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CÂMPUS DE JABOTICABAL

Sublethal effects of *Bacillus thuringiensis* Berliner in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

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2021

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Dissertation presented to the Faculty of Agricultural and Veterinary Sciences - Unesp, Jaboticabal Campus, as part of the requirements for obtaining the title of Master in Agronomy (Agricultural Entomology).

2021

M921s

Motta, Amanda Cristiane Queiroz
Sublethal effects of Bacillus thuringiensis Berliner in Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae) / Amanda Cristiane Queiroz Motta. -- Jaboticabal, 2021
116 p. : tabs., fotos

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal
Orientador: Ricardo Antonio Polanczyk
Coorientadora: Naymã Pinto Dias

1. Lethal concentration. 2. Cry poteins. 3. Fall armyworm. 4. Molecular biology. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

UNIVERSIDADE ESTADUAL PAULISTA

Câmpus de Jaboticabal

CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: SUBLETHAL EFFECTS OF *Bacillus Thuringiensis* BERLINER IN *Spodoptera Frugiperda* (J.E. SMITH) (LEPIDOPTERA: NOCTUIDAE)

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Aprovada como parte das exigências para obtenção do Título de Mestra em AGRONOMIA (ENTOMOLOGIA AGRÍCOLA), pela Comissão Examinadora:

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Jaboticabal, 25 de março de 2021

DADOS CURRICULARES DA AUTORA

AMANDA CRISTIANE QUEIROZ MOTTA – Natural de Sete Lagoas, MG, nascida em 21 de junho de 1996, filha de Jorge Eustáquio Pereira e Cléria Queiroz da Costa. Formada técnica em Meio Ambiente pela Escola Técnica Municipal de Sete Lagoas – Minas Gerais, no ano de 2014; formada Engenheira Agrônoma pela Universidade Federal de São João Del-Rei – *Campus* Sete Lagoas, no ano de 2018. Durante a graduação desenvolveu três projetos de Iniciação Científica na área de Entomologia Agrícola sendo dois sob financiamento do Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq, sob orientação da Profª. Drª. Cidália Gabriela Santos Marinho. Realizou o estágio de final de curso na empresa de Controle biológico JB Biotecnologia LTDA. Em março de 2019 iniciou o curso de mestrado no Programa de Pós-graduação em Agronomia (Entomologia Agrícola), na Universidade Estadual Paulista "Júlio de Mesquita Filho" – Faculdade de Ciências Agrárias e Veterinárias, *Campus* de Jaboticabal, São Paulo, desenvolvendo suas pesquisas na área de controle microbiano de artrópodes pragas, sob orientação do Prof. Dr. Ricardo Antônio Polanczyk e coorientação da Dra. Naymã Pinto Dias. Na oportunidade foi bolsista do Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq.

Dedico...

Ao meu marido **Pedro Henrique Braga Motta**, por ter sido parceiro e amigo nesse período tão importante da minha vida, por ser motivador e acreditar em mim e na minha pesquisa.

Aos meus familiares que me apoiaram e foram compreensíveis durante essa trajetória.

AGRADECIMENTOS

Ao concluir esse sonho me lembro de todas as pessoas que me ajudaram nessa trajetória e da contribuição direta ou indireta de cada uma delas.

Antes de todos eu agradeço primeiramente à Deus, nosso Eterno e Criador, obrigada Senhor por eu ter sonhado o sonho que o Senhor tinha para mim e por me sustentar durante essa caminhada.

Ao meu marido Pedro Henrique Braga Motta por viver esse sonho comigo, por ser tão paciente e amoroso em cada etapa e por me incentivar sempre.

À minha família por entenderem minha ausência, em especial aos meus pais Cléria Queiroz da Costa e Jorge Eustáquio Pereira, pelo zelo e o dom da vida, e meus queridos avós Elza Carlos Queiroz da Costa e Antônio João da Costa, por serem tão amorosos e pacientes comigo.

Ao meu orientador Prof. Dr. Ricardo Antônio Polanczyk, pelos ensinamentos, oportunidades, pela confiança e pelo auxílio no desenvolvimento desse trabalho e no meu desenvolvimento profissional.

À minha coorientadora Dra. Naymã Pinto Dias, pela valiosa orientação, ensinamentos, pela amizade e principalmente pelas palavras de incentivo em todos os momentos.

Ao meu coorientador Dr. Brad Duthie, da Universidade de Stirling na Escócia, pela parceria e paciência durante o desenvolvimento desse trabalho e por contribuir no meu desenvolvimento profissional.

A Pesquisadora Alejandra Bravo, do Instituto de Biotecnologia da Universidade Nacional Autônoma do México, por ter cedido as proteínas utilizadas nos bioensaios.

Ao Professor Carlos Alberto Labate, da Universidade de São Paulo, e sua equipe, por disponibilizarem o Laboratório Nacional Multiusuário de Proteômica e Metabolômica Aplicados à Agropecuária, Agroenergia e Biologia Geral para a realização das análises de protêomica deste trabalho.

À Universidade Estadual Paulista “Júlio de Mesquita Filho” e à Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal –FCAV/UNESP, juntamente ao Programa de Pós-Graduação em Agronomia - Entomologia Agrícola (PPGEA), por

toda infraestrutura e recursos recebidos para que fosse realizado o meu curso de Mestrado.

Ao corpo docente do Programa de Pós-Graduação em Agronomia Entomologia Agrícola pela contribuição de cada um na minha formação devido aos conhecimentos transmitidos.

Aos Professores Dr. Sérgio Antonio De Bortoli e Dr. Daniel Junior de Andrade pela participação e colaboração no meu Exame Geral de Qualificação.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), pela concessão da bolsa de estudos.

Aos companheiros do Laboratório de Controle Microbiano de Artrópodes Pragas – LCMAP, em especial ao Joacir do Nascimento, Matheus Borges e Igor Sena, pela contribuição na execução deste trabalho.

As minhas amigas Thaise Pascutti e Franciele Silva pelo companheirismo por tornarem essa caminhada mais leve e por dividirem momentos alegres.

A Dra. Janete Desidério pela disponibilização do Laboratório de Genética de Bactérias e Biotecnologia Aplicada (LGBBA) e aos integrantes do LGBBA pelo auxílio com as proteínas.

A todos os funcionários e demais estudantes do Programa de Pós-Graduação em Entomologia Agrícola- Unesp Jaboticabal, pela amizade e bom convívio, em especial ao Leonardo Thiessen e a Ana Letícia Zero pelo auxílio na execução desse trabalho.

Por fim, agradeço a todos que contribuíram para a realização desse trabalho, seja de forma direta ou indireta.

Muito Obrigada!

Efeitos subletais de *Bacillus thuringiensis* Berliner em *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

RESUMO- O *Bacillus thuringiensis* (Bt) tem se mostrado eficaz contra lepidópteros pragas, podendo ser utilizado como bioinseticida ou plantas transgênicas. As interações entre o organismo patogênico e o hospedeiro são geralmente caracterizadas pela patogenicidade e virulência. A atividade tóxica de Bt em lepidópteros-praga é mais bem elucidada para as proteínas Cry, em termos de patogenicidade e virulência. A virulência leva em consideração os efeitos subletais no organismo-alvo, o que pode contribuir para o conhecimento da dinâmica da população da praga. Os efeitos subletais são efeitos biológicos, fisiológicos, demográficos ou comportamentais em indivíduos ou populações que sobrevivem à exposição a alguma substância tóxica em dose ou concentração letal ou subletal. Os efeitos subletais nos lepidópteros podem ocorrer em todas as fases da vida. As proteínas Cry1Aa, Cr1Ab e Cry1Ac têm efeitos subletais em *S. frugiperda* devido à ligação reversível ao seu receptor. Os efeitos subletais do Bt em espécies de pragas podem ser avaliados em laboratório observando os parâmetros biológicos dos espécimes sobreviventes. O objetivo deste trabalho foi avaliar os efeitos subletais das proteínas Cry1Aa, Cry1Ab, Cry1Ac e de um produto comercial a base de Bt, em duas gerações de uma população suscetível de *S. frugiperda*. Após a submissão das larvas neonatas de *S. frugiperda* a CL_{25} estimada das proteínas e produto comercial, das larvas sobreviventes foram obtidos peso larval aos 7 e 10 dias de vida, peso de pré-pupa e deformação, peso pupal e deformação e sexo. Na fase adulta, 20 adultos foram utilizados para a montagem aleatória de casais e foram avaliados longevidade (dias de vida), fertilidade (% de ovos viáveis), fecundidade (número de ovos / dia) e número de adultos deformados. Para as análises proteômicas, dez casais de cada tratamento, e o mesmo para o controle, foram mantidos em gaiolas de PVC. Os ovos foram coletados de cada casal diariamente até a morte dos adultos e armazenados em freezer a -20ºC. Os mesmos procedimentos foram realizados para F2. A proteína foi extraída de cada amostra biológica usando o método fenólico e os peptídeos foram analisados em Synapt G2 HDMS (Waters, Manchester, UK). A análise de sobrevivência, independente do tratamento, não mostrou diferença em dez dias na F1. Os tratamentos apresentaram interferências negativas no desenvolvimento de *S. frugiperda* em F1 e F2. Dentre eles, o tratamento Cry1Ac apresentou interferência negativa em maior número de variáveis. As proteínas Cry não foram detectadas nos ovos de *S. frugiperda* em nenhuma das gerações avaliadas. Na segunda geração, foi identificado maior número total de proteínas nos ovos (1724) do que na primeira (1599). Em F1, 952 proteínas não redundantes foram identificadas e 989 proteínas não redundantes foram identificadas em F2. O produto comercial foi o tratamento que apresentou a maior quantidade de proteínas exclusivas em ambas as gerações, 74 na F1 e 210 na F2. Em sua maior parte, essas proteínas foram compartilhadas com Cry1Ac em ambas as gerações.

Palavras-chave: concentração letal, proteínas cry, lagarta-do-cartucho, biologia molecular

Sublethal effects of *Bacillus thuringiensis* Berliner in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae)

ABSTRACT- Among the effective biological control tactics applied against pest lepidopterans, the microorganism *Bacillus thuringiensis* (Bt) has been shown highly effective, and can be used as a bioinsecticide or transgenic plants. The interactions between the pathogenic organism and the host are generally characterized by pathogenicity and virulence. The toxic activity of Bt in pest lepidopterans is best elucidated for Cry proteins in terms of pathogenicity and virulence. Virulence takes into account the sublethal effects on the target organism, which can contribute to understanding the dynamics of the pest population. Sublethal effects are biological, physiological, demographic or behavioral effects in individuals or populations that survive exposure to some toxic substance in a lethal or sublethal dose or concentration. Sublethal effects on lepidoptera can occur at all stages of life. Cry1Aa, Cr1Ab and Cry1Ac proteins have sublethal effects on *S. frugiperda* due to reversible binding to their receptor. The sublethal effects of Bt in pest species can be evaluated in laboratory by assessing the biological parameters of the surviving specimens. The objective of this work was to evaluate the sublethal effects of proteins Cry1Aa, Cry1Ab, Cry1Ac and a commercial product based on Bt, in two generations of a susceptible population of *S. frugiperda*. After submitting neonate larvae of *S. frugiperda* to LC_{25} estimated for proteins and commercial product, to the survivor larvae were obtained larval weight at 7 and 10 days of life, pre-pupal weight and deformed, pupal weight and deformed and sex. In adulthood, 20 adults were used for the random assembly of couples and were evaluated longevity (days of life), fertility (% of viable eggs), fecundity (number of eggs/day) and number of adults deformed. To proteomic analyses ten couples for each treatment, and the same for the untreated, were set up in PVC cages. Eggs were collected from each couple daily until the death of adults and stored in a freezer at -20ºC. The same procedure was performed to F2. The protein was extracted from each biological sample using the phenolic method and peptides were analyzed in Synapt G2 HDMS (Waters, Manchester, UK). Survival analysis, independente of the treatment, did not showed difference over ten days in F1. The treatments showed negative interferences in the development of *S. frugiperda* in F1 and F2. Among them, the Cry1Ac treatment showed negative interference in the largest number of variables. The Cry proteins were not detected in the eggs of *S. frugiperda* in any of the evaluated generations. In the second generation, a greater total number of proteins in the eggs (1724) was identified than in the first (1599). In F1, 952 non-redundant proteins were identified and 989 non-redundant proteins were identified in F2. The commercial product was the treatment that presented the largest amount of exclusive proteins in both generations, 74 in F1 and 210 in F2. For the most part os these proteins were shared to Cry1Ac in both generations.

Keywords: lethal concentration, cry poteins, fall armyworm, molecular biology

SUMMARY

CAPÍTULO 1- Proteínas Cry de *Bacillus thuringiensis* : Uma visão geral dos efeitos subletais em lepidópteros pragas de cultivos commodities

RESUMO- Dentre as táticas eficazes de controle biológico aplicadas contra lepidópteros praga, o microrganismo *Bacillus thuringiensis* (Bt) tem se mostrado altamente eficaz no controle de pragas agrícolas e florestais, podendo ser utilizado como bioinseticida ou plantas transgênicas. As interações entre o organismo patogênico e o hospedeiro geralmente são caracterizadas pela patogenicidade e virulência, sendo a primeira quantitativa e a segundo qualitativa. A atividade tóxica do Bt, em lepidópteros pragas é mais bem elucidada para as proteínas Cry em termos de patogenicidade e virulência, mas deve-se levar em conta os efeitos subletais dessas toxinas sobre o organismo alvo, o que pode contribuir para o conhecimento da dinâmica populacional da praga e suas interações com fatores bióticos a abiótico. Os efeitos subletais são efeitos biológicos, fisiológicos, demográficos ou comportamentais em indivíduos ou populações que sobrevivem à exposição de alguma substância toxica em uma dose ou concentração letal ou subletal. Os efeitos subletais nos lepidópteros podem ocorrer em todas as fases da vida. O objetivo dessa revisão foi apresentar uma visão geral dos efeitos subletais das proteínas Cry de Bt em diferentes lepidópteros praga que ocorrem em commodities. Nossos critérios de seleção envolveram testes de laboratório realizados sob concentração letal, baixas doses ou efeitos subletais envolvendo parâmetros de vida e competição por sítios de ligação.

Palavras-chave: Bt, Microorganismo, Sítios de Ligação, Baixa dose

CHAPTER 1- Cry proteins from *Bacillus thuringiensis* : An overview of sublethal effects on Lepidopteran pests in crop commodities

ABSTRACT- Among the effective biological control tactics applied against pest lepidopterans, the microorganism *Bacillus thuringiensis* (Bt) has been shown highly effective in the control of agricultural and forest pests, and can be used as a bioinsecticide or transgenic plants. The interactions between the pathogenic organism and the host are generally characterized by pathogenicity and virulence, the first being quantitative and the second qualitative. The toxic activity of Bt in pest lepidopterans is best elucidated for Cry proteins in terms of pathogenicity and virulence, but one must take into account the sublethal effects of these toxins on the target organism, which can contribute to the knowledge of dinamine pest population and its interactions with biotic to abiotic factors. Sublethal effects are biological, physiological, demographic or behavioral effects in individuals or populations that survive exposure to some toxic substance in a lethal or sublethal dose or concentration. Sublethal effects on lepidoptera can occur at all stages of life. The purpose of this review was to present an overview of the sublethal effects of Bt Cry proteins in different pest lepidopterans that occur in commodities. Our selection criteria involved laboratory tests performed under lethal concentration, low doses or sublethal effects involving parameters of life and competition for binding sites.

Keywords: Bt, Microorganism, Binding-sites, Low-dose

1. INTRODUCTION

Insects from the Lepidoptera order, specifically the family Noctuidae, are considered one of the most important group of pests on crops worldwide (Tay et al., 2013). Among these pests, the most studied are those considered primary crops pests of great economic importance, such as the pests of maize, soybean, and cotton (Morales et al., 1995).

Chemical control is one of the most used methods for controlling these pests (Bel et al., 2017; Dudhbale et al., 2017). However, the inappropriate use of these chemical insecticides can cause a series of problems, both for man and the environment, such as disturbances in the human immune system, environmental pollution, deplay of natural enemies in the field, biological imbalance, and resistance of pest populations (Valicente, 1989; Poletti and Omoto, 2003; Bravo et al., 2011; Nicolopoulou-Stamati et al., 2016).

Among the effective biological control tactics applied in the field for these pests, the microorganism *Bacillus thurigiensis* (Bt) has been highly efficacious in pest management, mainly in corn crops and cotton (Lacey et al., 2015). This microbial control can be used as biopesticide or transgenic plants, consequently drastically reducing the spraying of chemical insecticides and safety for consumers and non-target organisms (Qaim and Zilberman, 2003; Rosas-García et al., 2009; Chattopadhyay et al., 2018).

Interactions between a pathogenic organism and a host can occur through pathogenicity and virulence. The pathogenicity is an all-or-none phenomenon, that is if the microorganism kills or not the host. However, virulence is variable and may depend on abiotic factors, and is characterized by the microorganism's ability to cause disease. In general, pathogenicity is applied to groups or species, whereas virulence is intended for within-group or species comparisons (Shapiro-Ilan et al., 2005).

The relation of pathogenicity and virulence in Bt strains, assayed, to lepidopteran pests is better elucidated to Cry proteins (Knowles, 1994; Bravo et

al., 2017; Heckel, 2020). In this review, we used the term pesticidal protein to refer to a Cry toxin; according to Crickmore et al., 2020,. The binding between the Cry protein and the insect receptor on the epithelial membrane may be reversible or irreversible. In the first, there is a dissociation of the protein receptor complex, and in the second, the insertion of the protein in the cell apical membrane and an irreversible binding (Van Rie et al., 1990; Rajamohan et al., 1998; Schnepf et al., 1998). Therefore, toxicity is a complex process in which binding is a necessary step but is insufficient to cause insecticidal activity. (Garczynski et al., 1991; Aranda et al., 1996). Insects that come into contact with the Cry protein and pass to the irreversible binding of the protein to the receptor may suffer the effects of the virulence of that microorganism and, consequently, can suffer the sublethal effects (Polanczyk et al., 2000; Polanczyk et al., 2003).

Sublethal effects are defined as biological, physiological, demographic, or behavioral effects on individuals or populations that survive to a substance at a lethal exposure, sublethal dose or concentration, or low doses (Desneux et al., 2007; Mohan et al., 2008; De França et al., 2017). The sublethal effects on lepidopterans can occur from a larval stage until the adult stage. Some of them are growth-inhibiting activity on larvae, inhibition of pupation, or interference on adult emergence (Asano et al., 1993; Babu et al., 2002; Li and Bouwer, 2012; Chauhan et al., 2017).

Some studies reported that adult insects surviving exposure to Cry proteins could transfer this protein to the next generation. Research performed by Paula et al. (2014) reported that females of the bordered patch, Chlosyne lacinia (Geyer) exposed to sublethal concentrations of Cry1Ac (100.0 and 2.0 ng/µl) were able totransfer this protein to offspring. The Bt treatment resulted in increased mortality and the development time of the offspring. Cry1F protein can also be transferred to generations of insects that feed on plants containing this protein (Souza et al., 2018).

This review have been described the sublethal effectsof Cry proteins in different lepidopteran pests in crop commodities. Our selection criteria involved laboratory tests estimating under lethal concentration, low doses, or sublethal

evidence effects, including life parameters and competition for binding aiming cross-resistance considerations.

2. REVIEW

2.1 Bt modes of action and structure

Bacillus thuringiensis during the sporulation phase produces pesticidal proteins (δ-endotoxins) as parasporal inclusions, which predominantly comprise one or more proteins, called crystalline (Cry) and Cytolytic (Cyt) proteins (Bravo et al., 2005). Cry proteins are considered toxic to the insect orders Lepidoptera, Coleoptera, Hymenoptera, and Diptera, and nematodes (Bravo et al., 2007). These proteins have been studied for many years to clarify their structure and mode of action.

The Cry structure is composed of three structural domains (3d-Cry), and each domain has a form and a function in the insecticidal activity. According to De Maagd et al. (2001), Domain I, N-terminal, comprises seven α-helices (six amphipathic helices around a central core helix) and is involved in the membrane insertion and pore formation. Domain II is a so-called ‘β-prism,’ with three-fold symmetry consisting of three β-sheets having a ‘Greek key’ conformation. The C-terminal domain III consists of two antiparallel β-sheets in a ‘jelly-roll’ formation. These two lasts are both involved in receptor recognition and binding. Additionally, a role has been found for domain III in the pore function.

Some proposals about the mode of action for Cry proteins have been created, but, nowadays the best-elucidated hypothesis is that Bt Cry proteins act by forming pores (PTF=pore forming toxin), but, as related in a review realized to Vachon et al. (2012), most events leading to their formation, following binding of the activated proteins to their receptors, remain relatively poorly understood. The mode of action involves solubilization of the crystal in the insect midgut, Bt proteins in Lepidoptera are soluble at pH above 9.5

(Knowless et al., 1993), proteolytic processing of the proprotein by midgut proteases, binding of the Cry protein to midgut receptors, and insertion of the protein into the apical membrane to create ion channels or pores (Schnepf et al., 1998).

Each step of the Cry mechanism could modulate activity against a particular insect and, therefore, the overall specificity of a protein (De Maagd et al., 2001). For example, the proprotein solubilization was related to some insect toxicity (Bravo et al., 2005), or the Cry-binding to midgut receptors is an important determinant of specificity (Pigott and Ellar, 2007). Therefore, the success of the Cry protein insecticidal activity depends on all steps of the mode of action must be specific to the target organism. In the midgut of *Spodoptera* species is possible inactivation the insecticidal protein by proteases (Rahman et al., 2012).

