		(*z* = +2)		697.5	y6
				569.4	y5
				456.3	y4
		466.8	22.1	819.5	y7
		(*z* = +2)		705.5	y6
				577.4	y5
				464.3	y4

	TLSNPTLR	451.3 (*z* = +2)	21.5	800.5 687.4 600.3 486.3 389.3	y7 y6 y5 y4 y3
		456.3 (*z* = +2)	21.5	810.5 697.4 610.4 496.3 399.3	y7 y6 y5 y4 y3
Cry1Da	NSMGELGTLR	539.3 (*z* = +2)	26.5	876.5 745.4 559.4 446.3	y8 y7 y5 y4
		544.3 (*z* = +2)	26.5	876.5 745.4 559.4 446.3	y8 y7 y5 y4
	EIILGEER	479.8 (*z* = +2)	23.1	716.4 603.3 490.2 433.2	y6 y5 y4 y3
		484.8 (*z* = +2)	23.1	726.4 613.3 500.2 443.2	y6 y5 y4 y3
	GPGFTGGDILTR	595.8 (*z* = +2)	29.7	731.4 674.4 502.3 389.3	y7 y6 y4 y3
		600.8 (*z* = +2)	29.7	741.4 684.4 512.3 399.3	y7 y6 y4 y3
Cry2Ab	VSSIGNSTIR	517.3 (*z* = +2)	25.2	934.5 847.5 647.3 590.3	y9 y8 y6 y5
		522.3 (*z* = +2)	25.2	944.5 857.5 657.4 600.3	y9 y8 y6 y5
	FGNQGDSLR	497.2 (*z* = +2)	24.1	846.4 547.3 490.3 375.2	y8 y5 y4 y3
		502.2 (*z* = +2)	24.1	856.4 557.3 500.3 385.2	y8 y5 y4 y3

Table S9. MRM parameters for the detection and quantification of proteotypic peptides used for the quantification of Cry proteins in VectoBac® 12AS parasporal crystals. The parameters were obtained after two LC-MS/MS-MRM analyses of two independent tryptic digestions of VectoBac® 12AS purified and solubilized crystals

Protein	Peptide sequence (N to C)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*) (*z* = +1)	Fragment ion
Cry4Aa	IEFLPITR	494.8 (*z* = +2)	23.9	875.5	y7
				746.5	y6
				599.4	y5
				486.3	y4
				389.3	y3
		499.8 (*z* = +2)	23.9	885.5	y7
				756.5	y6
				609.4	y5
				496.3	y4
				399.3	y3
	NIFGLPILK	507.8 (*z* = +2)	24.7	787.5	y7
				640.4	y6
				583.4	y5
				470.3	y4
				373.3	y3
		511.8 (*z* = +2)	24.7	795.5	y7
				648.5	y6
				591.4	y5
				478.3	y4
				381.3	y3
Cry4Ba	SNFLNATAK	483.3 (*z* = +2)	23.3	764.4	y7
				617.4	y6
				504.3	y5
				390.2	y4
				319.2	y3
		487.3 (*z* = +2)	23.3	772.4	y7
				625.4	y6
				512.3	y5
				398.2	y4
				327.2	y3
	TSIFNDPTR	525.8 (*z* = +2)	25.7	862.4	y7
				749.4	y6
				602.3	y5
				488.2	y4
				373.2	y3
		530.8 (*z* = +2)	25.7	872.5	y7
				759.4	y6
				612.3	y5
				498.3	y4
				383.2	y3
Cry11Aa	VNILNAEYR	546.3 (*z* = +2)	26.9	878.5	y7
				765.4	y6
				652.3	y5
				538.3	y4

				467.2	y3
		551.3 (*z* = +2)	26.9	888.5 775.4 662.3 548.3 477.2	y7 y6 y5 y4 y3
	LGTQTVLSSR	531.3 (*z* = +2)	26.0	790.4 662.4 561.3 462.3 349.2	y7 y6 y5 y4 y3
		536.3 (*z* = +2)	26.0	800.4 672.4 571.3 472.3 359.2	y7 y6 y5 y4 y3
	LNTGFNTATR	547.8 (*z* = +2)	27.0	867.4 766.4 709.4 562.3 448.3	y8 y7 y6 y5 y4
		552.8 (*z* = +2)	27.0	877.4 776.4 719.4 572.3 458.3	y8 y7 y6 y5 y4
Cry60Aa	YTETPLDR	497.7 (*z* = +2)	24.1	730.4 601.3 500.3 403.2 290.1	y6 y5 y4 y3 y2
		502.7 (*z* = +2)	24.1	740.4 611.3 510.3 413.2 300.2	y6 y5 y4 y3 y2
	TEMISPASR	496.2 (*z* = +2)	24.0	761.4 630.4 517.3 430.2	y7 y6 y5 y4
		501.3 (*z* = +2)	24.0	771.4 640.4 527.3 440.2	y7 y6 y5 y4
Cry60Ba	EIPGYINR	481.3 (*z* = +2)	23.2	719.4 622.3 565.3 402.2	y6 y5 y4 y3
		486.3 (*z* = +2)	23.2	729.4 632.3 575.3 412.3	y6 y5 y4 y3