2.2 Sublethal concentrations and effects on lepidoptera species

The inappropriate application of *Bacillus thuringiensis* formulations in the field can result in ingestion of sub-lethal doses of the biopesticide by a fraction of the pest population and thereby promotes the toxin tolerance and resistance in the long term (Chauhan et al., 2017). Several studies have been carried out evaluating sublethal effects of Cry proteins on the main lepidopteran pests. Most of these studies assessed the interference of Cry proteins in insect development, but few studies evaluated the protein-binding mechanisms and aspects related to resistance evolution. Among Lepidoptera species studied, most studies assessed species that cause great damage to commodities (Table 1).

Table 1. Effects of Cry proteins sublethal concentrations on commodity lepidopteran pests

Species	Family	Cry protein	Concentration	Main observed effects	Reference
Spodoptera frugiperda	Noctuidae	Cry1Aa, Cr1Ab, Cry1Ac and Cry1B	> 2000 ng cm^{-2}	No strict correlation between binding and toxicity, nontoxic of this δ-endotoxins	Aranda et al., 1996
		Cry1Ca	10, 8, 6, 4, 2 and 1 mg cm^{-2}	Changes in defense and oxidative stress-related genes were transcriptionally enhanced, and metabolic-related genes were repressed	Rodríguez-Cabrera et al., 2008
Spodoptera littoralis	Noctuidae	Cry1C	0.17, 2.40, 3.74, 5.39 and 5.46 µg g^{-1}	The protein was hydrolyzed more rapidly in the resistant line than the susceptible one	Moussa et al., 2020
Spodoptera eridania	Noctuidae	Cry1Ac, Cry1Fa, and Cry2Aa	>10000, >3000 and 11 ng cm^{-2} (LC_{50})	Did not cause any mortality or growth inhibition, caused only growth inhibition and growth inhibition plus mortality	Rabelo et al., 2020a
Spodoptera cosmioides	Noctuidae	Cry1Ac, Cry1Fa, and Cry2Aa	>10000, 853.4 and 1132.1 ng cm^{-2} (LC_{50})	Growth inhibition	Rabelo et al., 2020b
Helicoverpa armigera	Noctuidae	Cry1Ac	0.071 µg ml^{-1} (LC_{25}) and 0.119 µg ml^{-1} (LC_{50})	Decrease fertility, increase malformed adults, fecundity and fecundity period	Kannan and Uthamasamy, 2006
			2.5µg and 4µg g^{-1}	Growth rate of Knock out of HaREase gene was repressed significantly	Guan et al., 2019
Sesamia nonagrioides	Noctuidae	Cry1Aa, Cry1Ab, Cry1Ac and Cry2	0.35 and 0.035mg kg^{-1}	Higher mortality, longer developmental time, extra molts, and higher sensitivity to critical daylength for diapause induction	Eizaguirre et al., 2005

Species	Family	Cry protein	Concentration	Main observed effects	Reference
Sesamia nonagrioides	Noctuidae	Cry1Ab	0.35, 0.9, and 2 mg kg^{-1}	Higher levels of juvenile hormone, low level of ecdysteroids, consequently longer larval development, more larval molts, and pupation difficulty	Pérez-Hedo et al., 2011
Anticarsia gemmatalis	Noctuidae	Cry1Aa, Cry1Ab, Cry1Ac and Cry2	0.46 mg mL^{-1} (LC_{50})	Structural damage and death of the midgut epithelial cells of this insect	Castro et al., 2019
Ostrinia furnacalis	Crambidae	Cry1Ac	0.05, 0.2, 0.8, 3.2, 12.8 µg g^{-1}	Larval growth and development delayed, pupation, pupal weight, and adult emergency also decreased	Ma et al., 2008
Chlosyne lacinia	Nymphalidae	Cry1Ac	100 and 2.0 ng ml^{-1} (LC_{10})	F1 larvae had higher mortality and longer development time	Paula et al., 2014

Species studied included the complex *Spodoptera* [*Spodoptera littoralis* (Boisduval)*, S. litura* (Fabricius)*, S. cosmioides* (Walker), *S. frugiperda* (J.E. Smith), and *S. eridania* (Stoll)], that has been reported as critical pests in soybean, cotton and corn-producing regions in North America, South America, Asia and Africa (Hosny et al., 1986; Cruz et al., 1999; Santos et al., 2010; Aguirre et al., 2016; Dudhbale et al., 2017). Aranda et al. (1996) reported that some nontoxic δ- endotoxins (Cry1Aa, Cry1Ab, Cry1Ac, and Cry1B) could bound to the apical microvilli in the *S. frugiperda* midgut tissue sections.

Transcriptional studies have been performed to identify responses from midgut cells in Lepidoptera pests exposed to Bt proteins. Rodríguez-Cabrera et al. (2008) suggested that determining the transcriptional profiles of midgut cells early in Cry protein poisoning are crucial for understanding the biochemical and molecular aspects of insect detoxification. A study performed by these authors showed transcriptional responses from third-instar larvae of *S. frugiperda* exposed to Cry1Ca sublethal concentration (10, 8, 6, 4, 2,1, and 0 mg cm^{-2}). Sixteen genes were found in association with a known biological process in *S. frugiperda*. In protein-fed insects, defense (*serpin*) and oxidative stress-related (*catalase*) genes were transcriptionally up-regulated, while metabolic-related (*lipase 1, glycosyl hydrolase*) genes were down-regulated in this pest after 15 minutes of the protein treatment. Serpin regulates innate insect immunity via inhibition of serine proteinase cascades that initiate immune responses such as melanization and antimicrobial peptide production (Meekins et al., 2018). Catalase is a robust antioxidant enzyme that breaks down toxic reactive oxygen species (ROS) which are also actively released to respond against bacteria in insects (Molina-Cruz et al., 2008; Diaz-Albiter et al., 2011). While glycosyl hydrolase is a carbohydrate-active enzyme (Cantarel et al., 2008), and lipase has key roles in insect lipid acquisition, storage, and mobilization (Santana et al., 2017).

Laboratory approaches using low doses to assess sublethal effects have been increasingly comprehensive once the Cry δ-endotoxins from *B. thuringiensis* bioinsecticides are threatened by the possibility of pest resistance. A laboratory investigation was conducted by Moussa et al. (2020) to evaluate the resistance development in *S. littoralis* against Cry1C protein. Fourth-instar larvae were exposed to the Cry1C protein for subsequent 12 generations in an

artificial diet. The resistance ratio increased from generation to generation until it reached 32.12 folds in F12. The authors compared a resistant and susceptible line and showed that Cry1C protein was hydrolyzed quickly in the resistant population. *S. littoralis* could develop the resistance to Bt proteins while exposed to a diet mixed with the protein for subsequent generations. The explanation for this rapid development of resistance was spore/crystal mixture might contain associated particles that may delay resistance development in cotton leafworm strain compared to purified proteins used to Moussa et al. (2020).

Spodoptera eridania (Stoll), known as southern armyworm, is a pest under expansion in cotton and soybean fields, recently found in the African continent (Goergen, 2018). This defoliator had lower susceptibility to Cry1Ac and Cry1F than to Cry2Aa Bt protein. The highest Cry1Ac concentration tested (10000 ng cm^{-2}) did not cause mortality or growth inhibition, while the highest Cry1F concentration (3000 ng cm^{-2}) caused only growth inhibition. The authors detailed that greater rates of growth inhibition and mortality in Southern armyworm larvae exposed to Cry2Aa relative to Cry1Ac and Cry1F support the hypothesis that Cry2A protein does not share the same binding sites with Cry1A or Cry1F, critical for toxicity to armyworms (Rabelo et al., 2020a). These same proteins from Bt were tested on *S. cosmioides* (Rabelo et al., 2020b). This pest showed greater growth inhibition when exposed to Cry1Fa, concerning Cry1Ac, and Cry2Aa. Cry1Fa and Cry2Aa have similar toxicity, whereas Cry1Ac was at least 11.7 times less toxic than Cry1Fa. The effect of the Cry protein on the insect organism depends on the species.

Helicoverpa armigera (Hübner) is a highly polyphagous pest that mainly affects cotton crop (Abrahamson and Weis, 2000). The Cry1Ac protein has been used to control *H. armigera,* which was related that this protein cause decreased fertility, increase malformed adults, fecundity and fecundity period when this species is exposed to low (LC_{25}) and medium (LC_{50}) lethal concentrations (Kannan and Uthamasamy, 2006). That same protein can growth rate of knockout of *HaREase* gene was significantly repressed in second-instar larvae of *H. armigera* fed an artificial diet with Cry1Ac (2.5 or 4 mg g^{-1}) (Guan et al., 2019). These authors related that *HaREase* was involved in the lepidopteran immune stress processes and affected cotton bollworm

resistance to Bt toxicity. These results can provide a novel strategy to enhance the sensitivity of insects to Bt protein by inhibiting immune-related genes.

In another species, *Ostrinia furnacalis* (Guenée), an important corn pest in China, were evaluated growth, development, and mortality of neonates and third instar larvae on a diet with Cry1Ac. This protein harmed the growth and development of *O. furnacalis.* Increased concentrations of Cry1Ac on a diet reduced the development of larval, while third instars were >8 times more tolerant to Cry1Ac than neonates. After the larvae being fed on the Cry1Ac protein diet for ten days, the larval weight decreased significantly. Their developmental period was also prolonged. Pupation rate and pupal weight decreased significantly. However, the Cry1Ac protein did not affect the pupal period (Ma et al., 2008).

Sesamia nonagrioides (Lefebvre) is the major pest of corn in the Mediterranean Basin. Newly hatched (< 24 h) larvae were used to the evaluated effect of sublethal Bt concentrations (0.35 and 0.035 mg kg^{-1}) in an artificial diet on larval development and diapause induction. Larvae treated with commercial preparation Dipel DF (Cry1Aa, Cry1Ab, Cry1Ac, Cry2A, and Cry2B) increased the number of days needed to pupate in all instars and for all concentrations of protein that had not caused the total mortality of the larvae (except L6 fed 0.35 mg kg^{-1} diet), presented extra molts. The high Bt concentration caused a 41-min increase in *S. nonagrioides* larvae (from critical daylength 14 h 13 min found in untreated larvae to 14 h 54 min found in the larvae treated with the higher Bt concentration). To determine the effects of sublethal Bt concentrations on *S. nonagrioides*, were used neonate larvae fed on a diet with a commercial Bt preparation and diapausing larvae from a Bt commercial cornfield (event 176) both with Cry1Ab. Consistent with laboratory results, larvae collected in a Bt cornfield, and therefore fed on sublethal concentrations of the Bt protein Cry1Ab, also showed a higher number of supernumerary molts higher duration of diapause and postdiapause development. The author explains that delay the phenology of flights of adults emerging from Bt fields could decrease the efficacy of non-Bt refuges as adults from Bt fields would have less probability of mating with adults from non-Bt fields than with adults from the same fields (Eizaguirre et al., 2005).

Cry1Ab protein was tested for this same species in another work but was evaluated the hormonal balance of *S. nonagrioides* after was fed larvae until they molt to the pupa or the death of the larvae with a semiartificial diet with different sublethal amounts (0, 0.35, 0.9, and 2 mg/kg diet) of active Cry1Ab protein with trypsin. The larvae that survived presented higher levels of juvenile hormone (JH), whereas their level of ecdysteroids did not increase sufficiently to allow pupation, leading to a longer larval development and more larval molts. This response may be considered a defense mechanism that allows some larvae to survive protein ingestion (Pérez-Hedo et al., 2011).

Anticarsia gemmatalis (Hübner), one of the main defoliators in soybean plants in Brazil, was recently evaluated to assess the toxicity of *B. thuringiensis* subsp. *kurstaki* strain HD-1 (Castro et al., 2019). Bt median lethal concentrations (LC_{50} = 0.46 mg mL^{-1}) showed toxicity to *A. gemmatalis* fourth-instar caterpillars after 108 hours. Cytopathological changes mediated by these bacterial proteins in caterpillars midgut can cause cellular disorganization, microvillus degeneration, cell fragmentation and protrusion, peritrophic membrane rupture cell vacuolization. The cell presented a progressive increase of nuclei with condensed chromatin, and numerous lysosomes were found in the intestine of toxin-exposed insects. Apoptosis (a morphological pattern of programmed cell death) occurred in caterpillars' midgut cells exposed to Bt (Castro et al., 2019).

Paula et al., 2014 related that *C. lacinia* when exposed to sublethal or low concentrations of Cry1Ac, had adverse effects on the first filial generation (F1) offspring as higher mortality and longer development time compared to F1 larvae of parents that did not ingest Cry1Ac. Also, it was shown that this species could take up the protein and transfer it to eggs.

In addition to work carried out on a contaminated diet with Cry protein, some evaluate the sublethal effects of offering transgenic plants to larvae of pest lepidopterans. Souza et al. (2018) evaluated the resistance to Cry1F in non-aposematic larvae of *S. frugiperda* and the possibility of this species transferring Cry1F from a genetically engineered maize variety to their offspring. The authors found that Cry1F was transferred to offspring (1.47±4.42 ng Cry1F/10 eggs) in a toxin concentration about 28±83 times less than that detected in Cry1F Bt maize leaves.

2.3 Studies reporting lethal effects with Cry proteins

Sublethal effects or mortality on Lepidoptera pests can be usefully assessed in bioassays with diet-contamination or diet incorporation, different from specificity or binding bioassays. This topic will show some studies that have been used this method to evaluate lepidopteran pests. According to the method proposed by Bel et al. (2019), we did not include moribund insects (insects that were alive but that have not grown during the assay and did not respond to perturbation).

Cry1Ac4 was tested in a Spanish population of *H. armigera,* and this species was very susceptible to it and Cry2Aa1. However, when the insects were exposed to, Cry9Ca, Cry1Fa1, Cry1Ab3, Cry2Ab2, Cry1Da, and Cry1Ja1, it produced a significant growth inhibition whereas Cry1Aa3, Cry1Ca2, and Cry1Ea did not affect. Due to this, only Cry1Ac4 (3.5 g mL^{-1}) and Cry2Aa1 (6.3 g mL^{-1}) were able to estimate LC_{50}, range of concentrations tested, the rest of the proteins did not give enough mortality as to allow estimation (Avilla et al., 2005).

Bioassays with purified ICPs in *A. gemmatalis* showed that Cry1Aa, Cry1Ac, and Cry1Ba are the most active proteins on larvae of the *A. gemmatalis* caused 90% mortality race in 48 hours after treatment application; the Cry1Da and Cy1Ea caused 85% and 65% mortality, respectively, over a period between 144 and 168 hours after application of the proteins; and the Cry2Aa showed *n*o significant mortality compared to the untreated (Fiuza et al., 2013).

As previously mentioned, two populations *E. insulana* (Spanish and Egyptian), were used to test the toxicity of thirteen Cry proteins. In each treatment was used 100 µg/ml incorporated the protein into the diet fed to 25 neonate larvae. Six of the Cry proteins tested (Cry1Ca, Cry1Ea, Cry1Fa, Cry1Ja, Cry2Aa, and Cry2Ab) presented a lack of toxic activity to both populations. Cry1Aa, Cry1Ja, and Cry2Aa did not cause mortality but caused significant inhibition of growth. All other Cry proteins (Cry1Ab, Cry1Ac, Cry1Ba, Cry1Da, Cry1Ia, and Cry9Ca) were toxic against *E. insulana* for both populations and resulted in larval mortality, which increased with increasing

protein concentrations (Ibargutxi et al., 2006). After this bioassay, the authors realized competition binding experiments among these proteins to reported possible cross-resistance (more details are discussed in item 2.4).

In order to evaluated susceptibilities of the major pests of cotton in Australia, *H.armigera* and *H. punctigera,* to *B. thuringiensis* pesticidal proteins, were used an experimental formulation, DiPel ES and 2X (containing only Cry1Fa), and ten individual Cry purified pesticidal proteins were tested (Cry1Ac1, Cry1Ab3, Cry1Bb1, Cry1Ca1, Cry1Ea1, Cry1Fa1, Cry2Aa2, Cry2Ab1, Cry2Ac1, and Cry9Aa2) on diet incorporation and surface contamination bioassays. Both methods were deemed to be acceptable for *Helicoverpa* assays. The diet incorporation method was used to investigate the susceptibility of various instars because the later instars burrow into the diet more readily than the early instars. The surface contamination method was preferred for testing purified insecticidal proteins because the quantity of protein required for this method was 40-fold less. Cry1Ac, Cry2Aa, Cry2Ab and Cry1Ab were the most toxic d-endotoxins, respectively, to *H. armigera* and *H. punctigera*. Cry2Ac and Cry1Fa were not toxic to *H. punctigera*, but Cry9Aa was; the opposite happened to *H. armigera*. None of Cry1B, Cry1C, Cry1E, Cry1F, Cry2Ac, Cry9Aa, or Cry9Ca tested was toxic for *H. armigera*. This last species was consistently more tolerant to Bt pesticidal proteins than *H. punctigera*, but both were susceptible to only a limited range of these proteins (Liao et al., 2002).

Bioassays aiming to evaluate the toxicity or mortality of lepidopteran insects using Cry proteins are previous tests that generally will continue in another line of research. These studies are performed to evaluate binding site competition in the larval midgut; possible cross-resistance in a particular species; evaluation of the most favorable methodology for this type of experiment, associating with the development of larval instars, among others. Therefore, many studies use a large number of proteins in their tests to have a better comparison response between them, as we related above.

2.4 Studies assessing binding ability through low concentrations

Many studies have been performed to test the susceptibility of Lepidoptera pests to several *B. thuringiensis* insecticidal proteins. These studies correlated the ICPs toxicity to different processes to bind the midgut epithelial brush border (Table 2). There may be homologous or heterologous competition between Cry proteins in this protein binding process to the larval midgut larval receptor. Aranda et al. (1996) showed some ICPs interacting with the microvilli of epithelial cells of *S. frugiperda* in two different ways. The first includes a nonspecific interaction, where some nontoxic proteins (such as Cry1Ab to *S. frugiperda*) interact non-specifically with the microvilli since this binding was not affected by high concentrations of a homologous competitor. The second is highly specific and saturable, typical of highly toxic proteins (such as Cry1C and Cry1D to this Noctuidae).

Table 2. Binding sites of Cry proteins on commodity lepidopteran pests

Lepidopteran species	Family	Protein	Share binding sites	Not share binding sites	References
Anticarsia gemmatalis and *Chrysodeixis includens*	Noctuidae	Cry1Fa	Cry1Ac	Cry1Ca and Cry2Aa	Bel et al., 2017
		Cry1Ea		Cry1Ac and Cry1Fa	Bel et al., 2019
Spodoptera frugiperda	Noctuidae	Cr1Ea	NBSS	Cry1Ab, Cry1Ac, Cry1Ba, and Cry1Ca	Rang et al., 2004
		Cry1A.105	Cry1Ab, Cry1Ac, and Cry1Fa	Cry2Ab e Cry2A	Hernández-Rodriguez et al., 2013
Ostrinia nubilalis	Crambidae	Cry9Ca	NBSS	Cry1Ba	Hernández-Martínez et al., 2014
Chloridea virescens	Noctuidae	Cry1Aa	Cry1Ab, Cry1Ac, Cry1Fa, and Cry1Ja	-	Jurat-Fuentes and Andang, 2001
Chilo suppressalis and *Scirpophaga incertulas*	Pyralidae	Cry1Ac and Cy1Ab	Cry1Ab and Cry1Ac	Cry1C, Cry2A, and Cry9C	Alcantara et al., 2004
Helicoverpa armigera, *Spodoptera exígua*, *Spodoptera litura* and *Agrotis ipsilon*	Noctuidae	Cry2Ab	NBSS	Cry1Ab, Cry1Ac, Cry1B, Cry1C	Qiong et al., 2013
Earias insulana	Noctuidae	Cry1Ab	Cry1Ac	Cry1Aa3, Cry1Ba, Cry1Ca2, Cry1Da, Cry1Ea, Cry1Fa1, Cry1Ja1, Cry2Aa1, Cry2Ab2, Cry1Ia7 and Cry9Ca	Ibargutxi et al., 2006

NBSS= No binding sites shared

Competition-binding and binding inhibition with four Bt proteins (Cry1Ac, Cry1Fa, Cry1Ca, and Cry2Aa) in two important defoliation pests of soybeans [*A. gemmatalis* and *Chrysodeixis includens* (Walker)] was realized to Bel et al., (2017). Cry1Ac and Cry1Fa presented specific binding sites on the midgut Brush Border Membrane Vesicles (BBMVs) in both species in competition-binding. Conversely, this same protein can share some binding sites between them, as demonstrated in a competition-binding and binding inhibition, but these binding sites were not shared with Cy1Ca and Cry2Aa in either defoliation pests of soybeans. Two years later, a heterologous binding experiment was accomplished to *A. gemmatalis* and *C. includens*. The results showed that Cry1Ea does not share binding sites with Cry1Ac or Cry1Fa in either species (Bel et al., 2019). These studies indicated a potential risk of cross-resistance among Cry proteins and identify possible protein candidates for Bt-pyramided crops.

In another study, the authors related Cry1Ea does not compete for the binding site with any of the other proteins (Cry1Ab, Cry1Ac, Cry1Ba, Cry1Ca) in the brush border membrane fractions (BBMFs) of *S. frugiperda* (Rang et al., 2004). *Diatraea saccharalis* (Fabricius) and *Diatraea grandiosella* (Dyar) were also tested in homologous and heterologous competitions labeled and unlabeled Cry1Ea. In both *Diatraea* species, Cry1Ea does not bind to a specific receptor BBMVs, due to binding of labeled Cry1Ea in the presence of an excess of unlabeled protein, i.e., nonspecific binding was very high.

Binding studies using Cry9Ca and biotinylated Cry1Ba proteins (due to the difficulty of radiolabeling Cry1Ba without destroying its binding capacity) showed independent binding sites in *O.nubilalis,* indicating that these proteins do not share binding sites (Hernández-Martínez et al., 2014). This study shows the importance of using Bt-corn producing two or more Cry proteins with different binding sites to diminish the possibility for the appearance of resistant insects.

O. nubilalis and *S. frugiperda* can present high affinity to the same binding sites when exposed to Cry1A.105, Cry1Ab, Cry1Ac, and Cry1Fa. On the other hand, Cry2Ab and Cry2Ae did not compete for the binding sites with the Cry1 proteins (Hernández-Rodriguez et al., 2013). This study supports the importance of establishing binding models for Cry proteins as an essential tool

during the design of effective pyramided Bt-crops. The authors concluded that among Cry1Ab, Cry1Ac, Cry1A.105, and Cry1Fa proteins is a possible development of cross-resistance if the alteration of shared binding sites occurs in *O. nubilalis* and *S. frugiperda*, but between these proteins and Cry2A protein is very unlikely in such a case.

Chilo suppressalis (Walker) and *Scirpophaga incertulas* (Walker), important pests for rice were submitted to heterologous competition assays in order to estimate binding affinities to Bt proteins (Cry1Ab, Cry1Ac, Cry1C, Cry2A, and Cry9C) to BBMV (Alcantara et al., 2004). The protein Cry1Ab and Cry1Ac compete for the same binding sites in both species, but Cry1C, Cry2A presented weak bind, and Cry9C has no affinity Cry1Ab and Cry1Ac binding sites in both species. The authors suggested that Cry1Ab and Cr1Ac were not used together due to mutation in one receptor site that could reduce binding of both proteins, but they could be combined with Cry1C, Cry2A, or Cry9C for more durable resistance in transgenic rice.

Performance using BBMVs of *Chloridea virescens* (L.) for five Cry1 proteins (Cry1Ab, Cry1Ac, Cry1Ja, and Cry1Fa) showed that all of them competed with Cry1Aa. These proteins share homologies in domain II loops. In this case, in vivo potencies determined that all Cry proteins were highly toxic to *C. virescens*, except Cry1Ja that only killed larvae at high concentrations. Cry1Aa, Cry1Ab, and Cry1Ac competed with high affinity for Cry1Aa binding sites. Based on Cry1Fa and Cry1Ja competition with Cry1Aa and Cry1Ab, that those proteins recognize receptor A. Cry1Fa and Cry1Ja had a high affinity for receptor A in 125I-Cry1Aa binding assays. The Cry1Fa and Cry1Ja have a low affinity for the receptor shared with the proteins Cry1Ab and Cry1Ac (receptor A) (Jurat-Fuentes and Andang, 2001).

Toxicity of different Cry proteins activated (Cry1Ab, Cry1Ac, Cry1B, Cry1C, and Cry2Ab) were tested against *H. armigera*, *S. exígua*, *S. litura,* and *Agrotis ipsilon* (Hufnagel). Activated Cry1Ab, Cry1Ac, and Cry2Ab were found to be toxic to *H. armigera*. Cry1B, Cry1C, and Cry2Ab were toxic to *S. exigua* and *S. litura*, and Cry2Ab was toxic to *A. ipsilon*. The saturation binding results demonstrated that Cry1Ac, Cry1B, and Cry2Ab bind to BBMV with high affinities, and they are very potent proteins to the target pest larvae. Cry2Ab

showed a lower affinity and binding site concentration in the non-saturable binding, although Cry2Ab protein is more toxic to *S. litura* (Qiong et al., 2013).

Earias insulana (Boisduval), popularly known as spiny bollworm, is an important pest of cotton in Egypt, Africa, and Indian (Rajendran et al., 2018). Competition binding experiments among thirteen Cry proteins (Cry1Aa3, Cry1Ab3, Cry1Ac4, Cry1Ba, Cry1Ca2, Cry1Da, Cry1Ea, Cry1Fa1 e Cry1Ja1 Cry2Aa1, Cry2Ab2, Cry1Ia7, and Cry9Ca) were conducted in two populations of this species (Egyptian and Spanish). Among these proteins tested, only Cry1Ab and Cry1Ac competed for the same binding sites, indicating a high possibility that this insect may develop cross-resistance to Cry1Ab upon exposure Cry1Ac transgenic cotton but not to the other proteins tested (Ibargutxi et al., 2006).

Cry proteins can share or not the same binding sites at BBMVs. In some cases, a specific protein can possess two or more binding-receptors. Those studies are important to relate to each protein combined in transgenic crops or bioinsecticides to avoid new cross-resistance cases.

2.5 Prospects and challenges to Bt biopesticide

Currently, the Cry proteins constitute the largest group of insecticidal proteins produced by *Bacillus* species (Palma et al., 2014). Progress in molecular genetics has also made it possible to use Bt cry genes as a genetic resource for transgenesis and the construction of transgenic plants resistant to insects (Sanchis and Bourguet, 2008) and develop new products to use as sprays (Rosas-García, 2009) or endophytic as have been tested nowadays (Da Costa et al., 2020).

The most used Cry proteins to control lepidopteran pests (sprayable products or in transgenic plants) is Cry1 and Cry2 (Qiong et al., 2013). Cry1A proteins in Bt crops were designed to target mainly caterpillars of the eribid, gelechiid, heliothine, and plusiine taxa, but not those in the *Spodoptera* genus (Rabelo et al., 2020a). There are potential risks that evolved the resistance of insects to Cry protein owing to decreased binding of proteins to target sites in the BBMVs (Qiong et al., 2013).

Lepidoptera pests have been low-inherent susceptibility to Cry proteins; due to this problem, crops genetically modified have been pyramided with Cry proteins, and vegetative insecticidal protein (Vip) reportedly does not share binding sites or structural homology with the Cry proteins. For example, Vip3 (the only one produced by current commercialized Bt crops) and Cry2Ab did not detect significant positive cross-resistance (Tabashnik and Carrière, 2020). In addition to those proteins, Bt can produce proteins Cyt but mostly found in Bt strains active against Diptera (Bravo et al., 2007).

A new review investigating combinatorial effects among Bt Cry, Cyt and Vip proteins collected 118 different combinations, bioassayed against 38 invertebrate species (Lepidoptera order was 27 species). Most of this combinatorial effect was synergism (54%), then additive effect (32%), and antagonism (14%) was the rest. Synergism was noted most frequently for Cry/Cyt combinations, followed by Cyt/Vip and Cry/Cry, but, in Cry/Vip combinations, antagonism was more frequent and presented higher in magnitude than other categories (Baranek et al., 2020). Before combining differents Bt proteins, it is necessary to study how to occur the interact between them.

2.6 Final remarks

The evolution of insect resistance has been increasing over the years. This review presented different studies showing relations between Cry proteins and interaction with the midgut of lepidopteran species commonly in crop commodities. Cry proteins can cause sublethal effects when applied on a diet and offered to lepidopteran pests and can negatively influence the life parameters or mortality. In other cases, Cry proteins can compete for each other for the same binding site as heterologous or homologous competition and contribute to the development resistance of insects. To avoid cross-resistance, it is necessar to know the effect of a combination of Cry proteins (or others) on the midgut of Lepidoptera pests.

Transcriptional studies have been use mostly to identify and understand responses from midgut cells in Lepidoptera pests exposed to Bt proteins. The knowledge of which genes are repressed and enhanced after the Cry intoxication and how it can affect life parameters in lepidopteran are crucial to the increasing of the Bt adoption.

Studies evaluating the sublethal effect of Cry proteins on the offspring of insects exposed to treatments, are scarce on the literature. In addition, among the works that exist, there is none that assesses the sublethal effect on egg embryos, which had better to evaluated than those that assess larvae, due the fact is an earlier stage of pest development.

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CAPÍTULO 2 _ Efeito subletal de *Bacillus thuringiensis* Berliner em *Spodoptera frugiperda* (J.E. Smith)

RESUMO- *Bacillus thuringiensis* (Bt) apresenta efeitos subletais em *Spodoptera frugiperda*. Os efeitos subletais são definidos como efeitos biológicos, fisiológicos, demográficos ou comportamentais em indivíduos ou populações que sobrevivem à exposição a um tóxico em dose ou concentração letal, subletal ou em doses baixas, como é conhecido por alguns autores. Para *S. frugiperda* as proteínas Cry1Aa, Cr1Ab e Cry1Ac têm efeitos subletais devido à ligação reversível das proteínas ao seu receptor. Os efeitos subletais do Bt em espécies de pragas podem ser avaliados em laboratório por meio da avaliação dos parâmetros biológicos dos espécimes sobreviventes. Portanto, o objetivo deste trabalho foi avaliar os efeitos subletais das proteínas Cry1Aa, Cry1Ab, Cry1Ac e de um produto comercial em duas gerações de populações suscetíveis de *S. frugiperda*. Após estimativa da concentração letal de Dipel WP® e proteínas Cry1Aa, Cr1Ab e Cry1Ac que matam 25% (LC_{25}) da suscetível *S. frugiperda*, 360 larvas neonatos (24 horas de idade) foram individualizadas em um recipiente de plástico cilíndrico e transparente com superfície de dieta contaminada com 75 µl de cada solução correspondendo uniformemente a LC_{25}. A sobrevivência larval foi registrada diariamente até sete dias quando foi renovada a dieta sem contaminação em um recipiente plástico maior. Para as larvas sobreviventes foram obtidos peso larval aos 7 e 10 dias de vida, peso pré-pupal e deformado, peso pupal e deformado e sexo. Na idade adulta, 20 adultos foram utilizados para a montagem aleatória dos casais, sendo avaliados longevidade (dias de vida), fertilidade (% de ovos viáveis) e fecundidade (número de ovos / dia). O mesmo foi repetido para F2. A análise de sobrevivência, independente do tratamento, não mostrou diferença significativa ao longo de 10 dias na F1. Os tratamentos apresentaram diferentes interferências negativas no desenvolvimento de *S. frugiperda* em F1 e F2. Dentre eles, o tratamento Cry1Ac apresentou interferência negativa no maior número de variáveis. O Cry1Ac está sendo usado atualmente para desenvolver biopesticidas contra S. frugiperda. No entanto, deve-se levar em consideração se fisiologicamente indivíduos da espécie *S. fugiperda* são realmente capazes de transferir para seus descendentes as proteínas a que foram expostos quando jovens.

Palavras-chave: Bt, proteínas Cry, baixa dose, lagarta-do-cartucho

CHAPTER 2_ Sublethal effects of *Bacillus thuringiensis* Berliner on *Spodoptera frugiperda* (J.E. Smith)

ABSTRACT- *Bacillus thuringiensis (Bt)* has been shown sublethal effects on *Spodoptera frugiperda*. Sublethal effects are defined as biological, physiological, demographic, or behavioral effects on individuals or populations that survive exposure to a toxicant at a lethal, sublethal dose or concentration, or low doses.Cry1Aa, Cr1Ab and Cry1Ac proteins have sublethal effects on *S. frugiperda* due to reversible binding of proteins to their receptor. The sublethal effects of Bt in pest species can be evaluated in laboratory by assessing the biological parameters of the surviving specimens. Therefore, the objective of this work was to evaluate the sublethal effects of proteins Cry1Aa, Cry1Ab, Cry1Ac and a commercial product in two generations of a susceptible population of *S. frugiperda*. After estimated lethal concentration of Dipel WP® and proteins Cry1Aa, Cr1Ab, and Cry1Ac that kill 25% (LC_{25}) of the susceptible *S. frugiperda*, 360 neonate larvae (24 hr old) were individualized in a container with diet surface contaminated with 75µl of each solution, uniformly corresponding to LC_{25}. Larval survival was recorded daily until seven days when were renewed the diet without contamination in a larger plastic container. To the survivor larvae were obtained larval weight at 7 and 10 days of life, pre-pupal weight and deformed, pupal weight and deformed and sex. In adulthood, 20 adults were used for the random assembly of couples and were evaluated longevity (days of life), fertility (% of viable eggs) and fecundity (number of eggs /day). The same procedure was performed to F2. Survival analysis, independente of the treatment, did not showed difference over ten days in F1. The treatments showed negative interferences in the development of *S. frugiperda* in F1 and F2. Among them, the Cry1Ac treatment showed negative interference in the largest number of variables. Cry1Ac is currently being used to develop biopesticides against *S. frugiperda*. However, it should be taken into consideration if physiologically, individuals of *S. fugiperda* are able to transfer Cry proteins to their offspring.

Keywords: Bt, Cry proteins, low dose, fall armyworm

1. INTRODUCTION

Spodoptera frugiperda (J.E. Smith) (Lepidoptera: Noctuidae), historically known as fall armyworm (Luginbill, 1928; Blanco et al., 2016) is one of the most harmful pest species in Brazil due to its polyphagous and high habit biotic potential (Cruz, 1995; Waquil et al., 2008; Boregas et al., 2013). The fall armyworm has a wide geographical distribution (Boregas et al., 2013), and its importance is growing due to its recent finding in West and Central Africa (Goergen et al., 2016) and India (Sidana et al., 2018).

The large dispersion capacity of *S. frugiperda*, makes it difficult to control (Knipling,1980), and among the efficient forms to control which has been highlighting for this pest is the use of microbial control agents, especially the entomopathogenic bacteria *Bacillus thuringiensis* (Bt) (Lacey et al., 2015) which can be used in the form of bioinsecticide, transgenic plants (Glare et al., 2012; Dos Santos et al., 2018; Botha et al., 2019) and recent studies have been innovating in the use of this bacteria as endophytic (Praça et al ., 2012; Costa et al., 2020)

Bt has been shown to be safe to the environment, as the target pest is specific, innocuous to mammals and vertebrates, including humans (Bravo et al., 2011, Fiuza et al., 2017). These positive aspects help make agricultural production viable in an ecologically and economically sustainable way (Polanczyk et al., 2017; Dos Santos et al., 2018). In addition, this biotechnology, managed correctly, can prevent the emergence of new populations of resistant insects (Moussa et al., 2020; Baranek et al., 2020).

There are two different interactions between a pathogenic organism and a host, the pathogenicity that cause lethal effect and the virulence that could cause disease (Shapiro Illan et al., 2005), that is, the sublethal effects. Sublethal effects are defined as biological, physiological, demographic, or behavioral effects on individuals or populations that survive exposure to a toxicant at a lethal, sublethal dose or concentration, or low doses, as known by some authors (Desneux et al., 2007; Mohan et al., 2008; De França et al., 2017).

Aranda et al. (1996) evaluated the effects of Cry proteins on *S. frugiperda* and reported that the proteins Cry1Aa, Cr1Ab and Cry1Ac have reported in this species sublethal effects. The authors attribute this to a possible reversible binding of proteins to their receptors. The sublethal effects of Bt in pest species can be evaluated in the laboratory by assessing the biological parameters of the surviving specimens (Pedersen et al., 1997; Polanczyk & Alves, 2005; Storch et al., 2013; Costa Junior, 2017).

Therefore, the objective of this work was to evaluate the sublethal effects of proteins Cry1Aa, Cry1Ab, Cry1Ac, and a commercial product in two generations of a susceptible population of *S. frugiperda*.

2. MATERIAL AND METHODS

2.1 *Spodoptera frugiperda* colony rearing

The colony of *S. frugiperda* from a susceptible population to *B. thuringiensis* was obtained from Embrapa Milho e Sorgo, Sete Lagoas – MG and was rearing at the LCMAP - FCAV / UNESP under controlled conditions (25° ± 2° C, RH 70% ± 10 and photoperiod 14:10h.

Adult insects (males and females) were placed in a PVC tube cage (10 cm in diameter by 20 cm in height), covered with voile. Inside the cage, a soked cotton was offered with 10% of honey solution, as food, and was covered with A4 paper to provide a substrate for oviposition.

The eggs were removed three times a week and transferred to plastic cages (500 mL) that contained an artificial diet based on white beans, wheat germ, and beer yeast (Greene, 1976). After egg masses hatching, second-instar larvae were individualized in plastic containers (50 mL) with the same artificial diet.

When the insects reached the pupal stage, they were transferred to plastic cages (500 mL), lined with filter paper, where they were remained until the emergence of adults. Malformed adults were discarded from rearing.

2.2 Expression, purification, activation and quantification of Cry proteins

The proteins Cry1Aa, Cry1Ab and Cry1Ac, were obtained from the Institute of Biotechnology, National Autonomous University of Mexico.

Bt serotype *kurstaki* HD-73 strain expressing Cry1Ac, crystalliferous Bt 407- strain expressing Cry1Ab and Cry1Aa (Meza et al., 1996) were grown at 30 °C until complete sporulation for three days in nutrient broth sporulation medium (Schaeffer et al., 1965). For Bt 407- strain expressing Cry1Ab proteins, the growth medium was supplemented with erythromycin at 10 µg/ml. Spores/crystals were washed three times in 0.3 M NaCl, 10 mM EDTA, pH 8.0. Crystal inclusions were solubilized in an alkaline buffer (50 mM Na2CO3 and NaHCO3, 0.2% β-mercaptoethanol, pH 10.5) for 1 h at 37 °C.

Trypsin-activated toxins were obtained by treating soluble protoxins with trypsin (TPCK treated trypsin from bovine pancreas, SIGMA Aldrich, St. Louis, MO, USA) in a mass ratio of 1: 20 (trypsin: protein) for 1 h at 37 °C. Phenylmethylsulfonyl fluoride (PMSF) (1 mM final concentration) was added to stop proteolysis. Finally, the Cry proteins were purified with HP HiTrap ion exchange chromatographic column (GE Healthcare) according to the manufacturer's protocol. The final concentrations of the purified proteins were determined by Bradford (1976) method using bovine serum albumin (BSA) as a standard and separated by SDS-PAGE electrophoresis. After this procedure, the product was diluted to establish other concentrations to use in the toxicity bioassays.

2.3 Quantification of commercial product based on *B. thuringiensis*

The commercial product used was Dipel WP® (*B. thuringiensis* var. *kurstak*i), based on *B. thuringiensis,* expressing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2 proteins.

The concentrations of bioinsecticides and isolates were adjusted to $3x10^8$ spores mL^{-1} in a Neubauer chamber on the microscope light (1000 times magnification) (Alves and Morais, 1998). This concentration is considered

discriminatory in Bt pathogenicity tests for pest insects (Polanczyk et al., 2005). After this procedure, the product was diluted to establish other concentrations to use in the toxicity bioassays.

2.4 Toxicity bioassay with Bt on susceptible *S. frugiperda*

To perform the toxicity bioassay with the product were realized concentrations exponentially spaced ($3x10^3$, $3x10^4$, $3x10^5$, $3x10^6$, $3x10^7$, $3x10^8$, $3x10^9$, $3x10^{10}$ spores mL^{-1}). Different concentrations were tested for each protein: Cry1Aa (50 until 2000 ng cm^{-2}), Cry1Ab (5 until 5120 ng cm^{-2}) and Cry1Ac (12.5 until 3200 ng cm^{-2}).

Dipel WP® and the Cry proteins were diluted in sterile deionized water and 75µl were pipeted on the diet surface (the diet used was Greene, 1976 except antibiotics) in a cylindrical and transparent plastic container (Ø = 2.5 cm or 1.5 cm^3) (Ferré et al., 1991) (Figure 1). In all cases, the untreated was sterile and deionized water. The dilutions were applied uniformly over the diet surface in each container and allowed to dry.

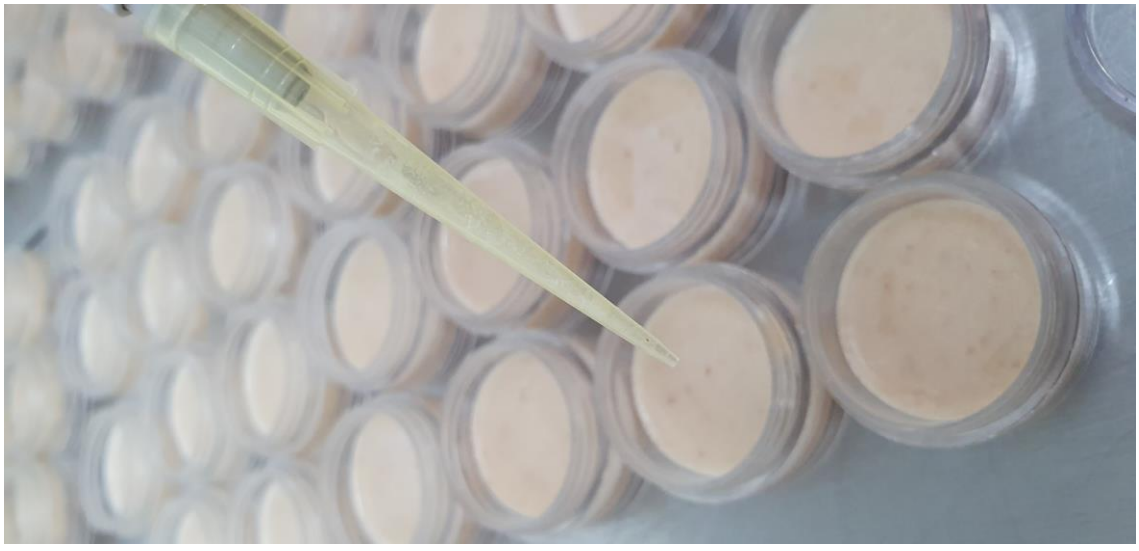

Figure 1. Application of Bt solution on the *S. frugiperda* surface diet.