	QDQTPLSGYPGETK	760.9 (z = +2)	39.1	751.4 694.3 531.3 434.2 377.2	y7 y6 y5 y4 y3
		764.9 (z = +2)	39.1	759.4 702.4 539.3 442.2 385.2	y7 y6 y5 y4 y3
	DYTNWDGTR	564.2 (z = +2)	27.9	849.4 748.3 634.3 448.2 333.2	y7 y6 y5 y4 y3
		569.2 (z = +2)	27.9	859.4 758.3 644.3 458.2 343.2	y7 y6 y5 y4 y3
Cyt1A	NEAWIFWGK	575.8 (z = +2)	28.6	907.5 836.4 650.4 537.3 390.2	y7 y6 y5 y4 y3
		579.8 (z = +2)	28.6	915.5 844.5 658.4 545.3 398.2	y7 y6 y5 y4 y3
	VLEVLK	350.7 (z = +2)	15.7	601.4 488.3 359.3 260.2	y5 y4 y3 y2
		354.7 (z = +2)	15.7	609.4 496.3 367.3 268.2	y5 y4 y3 y2

Table S10. MRM parameters for the detection and quantification of proteotypic peptides used for the quantification of Cry proteins in Novodor® parasporal crystals. The parameters were obtained after two LC-MS/MS-MRM analyses of two independent tryptic digestions of Novodor® purified and solubilized crystals

Protein	Peptide sequence (N to C)	Precursor ion (*m/z*)	Collision energy (V)	Product ion (*m/z*) (*z* = +1)	Fragment ion
Cry3Aa	WYNVGLDK	497.8 (*z*=+2)	24.1	808.4	y7
				645.4	y6
				531.3	y5
				432.2	y4
		501.8 (*z*=+2)	24.1	816.4	y7
				653.4	y6
				539.3	y5
				440.3	y4
	ELFSQAESHFR	675.8 (*z*=+2)	34.3	961.5	y8
				874.4	y7
				746.4	y6
				675.3	y5
				546.3	y4
		680.8 (*z*=+2)	34.3	971.5	y8
				884.4	y7
				756.4	y6
				685.3	y5
				556.3	y4
		450.9 (*z*=+3)	21.1	874.4	y7
				746.4	y6
				675.3	y5
				546.3	y4
				459.3	y3
		454.2 (*z*=+3)	21.1	884.4	y7
				756.4	y6
				685.3	y5
				556.3	y4
				469.3	y3
	GSSYESWVNFNR	723.3 (*z*=+2)	37.0	1301.6	y10
				1214.6	y9
				1051.5	y8
				922.5	y7
				835.4	y6
		728.3 (*z*=+2)	37.0	1311.6	y10
				1224.6	y9
				1061.5	y8
				932.5	y7
				845.4	y6
		482.6 (*z*=+3)	22.0	1051.5	y8
				922.5	y7
				835.4	y6
				649.3	y5
				550.3	y4

		485.9 (z=+3)	22.0	1061.5 932.5 845.4 659.3 560.3	y8 y7 y6 y5 y4
Cry23Aa	GSGFIEGAQGLR	596.3 (z=+2)	29.7	843.5 730.4 601.3 544.3 473.3	y8 y7 y6 y5 y4
		601.3 (z=+2)	29.7	853.5 740.4 611.3 554.3 483.3	y8 y7 y6 y5 y4
	EVYGATTVK	484.3 (z=+2)	23.3	739.4 576.3 519.3 448.3 347.2	y7 y6 y5 y4 y3
		488.3 (z=+2)	23.3	747.4 584.3 527.3 456.3 355.2	y7 y6 y5 y4 y3
	SIIQVTEYPLDDNK	545.6 (z=+3)	24.0	993.5 864.4 701.3 604.3 491.2	y8 y7 y6 y5 y4
		548.3 (z=+3)	24.0	1001.5 872.4 709.4 612.3 499.2	y8 y7 y6 y5 y4
Cry37Aa	VCSTPGVTVR†	538.3 (z=+2)	26.4	976.5 816.5 729.4 628.4	y9 y8 y7 y6
		543.3 (z=+2)	26.4	986.5 826.5 739.4 638.4	y9 y8 y7 y6
	EYDVGNADDILAYPSQK	949.4 (z=+2)	49.9	1149.6 806.4 693.4 622.3 459.3	y10 y7 y6 y5 y4
		953.5 (z=+2)	49.9	1157.6 814.5 701.4 630.3	y10 y7 y6 y5