Fifty neonate larvae (24 hr old) of susceptible *S. frugiperda* were used for each concentration. Larvae survival was recorded seven days after the treatment application.

2.5 Sublethal effect bioassay on susceptible *S. frugiperda*

After estimated lethal concentration of Dipel WP® and proteins Cry1Aa, Cr1Ab, and Cry1Ac that kill 25% (LC_{25}) of the susceptible *S. frugiperda*, 360 neonate larvae (24 hr old) were individualized in a cylindrical and transparent plastic container (Ø = 2.5 cm or 1.5 cm^3) with diet Greene (1976) (except antibiotics). The diet surface was contaminated with 75µl of each solution uniformly corresponding to LC_{25} allowed to dry (Ferré et al., 1991). Larval survival was recorded daily until seven days when the diet was renewed (2 cm^3) in a plastic container with 38 cm^2 (Figure 2).

Figure 2. Larvae in a plastic container with 38 cm^2.

Larvae weight was obtained from the larvae survivors at 7 and 10 days of life, pre-pupal weight and deformed pupal weight and deformed and sex. In adulthood, adults who did not present deformation were used for the random assembly of couples (Figure 3).

Figure 3: Differents life stages of *S. frugiperda*. A) pre-pupal; B) pre-pupal deformed; C) pupal deformed; and D) adults deformed.

Twenty couples for each treatmentwere set up in PVC cages (Ø = 10 cm; height = 20 cm), and were fed with sugar solution at 10% (Figure 4). The bioassay was conducted in controlled conditions of temperature (25° ± 2° C), relative humidity (70% ± 10), and photoperiod of 14:10h.

Figure 4. Couples individualized in PVC cages.

The parameters evaluated to the couples were longevity (days of life), fertility (% of viable eggs), and fecundity (number of eggs /day). In all cycles, stages were recorded the time (days) needs to change. After the eggs hatched, 18 neonate caterpillars were removed to the second posture of each treatment to continue evaluating the second generation (F2), totaling 360 individuals/treatment add untreated. The parameters evaluated in F2 were the same in F1, except survival in the first seven days.

2.6 Statistical analysis

The concentration-mortality data from the toxicity tests were submitted to Probit regression analysis (Finney, 1971) and the lethal concentration values (CL_{25}) were obtained using the SAS software (SAS Institute, 2015). Differences between the CL_{25} values were considered significant when the 95% confidence limit does not overlap.

The binary data were submitted to survival analysis using the Kaplan-Meier estimator with a general tendency to understand the dynamics of the study population compared to the pre-established time, for which the Survival Package was used. Then, each treatment was contrasted with the untreated using the stratification by groups. To determine which independent variables were essential for the establishment of sex, multiple logistic regression with a dichotomous variable was performed. Their significance was based on the chi-square test. Subsequently, the assumptions of the statistical model of a completely randomized design for the variables that comprised the assumptions of normality, homogeneity, and additivity were performed, then the analysis of variance with significance based on the F test at 5% probability was applied. The variables that showed significance were compared by Tukey's multiple comparison analysis of mean at 5% probability. To identify the linear association and the trend between the variables, correlations were made with significance based on the t-test at 5% probability, having these general and

stratified effects for the young and adult phases. Other packages used through the R (R Core Team) software were metam, ExpDes.pt, agricolae and ggplot2.

3. RESULTS

3.1 Toxicity bioassay with Bt on susceptible *S. frugiperda*

The LC_{25} of Cry1Aa was differing from Cry1Ab and Cry1Ac. However, Cry1Ab and Cry1Ac was not statisticaly diferent between them (Table 1).

Table 1. Lethal concentration (LC_{25}) of Dipel WP® (spores mL^{-1}), Cry1Aa, Cry1Ab and Cry1Ac (ng cm^{-2}) in a population of susceptible *Spodoptera frugiperda*.

Treatment	n	LC_{25} (95% C.I.)	Angular coefficient ± S.E.	χ^2	D.F.	P
Dipel WP®	350	$9.75x10^5$ ($4.76x10^5$ – $1.88x10^6$)	0.5959 ± 0.0631	7.3199	5	0.1979
Cry1Aa	300	1370 (1037 – 1573)	5.8343 ± 1.2956	8.0924	4	0.0883
Cry1Ab	350	57.33 (38.95-81.50)	1.1632 ± 0.0960	2.4474	5	0.7844
Cry1Ac	400	106.62 (67.15-156.10)	0.8631 ± 0.0350	4.6544	6	0.5888

n= Number of individuals tested per product, LC_{25} = Lethal concentration of 25% of the population, CI= Confidence interval, SE= Standard error, χ^2 = Pearson's chi-square value, DF= Degrees of freedom

Each LC_{25} estimated (Dipel WP®, Cry1Aa, Cry1Ab and Cry1Ac) was created the concentration-response curve (Figures 5 until 8).

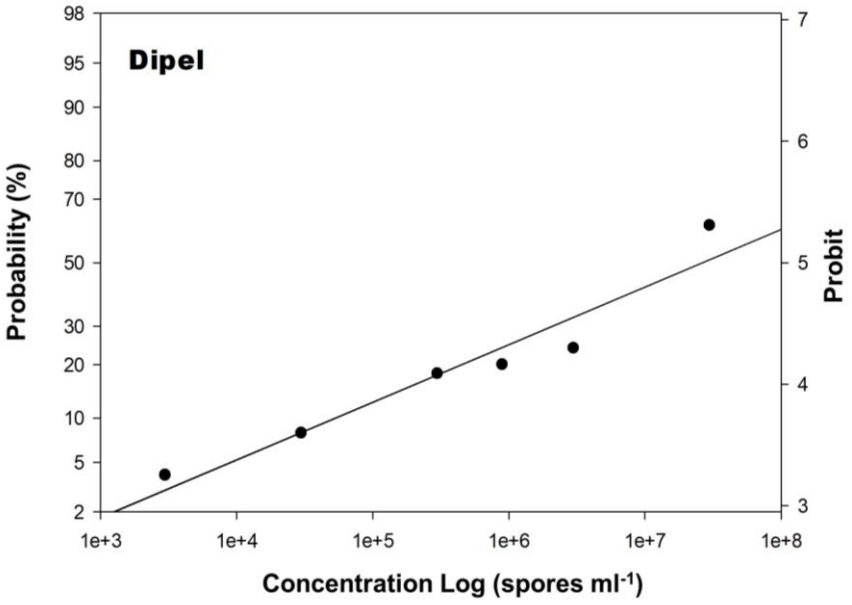

Figure 5: Concentration–response curve of Dipel WP® on suscetible *S. frugiperda*. For every six concentrations, 50 neonate larvae were tested by contaminated diet on the surface.

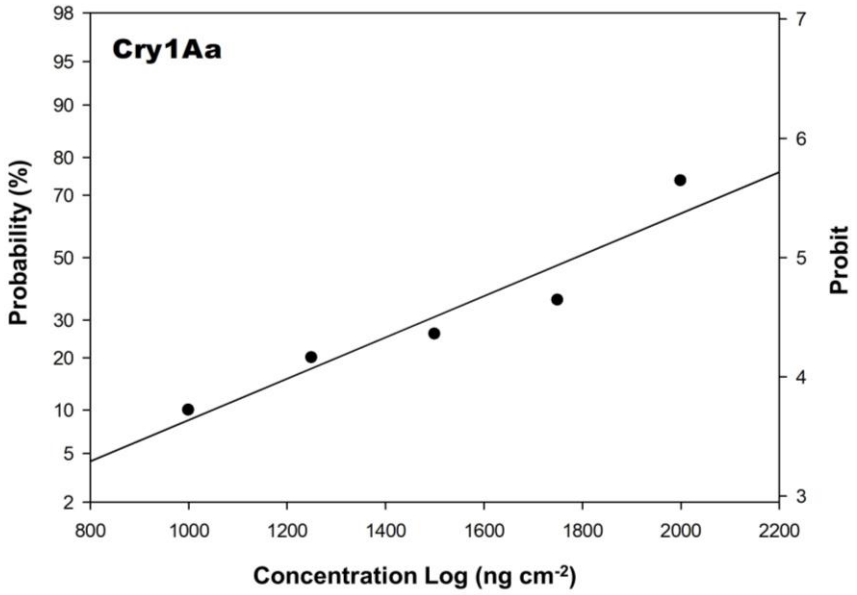

Figure 6: Concentration–response curve of Cry1Aa on suscetible *S. frugiperda*. For every five concentrations, 50 neonate larvae were tested by contaminated diet on the surface.

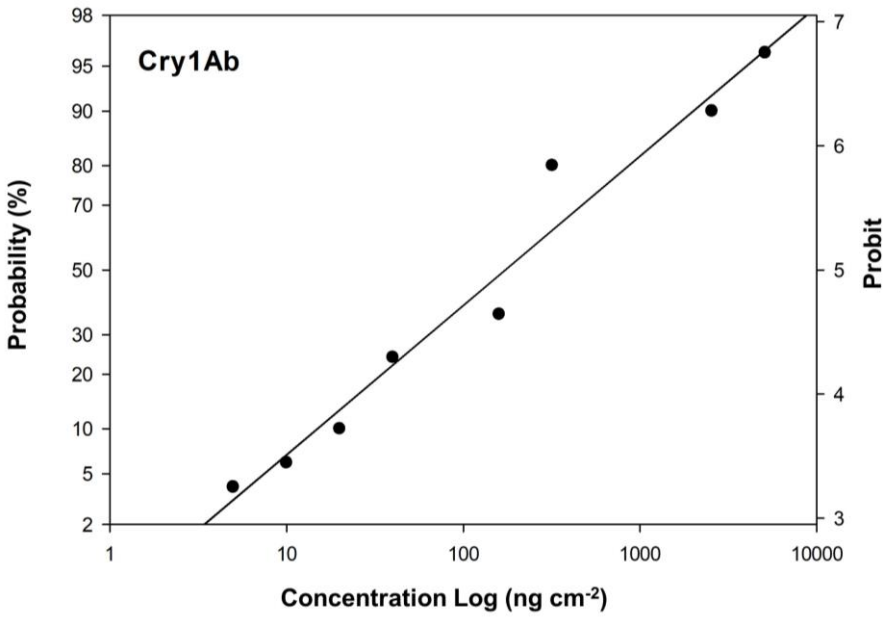

Figure 7: Concentration–response curve of Cry1Ab on suscetible *S. frugiperda*. For every eight concentrations, 50 neonate larvae were tested by contaminated diet on the surface.

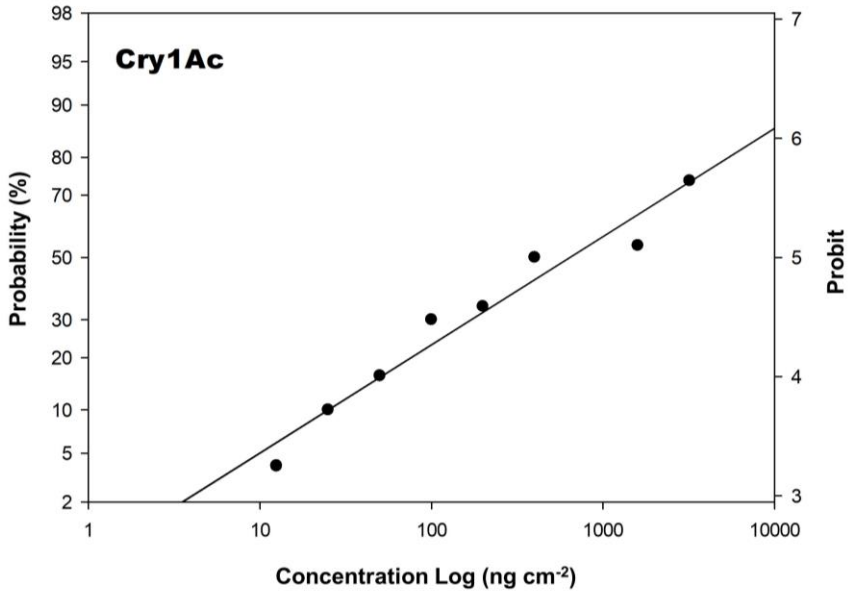

Figure 8: Concentration–response curve of Cry1Ac on suscetible *S. frugiperda*. For each eight concentration, 50 neonate larvae were tested by contaminated diet on the surface.

3.2 Sublethal effect bioassay of susceptible *S. frugiperda*

3.2.1 Survival analysis

Survival analysis, independent of the treatment, did not show a significant difference over ten days in the first Generation (F1) (Table 2). The same result was confirmed in the survival curve (Kaplan-Meier) (Figure 9).

Table 2. Survival analysis of *S. frugiperda*, independent of the treatment, over ten days to first generation (F1).

Time (Days)	n	Survival (95% C.I.)	Standard error
1	1800	0.9 (0.9044 - 0.89563)	0.002236
2	1800	0.8 (0.8059 - 0.79418)	0.002981
3	1800	0.7 (0.7067- 0.69334)	0.003416
4	1800	0.6 (0.6072 - 0.59289)	0.003651
5	1800	0.5 (0.5074 - 0.49275)	0.003727
6	1800	0.4 (0.4072 - 0.39291)	0.003651
7	1800	0.3 (0.3068 - 0.29338)	0.003416
8	1800	0.2 (0.2059 - 0.19424)	0.002981
9	1800	0.1 (0.1045 - 0.09571)	0.002236
10	1625	0.0097 (0.0113 - 0.00839)	0.000731

n= Number of individuals tested in all treatment that survival over the time, CI= Confidence interval

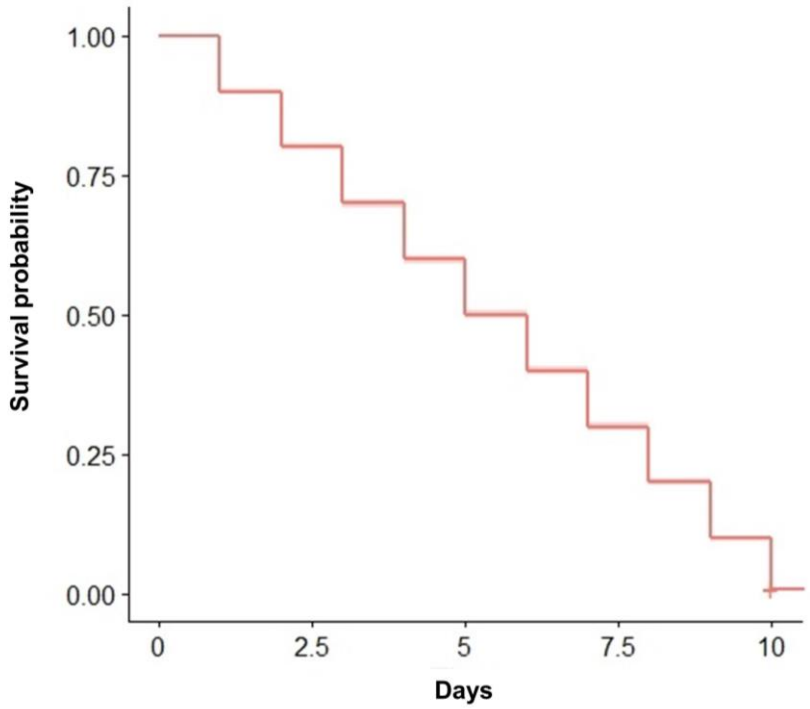

Figure 9: Survival curve of *S. frugiperda* to all treatment over ten days (F1) (Kaplan-Meier).

Through treatment stratification compared to the untreated was understood differences in mortality. Dipel WP® was the treatment that presented higher mortality (1.4722 %), followed the Cry1Ac (1.1944%). The untreated presented the lowest mortality (0.4722%). The mortality percentage value in ten days of Cry1Aa and Cry1Ab was 0.7778% and 0.9444%, respectively (Table 3).

Table 3. Survival analysis of each treatment over ten days (F1).

Treatment	n	events	mortality (%)
Untreated	3600	3583	0.4722
Cry1Aa	3600	3572	0.7778
Cry1Ab	3600	3566	0.9444
Cry1Ac	3600	3557	1.1944
Dipel WP®	3600	3547	1.4722

n= Number of individuals tested in each treatment versus untreated
CI= Confidence interval

However, there was no difference statistically between treatments (Figures 10 until 13).

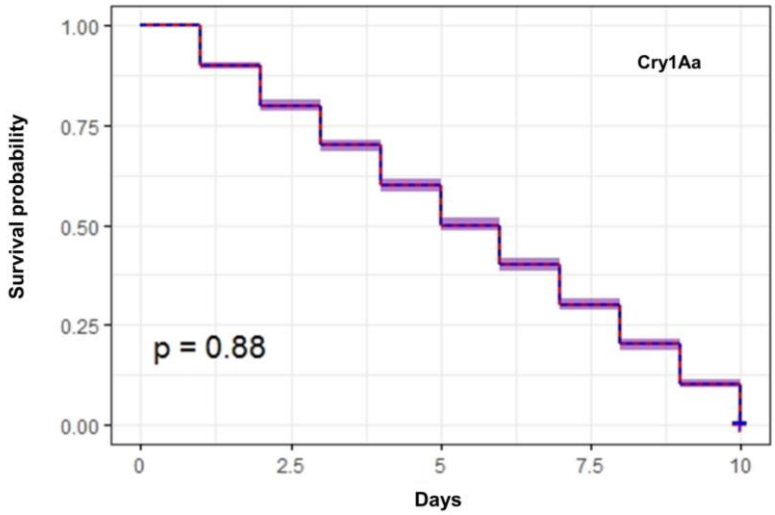

Figure 10: Survival curve to *S. frugiperda* exposed to Cry1Aa compared to untreated over ten days (F1) (Kaplan-Meier) (p=0.88).

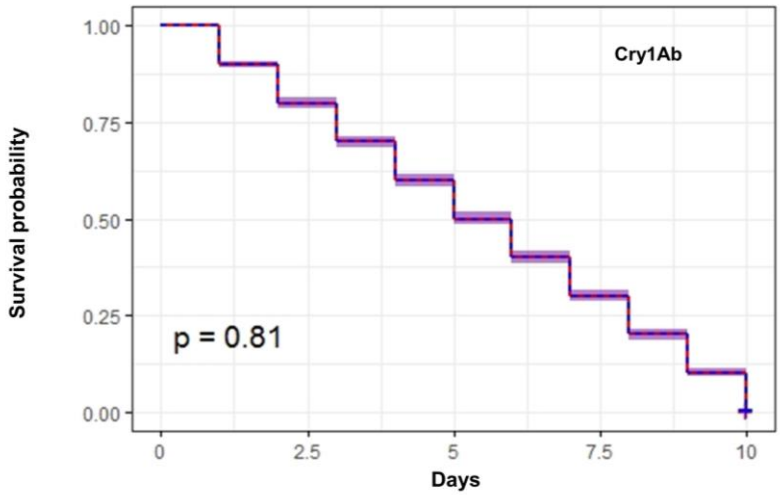

Figure 11: Survival curve to *S. frugiperda* exposed to Cry1Ab compared to untreated over ten days (F1) (Kaplan-Meier) (p=0.81).

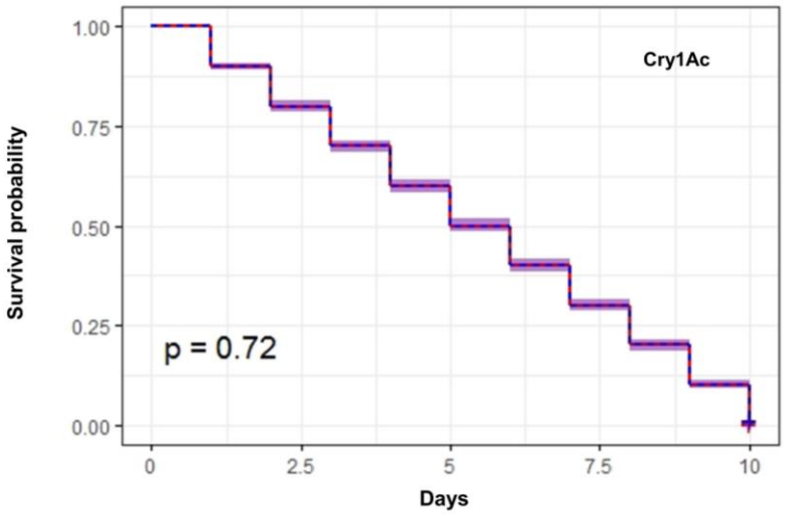

Figure 12: Survival curve to *S. frugiperda* exposed to Cry1Ac compared to untreated over ten days (Kaplan-Meier) (F1) (p=0.72).

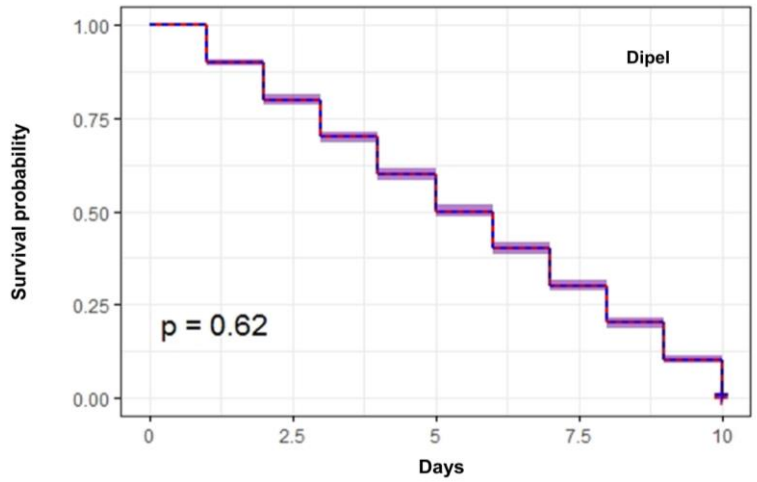

Figure 13: Survival curve to *S. frugiperda* exposed to Dipel WP® compared to untreated treatment over ten days (Kaplan-Meier) (F1) (p=0.62).

3.2.2 Descriptive analysis to larval variables

In the first generation, the lowest mean of larval weight in seven days of the evaluation was observed to larvae of the Cry1Ac treatment (0.004 g) and followed by the larvae exposed to the treatment Cry1Aa and Cry1Ab (0.011 g). The highest mean of larval weight was found for the untreated larvae (0.017 g).

The larvae treated with Dipel WP® presented a mean larval weight closest to the latter (0.013 g). In the second generation, the opposite occurred; the larval weight in seven days to Cry1Ac treatment was the biggest (0.044 g) followed by Cry1Ab (0.039 g), Dipel WP® (0.035 g) and Cry1Aa (0.030 g). The untreated presented the lowest mean to larval weight with seven days in F2 (0.022 g) (Figure 14).

The mean larval weight after ten days to Cry1Ac was lower (0.026 g). In sequence, larvae were treated with Dipel WP® (0.063 g), Cry1Ab (0.077 g) and Cry1Aa (0.086 g). After the tenth day of assembly of the bioassay, the highest mean of larval weight was to the larvae of the untreated (0.187 g) to F1. However, to F2, the untreated presented the lowest mean to larval weight in ten days (0.148 g). The larvae of the Cry1Ab treatment presented the biggest increase in larval weight among the generations in ten days of evaluation (0.221 g) followed by Cry1Ac (0.216 g), Dipel WP® (0,194 g) and Cry1Aa (0.189 g) (Figure 14).

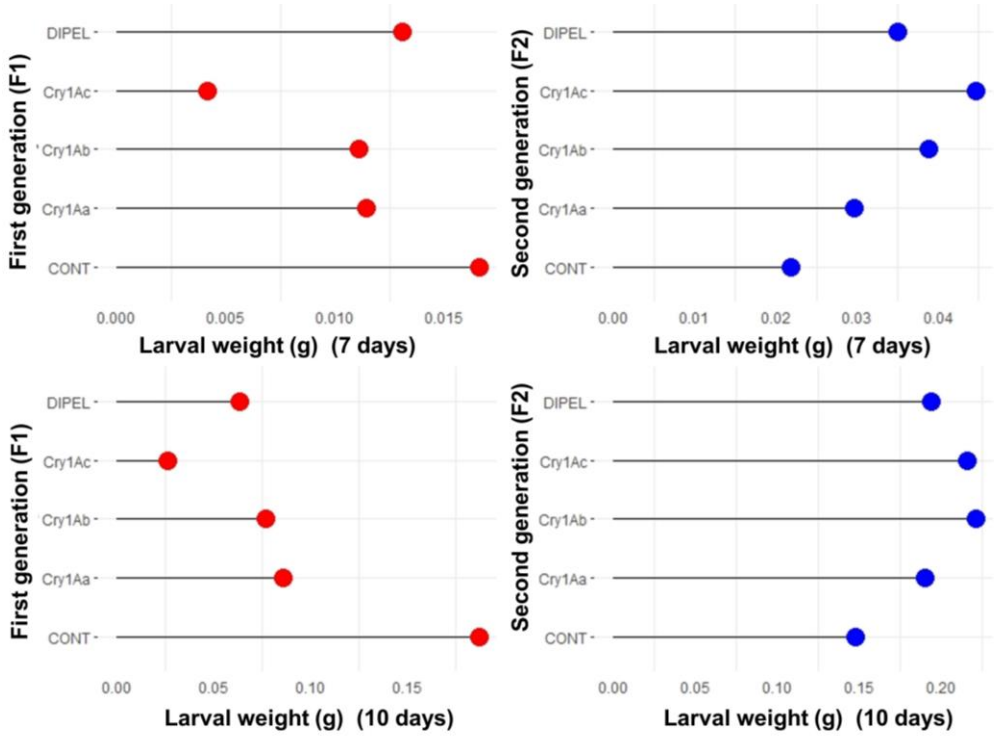

Figure 14. Descriptive analysis to larval weight after 7 and 10 days after setting up the sublethal effect bioassay, first (red) and second-generation (blue).

The highest mean to prepupal weight in F1 was observed for the larvae of the treatment with Cry1Aa (0.317 g) followed by Cry1Ac (0.311 g). In contrast, the untreated had the lowest mean pre-pupa weight (0.244 g). For Cry1Ab, the mean was 0.293 g and Dipel WP® 0.300 g (Figure 14). In the second generation, Dipel WP® presented the highest mean pre pupa weight (0.338 g), but the means of the treatments were very close to each other in the second generation: untreated 0,338 g, Cr1Aa 0,333 g, Cry1Ab 0,327 g and Cry1Ac 0,319 g (Figure 15).

The larvae submitted to treatment with Cry1Ac and Cry1Ab showed the highest mean of the larval period, being approximately 19 and 18 days, respectively. In the untreated, the duration of the larval period was ≈17 days, as well as for the treatment with Dipel WP®. Cry1Aa was the treatment that presented the lowest mean of the larval period (≈16 days). The results are repeated in F2. Compared to the F1, the larval period in the second generation in all treatment was faster: untreated ≈16 days, Dipel WP® and Cry1Aa ≈15 days, Cry1Ac and Cr1Ab ≈14 days (Figure 15).

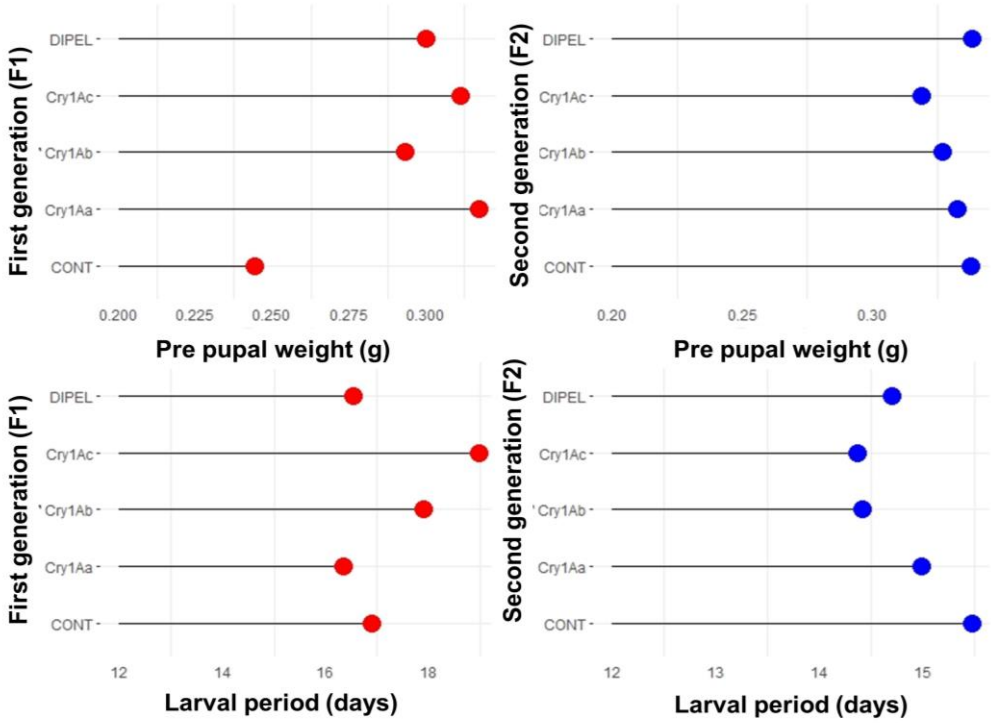

Figure 15. Descriptive analysis to prepupal weight (g) and time to larval become prepupae (days) to first (red) and second (blue) generation.

The prepupal period was shorter for the untreated larvae, Cry1Aa and Cry1Ab, about one day. The larvae of the Cry1Ac and Dipel WP® treatment showed a longer pre-pupa period when compared to the larvae of the other treatments (two days). The prepupal period was about two days in all for larvae in all treatments in F2 (Figure 16).

The untreated mean pupal weight was the lowest among the treatments (0.224 g), and the highest was the larvae of the Cry1Aa treatment (0.294 g). Cry1Ab treatment had a mean pupal weight of 0.260 g and those treated with Cry1Ac and Dipel WP® had a mean weight of 0.275 g in F1. In F2, the treatment individuals with Cry1Aa presented a reduced mean of pupal weight (0.291 g) and Cry1Ac remained with 0.275 g. Untreated increase the mean pupal weight to 0.294 g as well Cry1Ab (0.287 g). The pupal weight with Dipel WP® treatment in F2 was very close to F1 (0.277 g) (Figure 16).

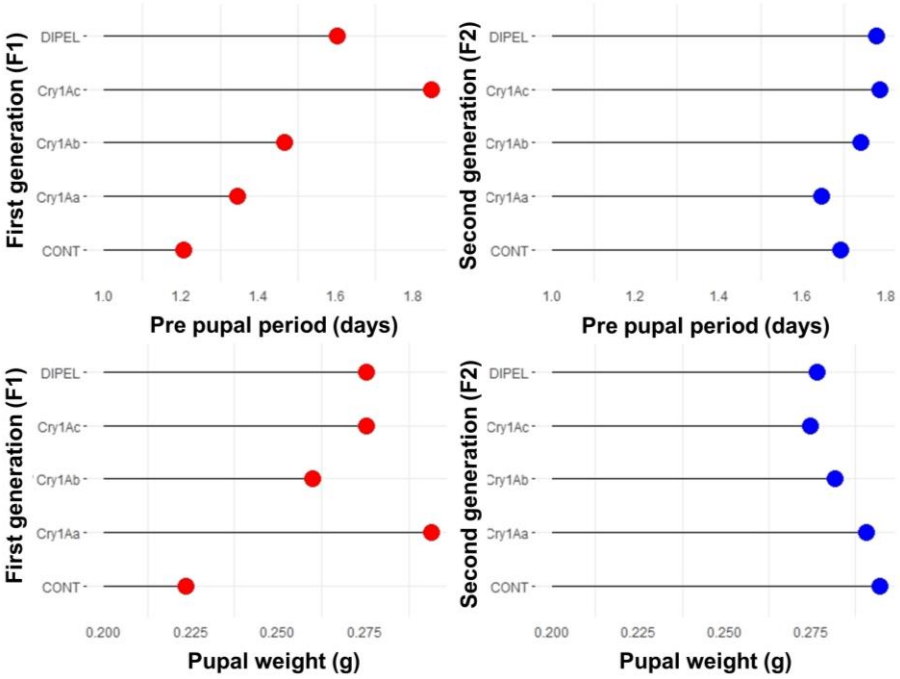

Figure 16. Descriptive analysis to prepupal period (days) and pupal weight (g) to first (red) and second (blue) generation.

The mean percentage of deformed pupae between the Cry1Ab and Dipel WP® treatments were similar (0.003%), followed by the mean of the untreated

(0.006%) and Cry1Aa (0.009%). The pupae of the Cry1Ac treatment not presented a considerable percentage of deformation in F1. However, in F2 the mean deformed pupae of the treatment was 0.02%. In F2, the deformed pupae by Dipel WP® treatment increased compared to F1 (0.04%), as well as the untreated (0.02%). Cry1Aa and Cry1Ab showed a mean reduction in the percentage of pupal deformation in F2, 0.005% and 0.0016%, respectively (Figure 17).

The lower pupal period was observed for the pupae submitted to Dipel WP®, Cry1Aa and untreated (≈ 9 days). The Cry1Ac and Cry1Ab treatments had the highest pupal period among the others (≈ 11 days) in F1. However, in F2, the Cry1Ac and Cry1Ab treatments showed a reduction in the mean pupal period (≈ 9 days) and the Dipel WP®, Cry1Aa, and untreated showed an increase of one day in the mean of the pupal period (≈ 10 days) (Figure 17).

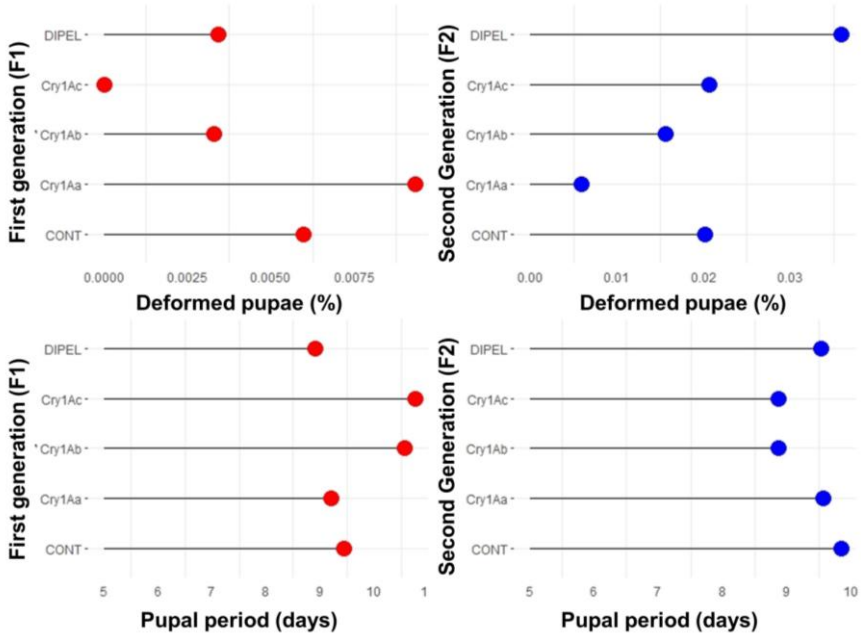

Figure 17. Descriptive analysis to deformed pupae (%) and pupal period (days) to first (red) and second (blue) generation.

The mean percentage of deformed adults from each treatment was lower for the untreated (0.0084%), followed by the Cry1Aa treatment (0.174%) and Dipel WP® (0.222%). The treatments with the highest mean number of

deformed adults were Cry1Ab (0.399%) and Cry1Ac (0.499%) in F1. In F2, the only treatment that had a reduction in the deformation percentage of adults was Cry1Ac (0.400%); untreated showed the same value; however, in this last case, it had an increase, as well as in the other treatments when compared to F1. The mean deformation of adults for the other treatments were: Cry1Aa 0.470%, Cry1Ab 0.490% and Dipel WP® 0.440% (Figure 18).

The treatment with the lowest mean number of adults who did not emerge from pupae was Cry1Ac (0.034%). The Cry1Aa treatment resulted in a higher mean of adults who did not emerge from the pupae (0.261%) followed by the untreated (0.245%). Cry1Ab and Dipel WP® presented intermediate means among the other treatments, 0.115% and 0.150%, respectively; in F In F2, there was an increase in the mean of deformed adults for all treatments. The untreated showed a mean of 0.250% as well as Dipel WP®. Cry1Ab and Cry1Ac presented means of 0.220 and 0.210%, respectively. Cry1Aa was the treatment with the lowest percentage of adults who did not emerge (0.180%) (Figure 18).

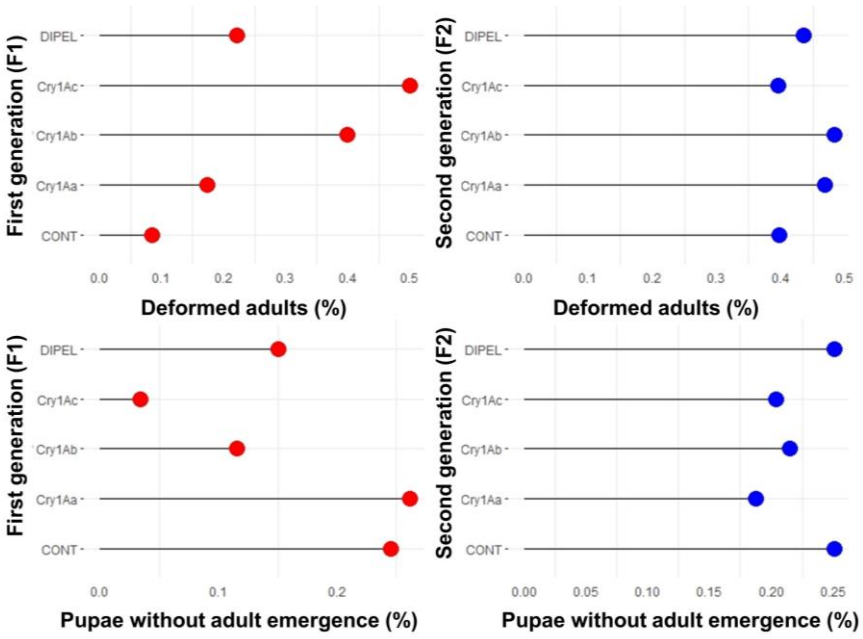

Figure 18. Descriptive analysis to deformed adults (%) and pupae without emergence (%) to first (red) and second (blue) generation.

3.2.3 Analysis of variance (ANOVA) and Tukey test to the larval stage

In the analysis of variance of larval weights with 7 and 10 days of life of the larvae submitted to treatments, there was a statistical difference in the first and second generations. The same was also verified for the weight of pre-pupae (Table 4).

Table 4. Means of larval weight with 7 and 10 days after set up the sublethal effect bioassay (LW7 and LW10) and prepupal weight (PPW), to first (F1) and second-generation (F2).

Treatments	LW7 (F1)	LW10 (F1)	LW7 (F2)	LW10 (F2)	PPW (F1)	PPW (F2)
Untreated	0.0166 a	0.1868 a	0.0219 d	0.1478 c	0.2441 c	0.3379 a
Cry1Aa	0.0114 b	0.0861 b	0.0297 c	0.1899 b	0.3171 a	0.3328 a
Cry1Ab	0.0111 b	0.0771 b	0.0388 b	0.2210 a	0.2930 b	0.3270 ab
Cry1Ac	0.0042 c	0.0260 d	0.0446 a	0.2162 a	0.3110 a	0.3187 b
Dipel WP®	0.0131 b	0.0635 c	0.0351 b	0.1939 b	0.3001 b	0.3380 a

LW7= Larval weight with 7 days after setted up the sublethal effect bioassay, LW10= Larval weight with 10 days after setted up the sublethal effect bioassay, PPW= Pre pupal weight. Means followed by the same letter in the column do not show the statistical difference by the Tukey test ($P \leq 0.05$).

In the first generation, the untreated mean larval weight was the highest in evaluating 7 (0.0166 g) and 10 (0.1868 g) days, differing from the other treatments. The lowest mean of larval weight was observed in the Cry1Ac treatment, both in the evaluation of 7 (0.0042 g) and ten days (0.0260 g). The larval weight of the treatments Cry1Aa and Cry1Ab showed no difference between them in the two evaluations; however, they differed from the other treatments, except for the Dipel WP® treatment 7-day evaluation. In the 10-day evaluation, the Dipel WP® treatment had a mean intermediate larval weight (0.0635 g). However, it was different from the other treatments (Table 5).

As opposed to F1, in F2, the mean larval weight of the untreated was the lowest among the other treatments; in 7 days, the mean weight was 0.0219 g, and in the 10-day evaluation, it was 0.1478 g, differing from the other treatments in both evaluations. In the 7-day evaluation, the Cry1Ab and Dipel WP® treatments showed no difference between them. However, they differed from the other treatments. The Cry1Ac treatment had the highest mean of larval weight (0.0446 g), differing from the others. The Cry1Aa treatment had a mean,

mean larval weight; however, it did not resemble any other treatment within seven days of evaluation. However, the mean larval weight value with ten days of evaluation for the Cry1Ac treatment was 0.2160 g, similar to the Cry1Ab treatment with a mean of 0.2210 g, and differing from the other treatments. The mean larval weight of the Cry1Aa and Dipel WP® treatments were similar to each other in the ten-day evaluation and differed from the other treatments (Table 5).

For the variable pre-pupa weight in the first generation, the untreated showed the lowest mean weight (0.2441), differing from the other treatments. In contrast, Cry1Aa and Cry1c had the highest means, 0.3171 g and 0.3110 g, respectively, both differing from the other treatments. Cry1Ab and Dipel WP® presented similar means and differed from other treatments in the first generation. In the second generation, the only treatment presented a statistically different mean among the others was the Cry1Ac treatment (0.3187), being the lowest mean. The Cry1Ab treatment showed a mean pre-pupa weight similar to the other treatments and to the Cry1Ac treatment (Table 5).

3.2.4 General linear correlation

In the linear correlation analysis description, only strong correlations between the parameters of the proteomic analysis (coefficients greater than 0.8) will be described (Figure 19).