		467.3	y4
633.3 (*z*=+3)	26.7	1034.6	y9
		919.5	y8
		806.4	y7
		693.4	y6
		459.3	y4
636.0 (*z*=+3)	26.7	1042.6	y9
		927.5	y8
		814.5	y7
		701.4	y6
		467.3	y4

Table S11. Quantification of the targeted peptides in proteins identified in DiPel® DF parasporal crystals. Peptides were obtained in the course of two separate tryptic digestions (D1 and D2), and each digested sample was analyzed twice (MS/MS injections I1 and I2)

Protein	Digestion and injection	Proteotypic peptide	Spiked-in SIL peptide (fmol)	Ratio*	Endogenous peptide (fmol)
Cry1Aa	D1, I1	APTFSWQHR	800	0.7	561
		TLSSPLYR	800	1.4	1107
	D1, I2	APTFSWQHR	800	0.8	674
		TLSSPLPLYR	800	1.0	836
	D2, I1	APTFSWQHR	800	0.8	652
		TLSSPL	800	1.1	874
	D2, I2	APTFSWQHR	800	1.4	1097
		TLSSPLYR	800	1.7	1336
Cry1Ab	D1, I1	HETVNVPGTGSLWPLSAP	800	NQ	NQ
		SPIGK	800	1.8	1400
		GSAQGIEGSIR			
	D1, I2	HETVNVPGTGSLWPLSAP	800	NQ	NQ
		SPIGK	800	1.2	952
		GSAQGIEGSIR			
	D2, I1	HETVNVPGTGSLWPLSAP	800	NQ	NQ
		SPIGK	800	1.1	890
		GSAQGIEGSIR			
	D2, I2	HETVNVPGTGSLWPLSAP	800	NQ	NQ
		SPIGK	800	1.7	1395
		GSAQGIEGSIR			
Cry1Ac	D1, I1	GYIEVPIHFPSTSTR	400	0.8	312
		GWGGSTGITIQGGDDVFK	400	1.0	416
	D1, I2	GYIEVPIHFPSTSTR	400	0.8	307
		GWGGSTGITIQGGDDVFK	400	1.0	420
	D2, I1	GYIEVPIHFPSTSTR	400	1.8	309
		GWGGSTGITIQGGDDVFK	400	1.3	529
	D2, I2	GYIEVPIHFPSTSTR	400	1.0	410
		GWGGSTGITIQGGDDVFK	400	1.2	475
Cry2Aa	D1, I1	VNAELIGLQANIR	2000	NQ	NQ
		VYTVSNVNTTTNNDGVND	2000	1.1	2152
		NGAR			
	D1, I2	VNAELIGLQANIR	2000	NQ	NQ
		VYTVSNVNTTTNNDGVND	2000	1.9	3760
		NGAR			
	D2, I1	VNAELIGLQANIR	2000	NQ	NQ
		VYTVSNVNTTTNNDGVND	2000	0.7	1372
		NGAR			
	D2, I2	VNAELIGLQANIR	2000	NQ	NQ
		VYTVSNVNTTTNNDGVND	2000	1.3	2523
		NGAR			

NQ = not quantified; *SIL peptide:endogenous peptide ratio

Table S12. Quantification of the targeted peptides in proteins identified in XenTari® GD parasporal crystals. Peptides were obtained in the course of two separate tryptic digestions (D1 and D2), and each digested sample was analysed twice (MS/MS injections I1 and I2)

Protein	Digestion and injection	Proteotypic peptide	Spiked-in SIL peptide (fmol)	Ratio*	Endogenous peptide (fmol)
Cry1Aa	D1, I1	APTFSWQHR	1200	0.59	713
		LIGNYTDYAVR	1200	0.28	334
		TLSSPLYR	1200	0.87	1043
	D1, I2	APTFSWQHR	1200	0.48	572
		LIGNYTDYAVR	1200	0.29	344
		TLSSPLYR	1200	0.78	932
	D1, I1	APTFSWQHR	1200	0.35	416
		LIGNYTDYAVR	1200	0.20	244
		TLSSPLYR	1200	0.53	634
	D1, I2	APTFSWQHR	1200	0.32	390
		LIGNYTDYAVR	1200	0.19	223
		TLSSPLYR	1200	0.60	723
Cry1Ab	D1, I1	HETVNVPGTGSLWPLSAPSPIGK	1200	1.52	1825
		IVAQLGQGVYR	1200	0.99	1188
		TLSSTLYR	1200	0.47	568
	D1, I2	HETVNVPGTGSLWPLSAPSPIGK	1200	1.35	1623
		IVAQLGQGVYR	1200	1.38	1651
		TLSSTLYR	1200	0.69	826
	D1, I1	HETVNVPGTGSLWPLSAPSPIGK	1200	1.20	1443
		IVAQLGQGVYR	1200	0.87	1047
		TLSSTLYR	1200	0.46	557
	D1, I2	HETVNVPGTGSLWPLSAPSPIGK	1200	1.24	1489
		IVAQLGQGVYR	1200	0.84	1005
		TLSSTLYR	1200	0.46	556
Cry1Ca	D1, I1	INQIPLVK	200	0.66	131
		SATLTNTIDPER	200	0.40	81
		TLSNPTLR	200	1.05	209
		YTDFSNPFSFR	200	0.84	169
	D1, I2	INQIPLVK	200	1.18	236
		SATLTNTIDPER	200	0.74	148
		TLSNPTLR	200	1.38	276
		YTDFSNPFSFR	200	1.54	307
	D1, I1	INQIPLVK	200	0.85	170
		SATLTNTIDPER	200	0.51	102
		TLSNPTLR	200	1.27	254
		YTDFSNPFSFR	200	0.99	197
	D1, I2	INQIPLVK	200	0.85	170
		SATLTNTIDPER	200	0.59	118
		TLSNPTLR	200	1.15	230
		YTDFSNPFSFR	200	1.06	213
Cry1Da	D1, I1	EIILGEER	80	1.91	153
		GPGFTGGDILTR	80	0.46	36

		NSMGELGTLR	80	0.39	31
	D1, I2	EIILGEER	80	1.97	158
		GPGFTGGDILTR	80	0.57	46
		NSMGELGTLR	80	0.37	29
	D1, I1	EIILGEER	80	1.45	116
		GPGFTGGDILTR	80	0.54	43
		NSMGELGTLR	80	0.34	27
	D1, I2	EIILGEER	80	1.35	108
		GPGFTGGDILTR	80	0.56	45
		NSMGELGTLR	80	0.41	33
Cry2Ab	D1, I1	FGNQGDSLR	20	NQ	NQ
		VSSIGNSTIR	20	NQ	NQ
	D1, I2	FGNQGDSLR	20	NQ	NQ
		VSSIGNSTIR	20	NQ	NQ
	D1, I1	FGNQGDSLR	20	NQ	NQ
		VSSIGNSTIR	20	NQ	NQ
	D1, I2	FGNQGDSLR	20	NQ	NQ
		VSSIGNSTIR	20	NQ	NQ

NQ = not quantified; ˆSIL peptide:endogenous peptide ratio

Table S13. Quantification of the targeted peptides in proteins identified in VectoBac® 12AS parasporal crystals. Peptides were obtained in the course of two separate tryptic digestions (D1 and D2), and each digested sample was analysed twice (MS/MS injections I1 and I2)