A positive correlation between generations was observed with variables larval weight of seven (LW7); as the generations advance, these variable show an increase

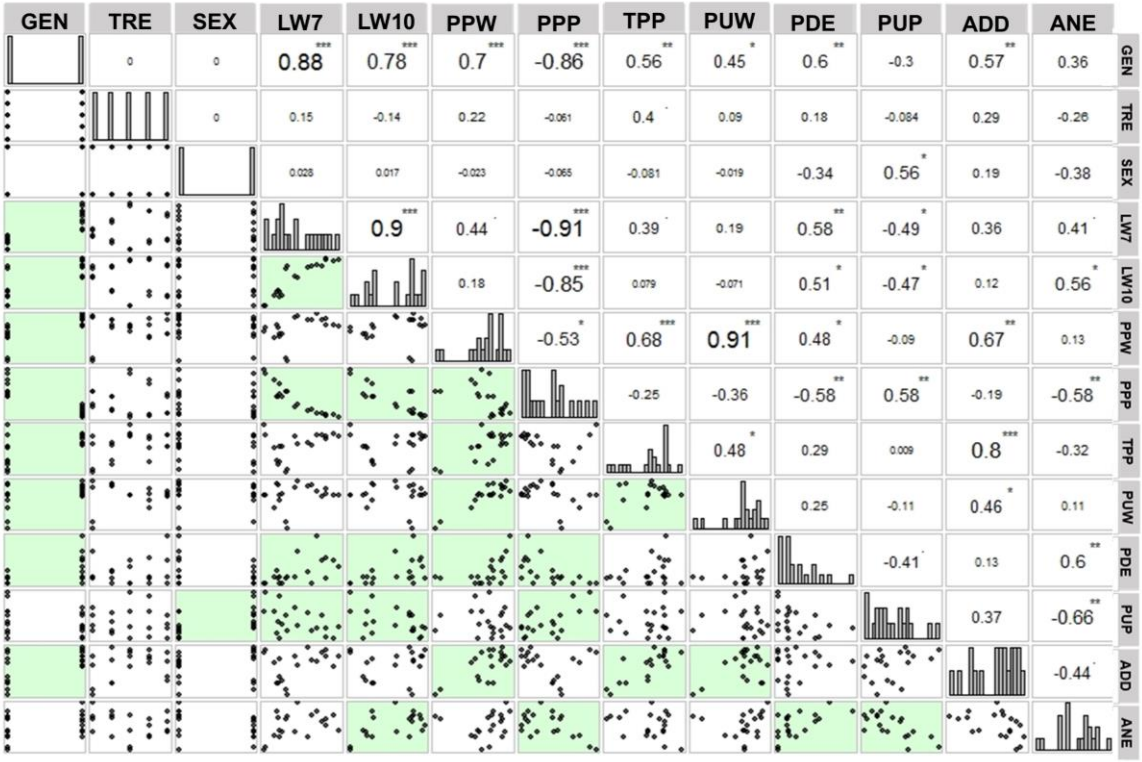

Figure 19. Linear correlation between treatment effect for all variables. GEN= generation; TER= treatment; SEX= sexo; LW7 and LW10= larval weight with 7 and 10 days, respectively; PPW= pre pupal weight; PPP= larval period; TPP= pre pupal period; PUW= pupal weight; PDE= pupal deformation; PUP= pupal period; ADD= adult deformation; ANE= adults did not emerge.

LW7 showed a strong positive correlation with LW10. Therefore, the greater the larval weight in seven days of life of the larvae, the greater the larval weight of ten days. PPW had a strong positive correlation with PUW. The greater the prepupal weight, the greater the pupal weight.

TPP has a strong and positive correlation with ADD and a positive correlation with PUW. Long periods of pre-pupa tend to generate more deformed adults and greater pupal weight. PUW is positively correlated with ADD, the higher the pupal weight, the greater the adult deformation.

3.2.5 Determinant variables for sex

In F1, the variables that determined the sex of *S. frugiperda* in the untreated treatment were pre-pupal weight (PPW), pupal weight (PUW) and

pupal period (PUP). Within them, the PPW has a positive effect for the female sex and the other negative effect, that is, increasing the weight of pre-pupae, increases the proportion of being females and the others reduce this proportion (Table 6).

Table 6. Determinant variables for sex (variable dependent) in F1 generation for each treatment (untreated, Cry1Aa, Cry1Ab, Cry1Ac and Dipel WP®).

Generation	Treatment	Determinant variables	Estimate	O.R.
F1	Untreated	Intercept	43.0011*	4.733084e+18
		PPW	32.7257*	1.631466e+14
		PUW	-39.2553*	8.946528e-18
		PUP	-4.5029*	1.107709e-02
F1	Cry1Aa	Intercept	11.7353*	1.249074e+05
		PUP	-1.3060*	2.708908e-01
F1	Cry1Ab	Intercept	25.8417*	1.670679e+11
		LW7	-135.1936*	1.932663e-59
		TPP	0.7820*	2.185799e+00
		PUP	-2.4141*	8.944787e-02
F1	Cry1Ac	Intercept	27.4483*	-
		PPP	0.3382*	1.402355e+00
		TPP	-0.6601*	5.168193e-01
		PUP	-3.0755*	4.616785e-02
F1	Dipel WP®	Intercept	16.3764*	-
		PUP	-1.8104*	1.635923e-01

F1 = first Generation, OR= odds ratios. LW7= larval weight with 7 days, PPW= pre pupal weight, TPP= pre pupal period, PUW= pupal weight, PUP= pupal period.

In the treatment of Cry1Ab, the variables that presented negative interference on the sex of *S. frugiperda* were the larval weight of seven days (LW7), and pupal period (PUP), that is, the higher the larval weights of seven days and the pupal period, the greater the probability that individuals were male. In the Cry1Aa treatment, the same happened for PUP, the only variable that affected the sex of individuals, however, in a negative way. For Cry1Ab, the only variable that had a positive influence was the pre-pupal period (TPP), which the longer the period was, the greater the probability that females occurred in this treatment.

Cry1Ac treatment, the variables that showed interference on the occurrence of sex in a negative way, that is, with a greater possibility of

individuals becoming male, were TPP and PUP. Moreover, the variable that had the greatest influence on the female sex was the larval period (PPP).

As in the other treatments, for the treatment with Dipel WP®, the variable that presented negative interference on sex was the PUP, being the only one.

In the second generation, the PUP variable was also common in all treatments as a negative determinant of sex, influencing the possibility of being male individuals. This variable was the only influential one in the Cry1Ac and Dipel WP® treatments (Table 7).

Table 7. Determinant variables for sex (dependent variable) in F2 for each treatment (Untreated, Cry1Aa, Cry1Ab, Cry1Ac and Dipel WP®).

Generation	Treatment	Determinant variables	Estimate	O.R.
F2	Untreated	Intercept	3.7390*	42.05481021
		PPP	1.2009*	3.32311792
		TPP	0.9803*	2.66531807
		PUP	-2.4305*	0.08799179
F2	Cry1Aa	Intercept	3.4781*	32.3975675
		PPP	0.5792*	1.7846066
		PUP	-1.2947*	0.2739757
F2	Cry1Ab	Intercept	8.6444*	5.678293e+03
		PPW	14.0393*	1.250755e+06
		TPP	-0.9530*	3.855831e-01
		PUP	-1.3365*	2.627625e-01
F2	Cry1Ac	Intercept	15.1716*	3.881171e+06
		PUP	-1.7323	1.768702e-01
F2	Dipel WP®	Intercept	9.0817*	8792.9239390
		PUP	-0.9745*	0.3773815

F2 = second generation, OR= odds ratios, PPW= pre pupal weight, PPP= larval period, TPP= pre pupal period, PUP= pupal period.

In the untreated and Cry1Aa treatment, the PPP variable showed a positive interference on sex, increasing the chances of females occurring. In the untreated, the TPP also positively influenced sex, and the opposite occurred in the Cry1Ab treatment for the same variable. Therefore, in the first case, the influence was positive for females and in the second case for the occurrence of males. In the Cry1Ab treatment, the variable that positively influenced sex was PPW, with a high chance of interference for females.

3.2.6 Linnear correlation for eggs

In the linear correlation of the data collected from the twenty couples of the first generation, the relationship that stood out the most for the high positive correlation with the number of eggs laid (NEG) with the number of eggs hatched (EGH). The greater the number of eggs laid by the females, the greater the number of caterpillars that hatched. NEG also had a positive correlation with the egg hatch percentage (HAT). In contrast, the correlation of the percentage of eggs that did not hatch (ENE) was negative with EGH and HAT, so the greater the number of eggs that did not hatch, the smaller the number of eggs that hatch and the percentage of eggs hatch. EGH had a high positive correlation with HAT. The more eggs hatched, the higher the hatching percentage (Figure 20).

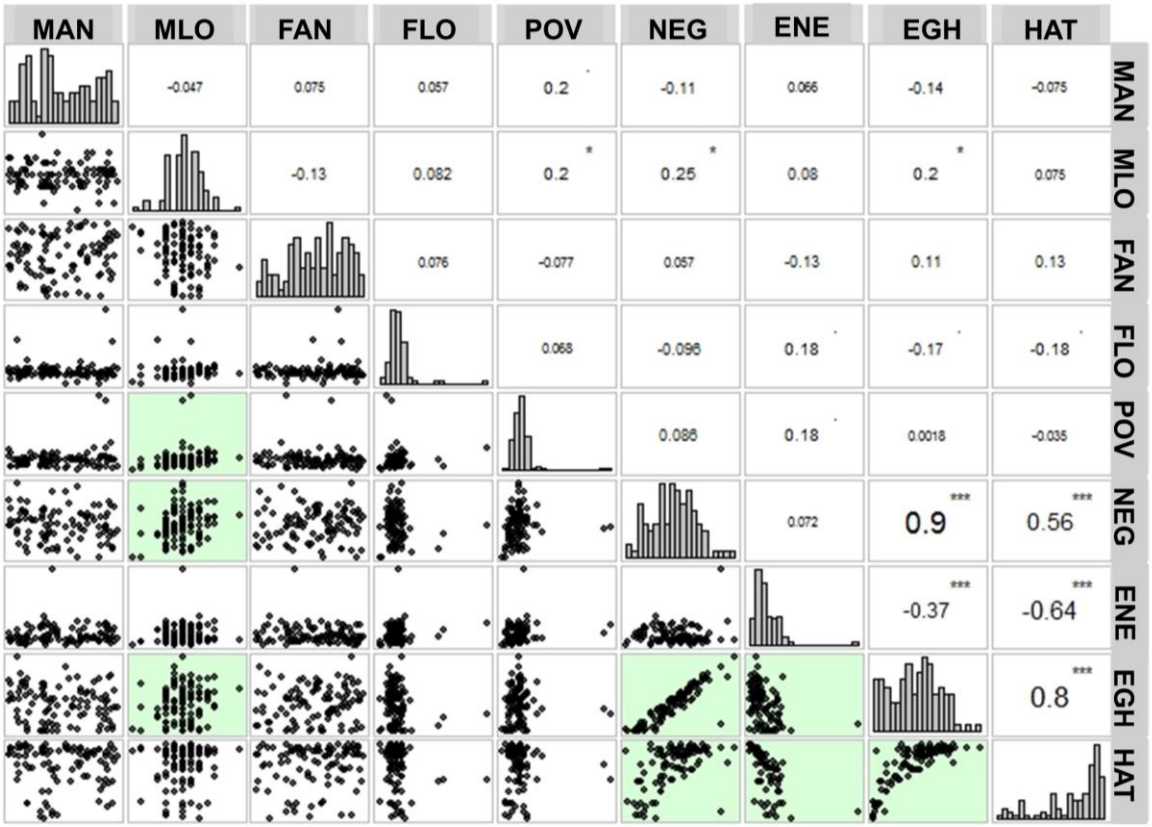

Figure 20. Linear correlation for egg effects in the first generation (F1). MAN= male number chosen to assemble the couple; MLO= adult male longevity; FAN= female number chosen to assemble the couple; FLO= adult female

longevity; POV= oviposition period; NEG= number of eggs placed; ENE= unviable eggs; EGH= viable eggs; HAT= hatching rate.

Male longevity (MLO) had weak and positive correlations with oviposition period (POV), NEG and EGH. The longer the male's life span, the longer the female's oviposition period could be, consequently the greater the number of eggs she would lay and hatch.

F2 generation showed a similar result to F1. The correlation between EGH and HAT was strong and positive. There was also a similarity in the correlation between NEG with EGH and HAT. The latter being a stronger correlation than in the first generation. However, in F2 NEG, it was also positively correlated with ENE, indicating that the greater the number of eggs laid in the second generation, the greater the number of eggs that did not hatch (Figure 21).

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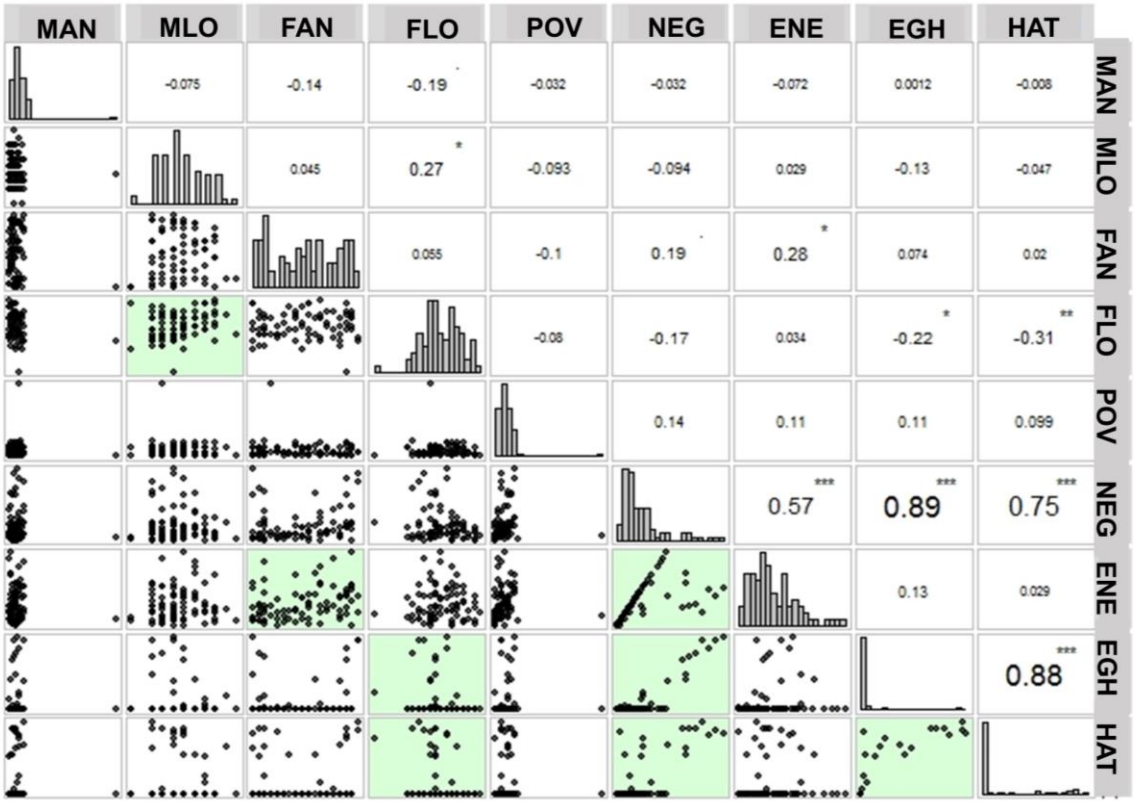

Figure 21. Linear correlation for egg effects in the second generation (F2). MAN= Male number chosen to assemble the couple; MLO= adult male longevity; FAN= Female number chosen to assemble the couple; FLO= adult female longevity; POV= Oviposition period; NEG= number of eggs placed; ENE= unviable eggs; EGH= viable eggs; HAT= hatching rate.

Females' longevity (FLO) had a weak and negative correlation with EGH and HAT, meaning that the longer the females had, the less chance of the eggs hatching and the percentage of hatching. The male longevity (MLO) also showed a weak and positive correlation with FLO; therefore, the possibility that the female has greater longevity may have been due to the male's high longevity. The fact that the low hatching of the eggs occurred (ENE) may have been due to the female chosen to form the couple (FAN), since the FAN had a weak and positive correlation with ENE.

3.2.7 Descriptive analysis to eggs variables

In the first generation, the mean male longevity was higher for the untreated, approximately 12 days. The individuals from the Cry1Aa and Dipel WP® treatments had mean longevity of ≈ 11 days, and the Cry1Ab and Cry1Ac treatments had the lowest mean longevity (≈ 10 days). In the second generation, the mean longevity for ≈ ten days in the Dipel WP® treatments ≈ 11 days in the untreated. The other treatments with mean longevity were similar to the first generation (Figures 22 and 23).

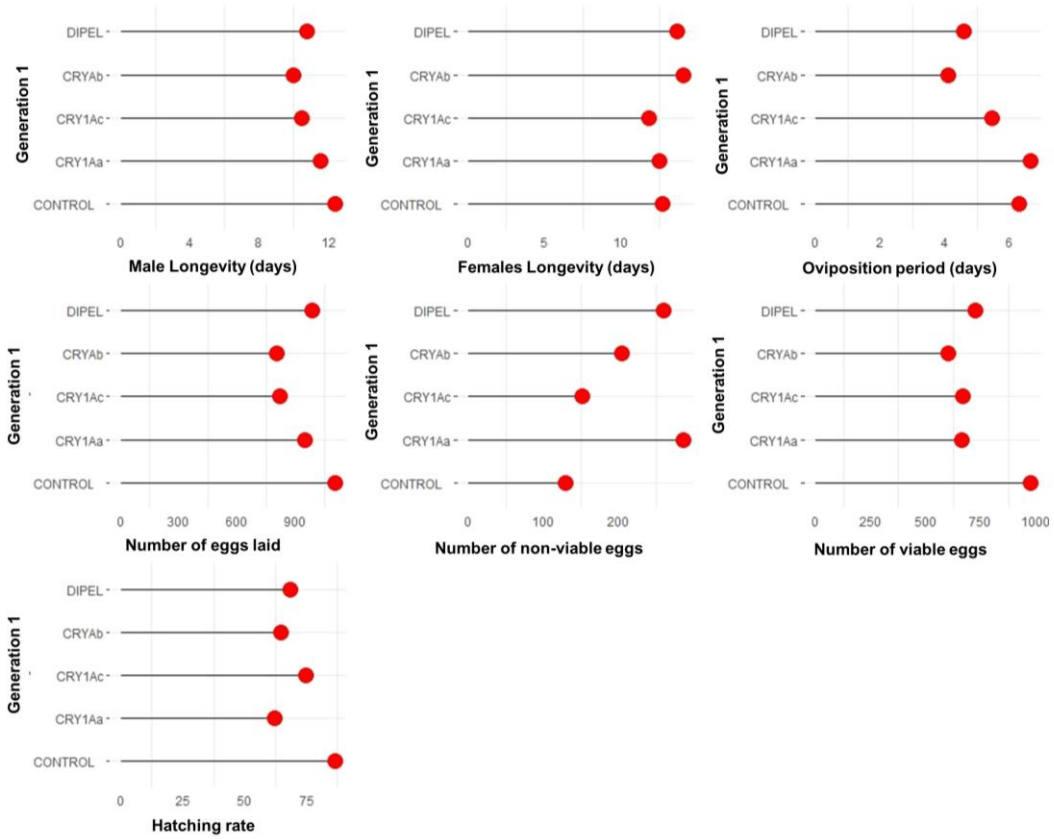

Figure 22. Descriptive analysis to eggs variable of *S. frugiperda* to the first generation.

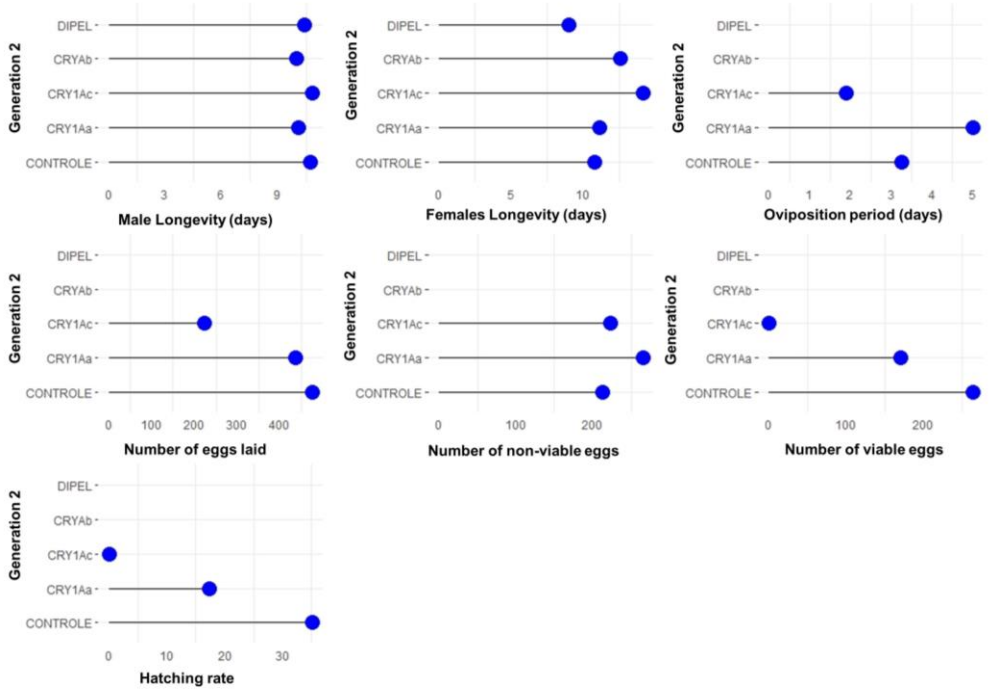

Figure 23. Descriptive analysis to eggs variable of *S. frugiperda* to the second generation.