Protein	Digestion and injection	Proteotypic peptide	Spiked-in SIL peptide (fmol)	Ratio*	Endogenous peptide (fmol)
Cry4Aa	D1, I1	IEFLPITR	100	0.3	31
		NIFGLPILK	100	0.7	65
	D1, I2	IEFLPITR	100	0.05	5
		NIFGLPILK	100	0.3	34
	D1, I1	IEFLPITR	100	1.0	100
		NIFGLPILK	100	1.1	110
	D1, I2	IEFLPITR	100	0.9	92
		NIFGLPILK	100	1.0	97
Cry4Ba	D1, I1	SNFLNATAK	200	0.7	142
		TSIFNDPTR	200	1.3	253
	D1, I2	SNFLNATAK	200	1.3	267
		TSIFNDPTR	200	1.8	363
	D1, I1	SNFLNATAK	200	1.1	228
		TSIFNDPTR	200	2.0	390
	D1, I2	SNFLNATAK	200	1.1	224
		TSIFNDPTR	200	1.9	383
Cry11Aa	D1, I1	VNILNAEYR	200	2.3	463
		LGTQTVLSSR	200	1.3	266
		LNTGFNTATR	200	1.2	238
	D1, I2	VNILNAEYR	200	1.0	207
		LGTQTVLSSR	200	0.4	84
		LNTGFNTATR	200	0.3	52
	D1, I1	VNILNAEYR	200	4.1	815
		LGTQTVLSSR	200	2.7	547
		LNTGFNTATR	200	2.3	460
	D1, I2	VNILNAEYR	200	3.6	729
		LGTQTVLSSR	200	2.8	563
		LNTGFNTATR	200	2.3	462
Cry60Aa	D1, I1	YTETPLDR	60	0.8	45
		TEMISPASR	60	0.8	49
	D1, I2	YTETPLDR	60	0.6	38
		TEMISPASR	60	0.6	39
	D1, I1	YTETPLDR	60	1.3	75
		TEMISPASR	60	1.4	85
	D1, I2	YTETPLDR	60	1.2	71
		TEMISPASR	60	1.3	80
Cry60Ba	D1, I1	EIPGYINR	200	0.5	109
		QDQTPLSGYPGETK	200	0.5	98
		DYTNWDGTR	200	0.8	160

	D1, I2	EIPGYINR	200	0.8	166
		QDQTPLSGYPGETK	200	0.5	92
		DYTNWDGTR	200	0.7	136
	D1, I1	EIPGYINR	200	0.8	153
		QDQTPLSGYPGETK	200	0.6	120
		DYTNWDGTR	200	1.1	214
	D1, I2	EIPGYINR	200	0.8	155
		QDQTPLSGYPGETK	200	0.6	129
		DYTNWDGTR	200	1.2	239
Cyt1A	D1, I1	NEAWIFWGK	400	1.6	631
		VLEVLK	400	0.7	261
	D1, I2	NEAWIFWGK	400	1.9	772
		VLEVLK	400	0.5	204
	D1, I1	NEAWIFWGK	400	6.7	2672
		VLEVLK	400	2.5	1014
	D1, I2	NEAWIFWGK	400	6.9	2756
		VLEVLK	400	2.7	1067

*SIL peptide:endogenous peptide ratio

Table S14. Quantification of the targeted peptides in proteins identified in Novodor® parasporal crystals. Peptides were obtained in the course of two separate tryptic digestions (D1 and D2), and each digested sample was analysed twice (MS/MS injections I1 and I2)

Protein	Digestion and injection	Proteotypic peptide	Spiked-in SIL peptide (fmol)	Ratio‡	Endogenous peptide (fmol)
Cry3Aa	D1, I1	WYNVGLDK	1600	0.2	282
		ELFSQAESHFR	1600	0.5	803
		GSSYESWVNFNR	1600	1.4	2234
	D1, I2	WYNVGLDK	1600	0.2	276
		ELFSQAESHFR	1600	0.5	810
		GSSYESWVNFNR	1600	1.5	2412
	D1, I1	WYNVGLDK	1600	0.5	765
		ELFSQAESHFR	1600	1.4	2180
		GSSYESWVNFNR	1600	2.8	4525
	D1, I2	WYNVGLDK	1600	0.5	776
		ELFSQAESHFR	1600	1.4	2302
		GSSYESWVNFNR	1600	3.0	4824
Cry23Aa	D1, I1	GSGFIEGAQGLR	400	1.1	455
		EVYGATTVK	400	0.3	114
		SIIQVTEYPLDDNK	400	0.4	177
	D1, I2	GSGFIEGAQGLR	400	1.2	484
		EVYGATTVK	400	0.3	107
		SIIQVTEYPLDDNK	400	0.5	187
	D1, I1	GSGFIEGAQGLR	400	2.0	798
		EVYGATTVK	400	0.5	181
		SIIQVTEYPLDDNK	400	1.1	423
	D1, I2	GSGFIEGAQGLR	400	2.2	894
		EVYGATTVK	400	0.4	157
		SIIQVTEYPLDDNK	400	1.2	473
Cry37Aa	D1, I1	VCSTPGVTVR	300	0.8	250
		EYDVGNADDILAYPSQK	300	0.6	176
	D1, I2	VCSTPGVTVR	300	0.9	278
		EYDVGNADDILAYPSQK	300	0.6	189
	D1, I1	VCSTPGVTVR	300	1.1	344
		EYDVGNADDILAYPSQK	300	1.2	348
	D1, I2	VCSTPGVTVR	300	1.3	398
		EYDVGNADDILAYPSQK	300	1.2	373

*SIL peptide:endogenous peptide ratio