The mean longevity of females in the first generation was higher for the treatments Cry1Ab and Dipel WP® (≈ 15 days), followed by the untreated and Cry1Aa treatments (≈ 13 days), and the lowest mean longevity was for Dipel WP® (≈ 12 days). In the second generation, the only treatment that had an increase in the mean longevity of the females was Cry1Ac (≈ 14 days). The untreated and Cry1Aa treatments reduced the mean longevity of the females to ≈ 11 days, Cry1Ab ≈ 12 days and Dipel WP® ≈ 9 days.

The highest mean oviposition period was for Cry1Aa (≈ 7 days) and untreated (≈ 6 days) treatments in the first generation. Dipel WP® and Cry1Ac showed similar mean oviposition (≈ 5 days). The lowest mean oviposition was obtained by insects exposed to the Cry1Ab (≈ 4 days). In the second generation, Dipel WP® and Cry1Ab treatments did not present mean oviposition. The Cry1Aa treatment had the highest mean oviposition (≈ 5 days), the untreated ≈ three days and Cry1Ac ≈ 2 days.

3.2.8 Analysis of variance (ANOVA) and Tukey test to eggs variables

The analysis of variance was performed for the egg variables of the treatments that showed less data variability: MLO = male adult life period (days); FLO = female adult life period (days); POV = oviposition period (days); NEG = number of eggs laid; ENE = number of non-viable eggs; EGH = number of viable eggs and HAT = hatching rate, in the F1 and F2. In the F1, the variables that showed a significant difference were: MLO, ENE and HAT. In the F2 only MLO (Table 8).

Table 8. Analysis of variance (ANOVA) for eggs variables, to first (F1) and second-generation (F2).

Treatments	MLO (F1)	ENE (F1)	HAT (F1)	FLO (F2)
Untreated	12.40 a	128.90 a	86.64 a	10.80 bc
Cry1Aa	11.55 ab	285.40 a	62.24 b	11.18 bc
Cry1Ab	9.95 b	203.50 a	64.53 ab	12.60 ab
Cry1Ac	10.45 b	151.05 a	74.91 ab	14.15 a
Dipel WP®	10.75 ab	258.95 a	68.32 ab	9.0 c

MLO= Male adult life period (days), ENE= number of non-viable eggs, HAT= Hatching rate (%), FLO= Female adult life period (days). Means followed by the same letter in column do not show statistical difference by the Tukey test ($P \leq 0.05$).

The mean percentage of hatching of the eggs was higher in the untreated (≈ 86.64%). However, it only differed from the Cry1Aa treatment, which presented a lower mean (≈ 62.24%); the other treatments had similarities with both.

In F2, the females of the Cry1Ac treatment had the highest mean of longevity (14 days), and the females of the Dipel WP® treatment had the lowest mean (9 days); both differed from each other. The females of the treatments and Cry1Ab, untreated and Cry1Aa had similar means; however, the last two were similar to the Dipel WP® treatment. Cry1Ab had a similar mean to Cry1Ac.

4 DISCUSSION

4.1 Concentration-response

Concentration-response bioassay with Cry proteins showed that *S. frugiperda* had low susceptibility to all proteins tested. The *S. frugiperda* (susceptible population from Sete Lagoas - MG) was less susceptible to Cry1Ab (LC_{25} = 57.33 ng cm^{-2}). Cry1Aa was treatment estimated the highest lethal concentration (LC_{25} = 1370 ng cm^{-2}). Cry1Ac presented an intermediate estimate of LC_{25} (106.62 ng cm^{-2}).

These same proteins were tested to *Ostrinia furnacalis*, *O. nubilalis* and *Diatraea saccharalis* and the highest LC_{50} value obtained among all proteins was 11.5 ng cm^{-2} to Cry1Ab in *O. nubilalis* (Tan et al., 2011). Lethal concentration values were low as those observed in this work. Therefore, these

Crambidaes presented high susceptibility to these proteins compared to *S. frugiperda.*

High values for lethal concentrations to Cry proteins are common for the genus *Spodoptera* due to the low susceptibility (Rabelo et al., 2020a). Furthermore, to Cry1Aa, Cry1Ab and Cry1Ac proteins and the product Dipel WP® (containing these proteins plus Cry2) presented no strict correlation between binding and toxicity in *S. frugiperda.* To Cry1 proteins were related weak interaction with epithelial brush border membrane on larvae of this specie fed to them (Aranda et al., 1996). Due to this fact they were chosen to conduct this work.

Spodoptera frugiperda is a species that presents genetic variability within populations and populations (Monnerat et al., 2006; Clark et al., 2007). This characteristic can be one reason for values so different in the estimates of lethal concentrations between works. In addition, another fact that could interfere with the lethal concentration estimation is test methodology. The two most used methodologies are diet-contamination or diet incorporation (Asano et al., 1993; Ibargutxi et al., 2006; Li and Bouwer 2012; Rabelo et al.,2020a). Liao et al. related to the genus *Helicoverpa* that the diet incorporation method was used to investigate the susceptibility of various instars because the later instars burrow into the diet more readily than the early instars. The surface contamination method was preferred for testing purified insecticidal proteins because the quantity of protein required for this method was 40-fold less. (Liao et al., 2002).

4.2 Larvae survival

No significant differences were observed in the larval survival in the first ten days of evaluation between all treatments and untreated in F1. This result was probably due to the estimated lethal concentration being very low, causing few deaths in the first days of evaluation. In other studies, when *S. frugiperda* was submitted to survival tests in Bt plants, there was a negative interference in the survival of individuals exposed to plants that express the proteins, with the shortest evaluation time (Mendes et al., 2011; Machado et al., 2020).

Physiologically, some authors list that among the factors that can influence larval survival after Bt exposure the larvae adaptation to these

proteins can be due to some mechanism that does not activate protoxin correctly due to lack of proteinase (Oppert et al., 1997; Zhu et al., 2015).

4.3 Sublethal effects on larvae, pupae, and adults

Sublethal effects (developmental time and growth, or cumulative lethal effects over the entire juvenile life stage (“slow kill”) or reproductive effects) the more sensitive indicators of crystal protein toxicity than lethal effects (“quick kill”) (van Frankenhuyzen, 2009; Hilbeck and Otto, 2015).

In general, the mean of sublethal parameters evaluated in the Dipel WP® treatment intermediate among the other treatments, except that pupal deformation in the second generation was higher among all treatments. Salama et al. (1986) determine the effect of *B. thuringiensis kurstaki* (Btk) HD-1 (Dipel WP®) on the prepupal and pupal stages of the *Spodoptera littoralis.* The duration of the prepupal stage was affected by Btk at high concentration (5%) when sprayed or kept in soil treated with the pathogen, and the number of malformed adults increased. The authors did not evaluate pupal deformation, but these results show that Btk can cause negatively influence the development of the *Spodoptera* genus, even though, Bohorova et al. (1996) reports that *S. frugiperda* shows low susceptibility to products based on *B. thuringiensis* subsp. *kurstaki* strain HD-1.

Different results were found in the species *Choristoneura fumiferana* when it was offered to low dose (≤ 50% lethal dose) of *B. thuringiensis* subsp. *kurstaki* at various stages of their development. Significantly increased development time to the pupal stage and reduced pupal size and number of eggs laid per female did not affect the proportion of embryonated eggs (Pedersen et al., 1997). To neonate larvae of *Helicoverpa armigera* that survived after treatment with Cry1Aa, Cry1Ab and Cry1Ac, showed significant stunting in their growth compared to the untreated larvae (Li and Bouwer, 2012).

Fathipour et al. (2019) fed larvae of *H. armigera* with an artificial diet containing sublethal Btk doses (LC_5, LC_{10}, LC_{15}, LC_{20} and LC_{25}). *H. armigera* larvae compensated for any damage caused by the treatment; therefore, it was concluded that Btk was not toxic to this species.

The media of larval weights with 7 and 10 days to Cry1Ab treatment of evaluation in the second generation were higher than Untreated. Sousa et al. (2016) showed similar results in three different populations of *S. frugiperda* derived and reared on Cry1Ab maize. They related higher larval weight, faster larval development, and better reproductive performance than the individuals derived from non-Bt maize, and one of these populations showed better performance on both Cry1Ab and untreated diets, indicating no fitness cost of the resistance trait.The authors related that this better performance to Cry1Ab is due to resistance alleles in these populations, what may also have happened in this work between generatios.

Cry1Aa was the treatment that caused the major median of deformed pupae in F1, but, the median of larval weight was lower than untreated in F1 and the evaluation with seven days in F2, only the median larval weight with 10 days of the evaluation presented higher to the median weight of untreated. A similar result was found in the second-instar of *Choristoneura fumiferana* related to larval weight that presented gain decreased at sublethal concentrations of Cry1Aa (Ramachandran et al., 1993). The pupal weights were not affected, regardless of the duration of exposure to toxic diet, as well, found in this work, the pupal weight media in F1 was higher than in untreated.

In *Lymantria dispar* (Noctuidae: Erebidae), were injected Cry1Aa, Cry1Ab and Cry1Ac to evaluated food intake and relative growth. This species was only weakly sensitive to Cry1Aa and Cry1Ab and not sensitive at all toward Cry1Ac. Cry1Aa e Cry1Ab affected growth and food intake (Cerstiaens et al., 2001). In the population of *S. frugiperda* used in this work, the Cry1Ac treatment was the one that most interfered in the development of the species. In F1 presented the lowest mean larval weight in 7 and 10 days of evaluation and one of the highest mean pre-pupal weights. In F2, both larval weights showed the highest, statistically, so the pre-pupal weight was the lowest among the treatments.

A bioassay realized with a susceptible strain of *S. frugiperda* found high mortality (97 and 82%), stunting, and weight reduction on Cry1Ac/Cry1F-soybean than on non-Bt soybean (2 and 8% mortality and stunting, respectively) (Machado et al., 2020). Furthermore, susceptible insects did not survive until the adult stage on Cry1Ac/Cry1F-soybean, different from the

results of this work, where were used low dose to evaluate the sublethal effects for two generations, and the insects have reached adulthood.

To another noctuid, *Helicoverpa armigera*, was related similar results to Cry1Ac in a diet incorporated method that the weight gained in seven and eleven days of exposure were less compared untreated, prolonged developmental time larval, retardation in larval growth, development and a higher number of adults malformed (Kannan and Uthamasamy, 2006). Cry1Ac presented a negative impact on fitness costs in both species.

In other lepidopteran family were related some sublethal effects similar to found in this work. Ma et al. (2008) fed *O. furnacalis* on an artificial diet containing purified Cry1Ac protein and they found that larval growth and development delayed, pupation, pupal weight and adult emergency decreased. Bernardi et al. (2014) tested this same protein at the maximum concentration (100 mg Cry1Ac mL^{-1} diet) and found intermediate mortality of *S. frugiperda* (50%).

Although sublethal effects previous study reports that Cry1Ac is atoxic against *S. frugiperda* larvae (Luo et al., 1999) and for the same protein, it has already been reported that in this species, it has a weak binding in the larvae midgut (Aranda et al., 1996) Cry1Ac is currently being used for the development of new products to untreated to *S. frugiperda*.

The effect of a single, sublethal dose of Bt on the midgut of the moth *Manduca sexta* larvae was monitored during acute and recovery stages. The authors related that after the acute stage, the midgut tissue recovered and the insects appeared to recover entirely and continue normal development (Spies and Spance, 1985). Some works have been relating the capability of resistants strains of *Heliothis virescens* to repair (or substitute) more readily the damaged cells (Martinez-Ramirez et al., 1999; Forcada et al., 1999).

In *Spodoptera exigua,* the expression of genes *REPAT1* can reduce the virulence of baculovirus to the infected larvae and *mitigating* pathological effects (Herrero et al., 2007). As well as, *H. virescens* larvae feeding of purified alpha-arylphorin resulted in midgut hyperplasia and significantly reduced susceptibility to Cry1Ac (Castagnola et al., 2017). *Arylphorin* genes is a candidate protein to regulate regeneration after intoxication, given that it is expressed by midgut cells, has a mitogenic effect on gut stem cells, and displays altered expression.

During infective processes (Castagnola and Jurat-Fuentes, 2016). This last review related that insects in each insect group have a defensive response to the damage caused by entomopathogens and their toxins activates into the digestive system of insects. The susceptible population of *S. frugiperda* in this work could present some defense mechanisms that helped develop after exposure to treatments.

Retnakaran et al., 1983 related that a low dose of Bt protein can induced feeding inhibition in the *C. fumiferana* permit the larva to degrade the toxin and repair the midgut by replacing the destroyed cells with cells from the regenerative nidi. Once the toxin (or toxin-induced factors) responsible for the feeding inhibition is cleared from the system, feeding commences. The authors show there is complete recovery from this feeding inhibition in 8 hr or less, This fact can explain why many parameters evaluated in this work were similar to the untreated.

4.4 Sublethal effects on eggs

The mean number of days of the female's adult period in F2 was lower for those submitted to the Dipel WP® treatment, and the number of eggs laid by these females was practically zero in the same generation. Similar results were found to *Spodoptera littoralis* submitted to the same treatment; the emerged moths showed short longevity associated with low egg production and low fertility. Moreover, malformed individuals was observed (Salaman and Zaki, 1986). *Choristoneura fumiferana* presented the percent of egg hatch, and first-instar survival as negatively affected by Dipel WP® due to stress spruce budworms parents underwent after applying the product (Bauce et al., 2006). These results showed that the reproduction of lepidopteran species could be affected by Bt.

Sedaratian et al. (2013) showed as the increased low dose of Btk offered for *H. armigera* larvae, the percentage of eggs that did not hatch decreased. Therefore, Btk has a direct effect on the hatchability of these eggs.

The females of Cry1Ab treatment did not present a number of eggs enough to be evaluated in the second generation; in other words, the oviposition was affected by the treatment. In the first generation, the same

treatment presented a mean of 4 days of oviposition, the smallest among the others. Erasmus et al. (2010) determined the effects of Bt maize on fecundity of *Agrotis segetum* and related the fewer eggs were laid by moths fed as larvae on maize event Bt11.

Moawad and Nasr (1983) related that *A. ipsilon* there was also a positive relationship between the number of eggs laid and the weight of the female pupae. Cry1Aa was the treatment that presented the higher pupal weight and means of adults who did not emerge from the pupae in F1, but, in both generations presented the higher oviposition period, intermediated number of eggs laid higher and a number of non-viable eggs. These results corroborate those found for *A. ipsilon.*

The energy reserves (triglycerides and glycogen) that mothers allocated to their progenies enhance or decrease the survival of diapausing second-instar larva of Spruce budworm (Bauce et al., 2006). According to the results shown in this work, this problem related to energy reserves could affect the reproductive stage.

4.5 Interference on sex

Some studies that have been using *B. thuringiensis* to evaluate the sublethal effect found that the agent interferes with the sex of the exposed organism (Pedersen et al., 1997; Fernandes, 2020). However, few studies assess the variables and the development of the insect that are directly related to this influence.

To Cry1Ab treatment, the one determinant variable for sex was larval weight with seven days of evaluation. In the first case was a negative relation; that is, the greater the larval weight in seven days of evaluation, the greater the chances of individuals being male. Quezada-García et al. (2014) related that Males and females of *C. fumiferana* were submitted on a “stress” diet (deficient in sugars and slightly higher in nitrogen), result in fewer females, showing that nutritional variation causes differential mortality between sexes, and suggesting that females are more sensitive to nutritional stress. The males of the Cry1Ab treatment were probably less sensitive to feeding on an artificial diet contaminated with protein, resulting in greater larval weight. In F2 of this same

treatment, when the larvae were submitted on an artificial diet without contamination, the opposite happened for the prepupal weight, which presented a positive relationship in determining sex, the heaviest pupae being female.

The pupal period was the variable common in all treatment in both generations as a negative sex determinant; in other words, male individuals tend to have a longer pupal period than females. To untreated, one of the determinant variables for females in F1 was pre pupal weight, heavier the prepupa greater the chance of being female, but, in this same treatment and generation heavier pupae, greater the chance of being male. Different results were found to *S. albula and S. litura,* fed only with artificial diet and plants hosts, respectively (Montezano et al. 2013; Xue et al., 2010)

Most studies with sublethal effect on lepidopteran pests evaluate only the sex ratio and not which variables during the pest development can influence the sex determination during the metamorphosis phase. It is important to know which variables can change the sex ratio so that new studies are oriented to change any of these parameters of larval development in order for the population in the field to have a deficiency of one of the sexes, for example.

5 CONCLUSION

The proteins Cry1Aa, Cry1Ab, Cry1Ac, and a commercial product based on these proteins can cause sublethal effects in different life stages on susceptible of *S. frugiperda* for two generations.

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CAPÍTULO 3 – Detecção de *Bacillus thuringiesis* Berliner em ovos de *Spodoptera frugiperda* (J.E. Smith) através de análise de proteômica

RESUMO- Poucos estudos observam se o parental que foi submetido a uma exposição prévia à proteína Cry pode transferir a mesma de uma geração para outra. Devido à evolução na pesquisa entomológica, muitos trabalhos têm utilizado a análise proteômica para o sequenciamento de insetos e para realizar análises nas posturas de ovos com diferentes objetivos. Neste trabalho, a F1 de *S. frugiperda* foram expostos a baixas doses das proteínas que já apresentaram relatos de efeitos subletais nesta espécie: Cry Cry1Aa, Cry1Ab, Cry1Ac e Dipel WP®. Além disso, foi verificado por meio de análises proteômicas se os pais conseguem passar essa proteína para os ovos. As concentrações de CL_{25} obtidas foram: 9,75x105 esporos ml^{-1} (Dipel WP®), 1370 ng cm^{-2} (Cry1Aa), 57,33 ng cm^{-2} (Cry1Ab) e 106,62 ng cm^{-2} (Cry1Ac). Os produtos foram diluídos em água desionizada estéril e micropipetados 75µl na superfície da dieta e oferecidos a 360 larvas neonatos (24h de idade). Em todos os casos, o tratamento controle era com água esterilizada e desionizada. As larvas foram expostas ao Bt por sete dias e após foram renovadas a dieta (2 cm^3) sem contaminação em um recipiente plástico de 7,5 cm^2, onde permaneceram até a idade adulta. Na idade adulta, adultos que não apresentavam deformação foram utilizados para a montagem aleatória dos casais. Dez casais para cada tratamento, e o mesmo para o tratamento controle, foram colocados em gaiolas de PVC. Os ovos foram coletados diariamente até a morte dos adultos e armazenados em freezer a -20ºC. A proteína foi extraída de cada amostra biológica, utilizando o método fenólico e os peptídeos foram analisados em Synapt G2 HDMS (Waters, Manchester, UK). Na segunda geração, foi identificado um maior número total de proteínas nos ovos (1724) do que na primeira (1599). Em F1 952 proteínas não redundantes foram identificadas e 989 em F2. Dipel WP® foi o tratamento que apresentou a maior quantidade de proteínas exclusivas em ambas as gerações 74 em F1 e 210 em F2. A maior parte dessas proteínas foi compartilhada com Cry1Ac em ambas as gerações. Entre as proteínas compartilhadas nos tratamentos Dipel WP® e Cry1Ac em F2, dezesseis proteínas eram de choque. Proteínas que apresentam rápido aumento coletivamente nos polipeptídeos conservados ajudam os insetos a responder à temperatura elevada e a uma variedade de estresses químicos e físicos. No entanto, as proteínas Cry não foram detectadas neste trabalho.

Palavras-chave: Identificação de proteínas, proteínas Cry, Efeito subletal, Noctuidae

CHAPTER 3_ Detection of *Bacillus thuringiesis* Berliner in eggs of *Spodoptera frugiperda* (J.E. Smith) through proteomics analysis

ABSTRACT-. Few studies observe whether the parental that was submitted a previous exposure to Cry protein can transfer sublethal effects from one generation to another. Due to evolution in entomological research, many works have been using proteomic analysis for insect sequencing and to perform analysis in the egg masses laid with different aims. In this work, *S. frugiperda*'s parents were exposed to low doses of Cry proteins Cry1Aa, Cry1Ab, Cry1Ac and Dipel WP® that have already presented reports of sublethal effects in this species. In addition, it was verified through proteomics analysis if the parents can pass this protein to the eggs. In bioassay were used LC_{25}= concentration that kills 25% of the susceptible *S. frugiperda* population. The concentrations obtained were: $9.75x10^5$ spores ml^{-1} (Dipel WP®), 1370 ng cm^{-2} (Cry1Aa), 57.33 ng cm^{-2} (Cry1Ab) and 106.62 (Cry1Ac). The products were diluted in sterile deionized water and micropipeted 75µl on the diet surface and offered to 360 neonate larvae (24 hr old). In all cases, the untreated was sterile and deionized water. The larvae were exposed to Bt for seven days and renewed the diet (2 cm^3) without contamination in a plastic container with 7.5 cm^3, where they stayed until adulthood. In adulthood, adults who did not present deformation were used for the random assembly of couples. Ten couples for each treatment, and the same for the untreated, were set up in PVC cages. Eggs were collected from each couple daily until the death of adults and stored in a freezer at -20ºC. The protein was extracted from each biological sample using the phenolic method and peptides were analyzed in Synapt G2 HDMS (Waters, Manchester, UK). In the second generation, a greater total number of proteins in the eggs (1724) was identified than in the first (1599). In F1, 952 non-redundant proteins were identified and 989 non-redundant proteins were identified in F2. Dipel WP® was the treatment that presented the largest amount of exclusive proteins in both generations, 74 in F1 and 210 in F2. For the most part os these proteins were shared to Cry1Ac in both generations. Among the proteins shared in the Dipel WP® and Cry1Ac treatments in F2, sixteen heat shock proteins (Hsps). Hsps present rapid increase collectively in these conserved polypeptides help insects respond to elevated temperature and a variety of chemical and physical stresses. However, Cry proteins were not detected in this work.

Keywords: protein identification, Cry protein, sublethal effects, Noctuidae

1. INTRODUCTION

In Brazil, corn is one of the main annual crops. Expectations for the corn crop in the 2020/21 harvest is for a total area of 18,463.5 thousand hectares and an estimated production of 102.3 million tons. (Conab, 2021). Among the factors that can negatively influence the reduction of these numbers are insect pests. *Spodoptera frugiperda* (J.E. Smith), affects crops not only in Brazil but also in other countries and continents, as recently (Cruz 1995; FAO, 2018). Despite being the main pest corn crop, *S. frugiperda* is polyphagous and bringing significant losses in other crops of economic importance (Cruz, 1995; Barros et al., 2010; Kuate et al., 2019;).

The use of chemical control and transgenic plants are the most common form to control *S. frugiperda* (Carvalho et al., 2013; Botha et al., 2019). However, has been increasing the use of biopesticides to control this pest, and most of these products are developed based on *Bacillus thuringiensis* (Bt) (Berliner), as well as transgenic plants (Olson, 2015; Damalas and Koutroubas, 2018; Qiong et al., 2013). The Bt is a Gram-positive bacterium that produces insecticidal proteins as crystal inclusions during its sporulation phase of growth, known as Cry or Cyt toxins (Bravo et al., 2011).

Several studies have been carried out observing the negative or positive changes in the development of insects caused by Cry proteins, which are called sublethal effects (Eizaguirre et al., 2005; Kannan and Uthamasamy, 2006; Rabelo et al., 2020b; Castro et al., 2019). However, few studies observe whether the parental that was submitted a previous exposure to Cry protein can transfer sublethal effects from one generation to another.

Paula et al. (2014) realized a study demonstrating that *Closyne lacinia* can be taking up Cry1Ac when exposed to low concentrations and transferring the entomotoxin to larvae that presented adverse effects. Souza et al. (2018) tested a low dose of Cry1F in *S. frugiperda* (with resistance to Cry1F) and related that this species could transfer Cry1F from a genetically engineered maize variety to their offspring. Both of these works were used Elisa (Enzyme-

Linked Immunosorbent Assay) for toxin detection, and Cry1F was used ECL Western Blot.

Due to evolution in entomological research, many works have been using proteomic analysis for insect sequencing and to perform analysis in the egg masses laid with different aims. To *Bombyx mori* were used shotgun liquid chromatography mass spectrometry (LC-MS/MS) approach combined with bioinformatics analysis to illuminate the differences among protein identification profiles of the diapause and nondiapause eggs (Fan et al., 2013). To the same aim, the proteomes (iTRAQ) were used in *Locusta migratoria* (Orthoptera) eggs (Kun et al., 2017). In *Ericerus pela* (Hemiptera) were used high-throughput proteomics to characterize protein expression in eggs (Hu et al., 2017).

In this work, *S. frugiperda*'s parents were exposed to low doses of Cry proteins Cry1Aa, Cry1Ab, Cry1Ac and Dipel WP® that have already presented reports of sublethal effects in this species (Aranda et al., 1996). In addition, it was verified through proteomics analysis if the parents can pass this protein to the eggs.

2. MATERIAL AND METHODS

2.1 *Spodoptera frugiperda* colony rearing

The colony of *S. frugiperda* from a susceptible population to *B. thuringiensis* was obtained from Embrapa Milho e Sorgo, Sete Lagoas – MG and was rearing at the LCMAP - FCAV / UNESP under controlled conditions (25° ± 2° C, RH 70% ± 10 and photoperiod 14:10h.

Adult insects (males and females) were placed in a PVC tube cage (10 cm in diameter by 20 cm in height), covered with voile. Inside the cage, a soked cotton was offered with 10% of honey solution as food and was covered with A4 paper to provide a substrate for oviposition.

The eggs were removed three times a week and transferred to plastic cages (500 mL) that contained an artificial diet based on white beans, wheat germ, and beer yeast (Greene, 1976). After egg masses hatching, second-

instar larvae were individualized in plastic containers (50 mL) with the same artificial diet.

When the insects reached the pupal stage, they were transferred to plastic cages (500 mL), lined with filter paper, where they remained until the emergence of adults. Malformed adults were discarded from rearing.

2.2 Expression, purification, activation and quantification of Cry proteins

The proteins Cry1Aa, Cry1Ab and Cry1Ac, were obtained from the Institute of Biotechnology, National Autonomous University of Mexico. Bt serotype *kurstaki* HD-73 strain expressing Cry1Ac, crystalliferous Bt 407- strain expressing Cry1Ab and Cry1Aa (Meza et al., 1996) were grown at 30 °C until complete sporulation for three days in nutrient broth sporulation medium (Schaeffer et al., 1965). For Bt 407- strain expressing Cry1Ab proteins, the growth medium was supplemented with erythromycin at 10 µg/ml. Spores/crystals were washed three times in 0.3 M NaCl, 10 mM EDTA, pH 8.0. Crystal inclusions were solubilized in an alkaline buffer (50 mM Na2CO3 and NaHCO3, 0.2% β-mercaptoethanol, pH 10.5) for 1 h at 37 °C.

Trypsin-activated toxins were obtained by treating soluble protoxins with trypsin (TPCK treated trypsin from bovine pancreas, SIGMA Aldrich, St. Louis, MO, USA) in a mass ratio of 1: 20 (trypsin: protein) for 1 h at 37 °C. Phenylmethylsulfonyl fluoride (PMSF) (1 mM final concentration) was added to stop proteolysis. Finally, the Cry proteins were purified with HP HiTrap ion exchange chromatographic column (GE Healthcare) according to the manufacturer's protocol. The final concentrations of the purified proteins were determined by Bradford (1976) method using bovine serum albumin (BSA) as a standard and separated by SDS-PAGE electrophoresis. After this procedure, the product was diluted to establish other concentrations to use in the toxicity bioassays.

2.3 Quantification of commercial product based on *B. thuringiensis*

The commercial product is based on *B. thuringiensis,* Dipel WP® (*B. thuringiensis* var. *kurstaki*), which contain Cry1Aa, Cry1Ab, Cry1Ac and Cry2 proteins, is not recommended to control *S. frugiperda*, was used.

The concentrations of bioinsecticides and isolates were adjusted to $3x10^8$ spores mL^{-1} in a Neubauer chamber on the microscope light (1000 times magnification) (Alves and Morais, 1998). This concentration is considered discriminatory in Bt pathogenicity tests for pest insects (Polanczyk et al., 2005). After this procedure, the product was diluted to establish other concentrations to use in the toxicity bioassays.

2.4 Bioassay with Bt on susceptible *S. frugiperda*

Tests to estimated the low dose (LC_{25}= concentration that kills 25% of the susceptible *S. frugiperda* population) were performed previously . The concentrations obtained were: $9.75x10^5$ spores ml^{-1} (Dipel WP®), 1370 ng cm^{-2} (Cry1Aa), 57.33 ng cm^{-2} (Cry1Ab) and 106.62 (Cry1Ac). The products were diluted in sterile deionized water and micropipeted 75µl on the diet surface (Greene et al., 1976) in a cylindrical and transparent plastic container (Ø = 2.5 cm or 1.5 cm^3) and offered to 360 neonate larvae (24 hr old). In all cases, the untreated was sterile and deionized water. The larvae were exposed to Bt for seven days and after were renewed the diet (2 cm^3) without contamination in a plastic container with 7.5 cm^3, where they stayed until adulthood.

In adulthood, adults who did not present deformation were used for the random assembly of couples. Ten couples for each treatment, and the same for the untreated, were set up in PVC cages (Ø = 10 cm; height = 20 cm), and they were fed with sugar solution at 10%. Eggs were collected daily from each couple until the death of adults and stored in a freezer at -20ºC. The bioassay was conducted in controlled conditions of temperature (25° ± 2° C), relative humidity (70% ± 10), and 14 hours photophase.

2.5 Total protein extraction and trypsin digestion

Total protein was extracted from each biological sample, using the phenolic method according to Hurkman and Tanaka (1986), with minor modifications. Eggs were removed from the oviposition substrates after submersion in nitrogen to facilitate the handling. Three samples of each treatment were used as biological repetitions, and in each sample, there were eggs from three different couples. The same was repeated for both generations. Eggs were ground into a fine powder with the aid of the ball mill (Figure 1).

Figure 1. Egg's maceration process. A) For each sample, were used five spheres; B) ball mill; C) Fine powder of eggs.

The fine powder of eggs was homogenized in 1 ml of extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, pH 7.5, 50 mM EDTA, 0.1 M KCl, 1% w/v polyvinylpolypyrrolidone (PVPP), 2% v/v 2-mercaptoethanol, and 2 mM PMSF), by shaking (70 rpm) for 30 min at 4°C. After, the same volume of 10 mM Tris-HCl (pH 8.0) saturated phenol was added to the protein suspension and samples were shaken for 30 min at 4°C; the phases were separated by centrifugation (10,000 g for 30 min at 4°C). The supernatant was recovered and

re-extracted with an equal volume of extraction buffer plus 0.15 g of PVPP (10,000 g for 30 min at 4°C). This step was repeated without PVPP. Proteins were precipitated by adding 5 vol of 0.1 M ammonium acetate in methanol (100%) and incubated overnight at -20°C. The samples were then centrifuged (16,000 g, 30 min at 4°C) and the resulting pellets were washed three times with 0.1 M ammonium acetate in methanol, followed by a final wash with acetone. The evaporation of acetone was achieved by leaving the samples in boxes containing silica (4°C). After complete drying the protein pellets were suspended in 500 µl of solubilization buffer [2,1 M urea, 0,76 M thiourea, 0.005% v/v Triton X-100, 50 µl dithiothreitol (DTT) and 5 mL milliQ]. Complete protein solubilization was achieved by vigorous shaking using a vortex for 2 min. Protein extracts were desalinized using Amicon Ultra-0.5 ml 3K-NMWL filter devices (Millipore Corporation).

Total proteins were quantified using the Bradford method (Bradford, 1976) and polyacrylamide gel electrophoresis (SDS-PAGE) (Except one sample of Cry1Aa in F2, which did not have enough eggs for protein extraction) (Figures 2, 3 and 4).

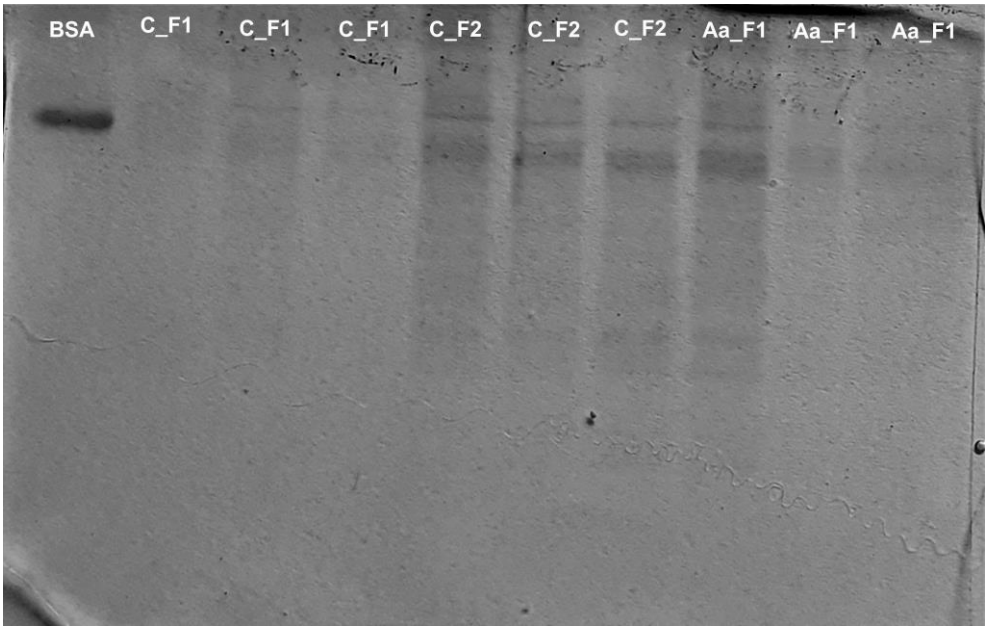

Figure 2. Polyacrylamide gel electrophoresis (SDS-PAGE) of control (F1 and F2) and Cry1Aa (F1) samples. BSA = Bovine Serum Albumin, molecular weight 66.4 kDa.

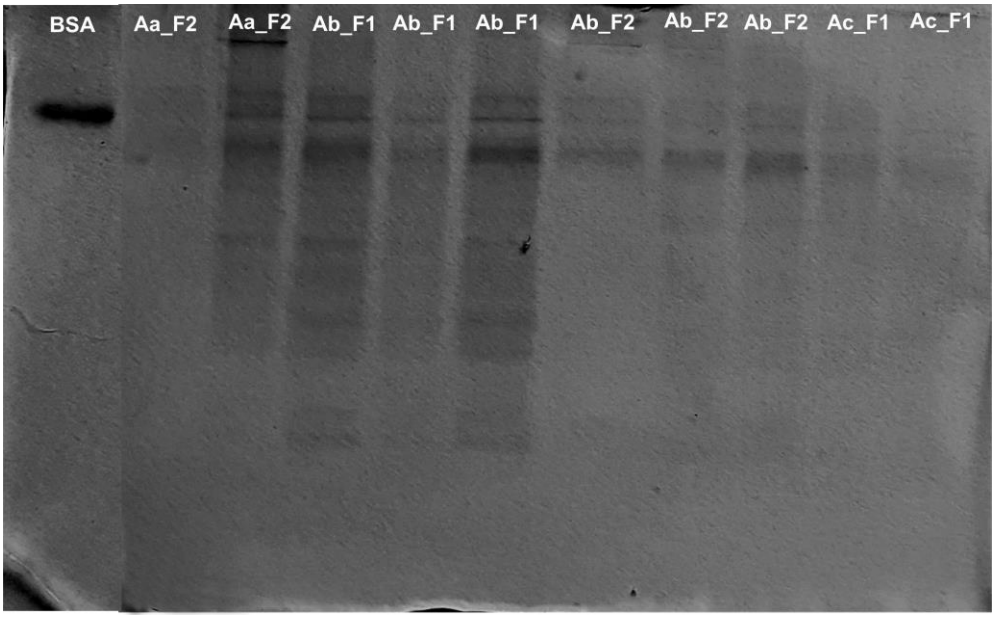

Figure 3. Polyacrylamide gel electrophoresis (SDS-PAGE) to Cry1Aa (F1 and F2), Cry1Ab (F1 and F2) and Cry1Ac (F1). BSA = Bovine Serum Albumin, molecular weight 66.4 kDa.

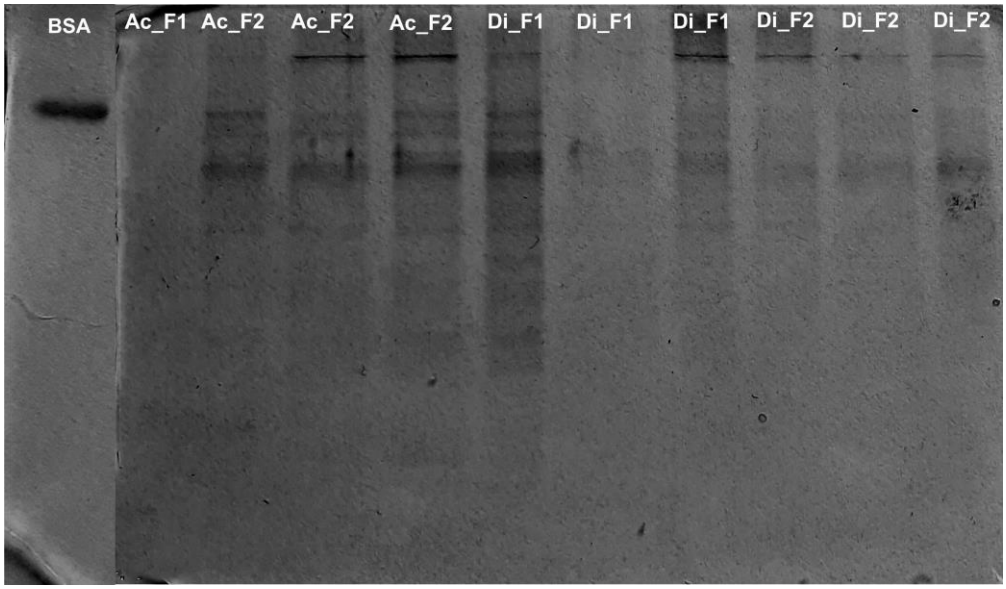

Figure 4. Polyacrylamide gel electrophoresis (SDS-PAGE) of Cry1Ac (F1 and F2), and Dipel WP® (F1 and F2) samples. BSA = Bovine Serum Albumin, molecular weight 66.4 kDa.

Fifty micrograms of total protein of each sample were denatured with 25 µl of 0.2% RapiGest (Waters, USA), reduced with 2.5 µl of 100 mM DTT and alkylated with 2.5 µl of 300 mM iodoacetamide. Trypsin digestion was performed with sequencing Grade Modified Trypsin (Promega) at a 1:100 (w/w)

enzyme: protein ratio and proteins were incubated at 37°C overnight. After, 10 µl of 5% (v/v) trifluoroacetic acid (TFA) was added to the digested mixture to hydrolyze the RapiGest (Waters, USA). The peptide mixture was then desalted using ZipTip C18-columns (Millipore Corporation). The final volume of 40 µl was obtained by adding a 20 mM ammonium formate (pH 10) solution containing 200 fmol/µl of rabbit phosphorylase (internal standard to data normalization and label-free protein quantification P00489) to the lyophilized, desalted peptide sample.

2.6 Mass spectrometry (LC-MS/MS)

The peptides mixture was analyzed by reverse-phase ultraperformance liquid chromatography (ACQUITY UPLC M-Class System with 2D Technology—Waters, USA) using a Synapt G2 HDMS (Waters, Manchester, UK). First dimension chromatographic separation was achieved using an AQUITY UPLC M-Class peptide BECH C18 columns (5 µm, 300 µm x 50 mm). Elution was performed using five different binary gradients with 20 mM pH 10 ammonium formate in acetonitrile at a flow rate of 2 µl min-1. Eluted peptides from the first-dimension column were trapped in a Symmetry 2D C18 column (5 µm, 180 µm x 20 mm) and diluted, online, with acetonitrile containing 0.1% formic acid. Second dimension separation was performed in an AQUITY UPLC M-Class CSH C18 column (1.7 µm, 75 µm x 150 mm), using a binary gradient from 7% to 85% acetonitrile with 0.1% formic acid, during 75 min, at a flow rate of 400 µl min-1. Mass spectrometry acquisition was achieved in a Synapt G2 HDMS (multiplexed DIA—data-independent acquisition) (Waters, Manchester, UK) mass spectrometer equipped with ion mobility cell and a nanolockspray source in the positive ion and “V” mode. The doubly-charged ion [(M + 2H)2+] was used for single initial point and MS/MS fragment ions of GFP (Glu 1)-Fibrinopeptide B m/z 785,84,206 [(M + 2H)2+] (Waters, Corp., Milford, USA) were used as lock masses and instrument calibration, respectively. Data-independent scanning (MSE) experiments were performed by switching between low (3 eV) and high collision energies HDMSE (19–45 eV), applied to the trap “T-wave” CID (collision-induced dissociation) cell filled with argon gas.

The scan time of 0.8 s was used for low and high energy scans from m/z 50 to 2000 (Silva et al., 2006).

2.7 Processing parameters and database search (LC-MS/MS)

The raw data processing, protein identification and relative quantitative analyses were all performed using ProteinLynx Global Server v2.5.1 (PLGS, Waters). Protein identifications were performed using the sequences obtained from Uniprot: Cry1Aa (Uniprot entry: P0A366), Cry1Ab (Uniprot entry: P0A370), Cry1Ac (Uniprot entry: P05068) *Bacillus thuringiensis* var*. kurstaki*, which were attached to the Spodoptera frugiperda databank from Uniprot).The sequence of the internal standard rabbit phosphorylase (Uniprot entry: P00489) was also included in the dataset of each treatment,to identify and quantify the proteins. The intensities of the spectra were calculated by the stoichiometric method according to MSE analysis (Silva et al., 2006) and normalized using the PLGS auto normalization function.

The processing parameters included: automatic tolerance for precursor and product ions, minimum of three fragment ions matched per peptide, minimum of seven fragment ions matched per protein, minimum of two peptides matched per protein, one possible trypsin missed cleavage, carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification, and a maximum false-positive discovery rate (FDR) lower than 1%, determined based on the search of a reversed database, which was generated automatically using PLGS by reversing the sequence of each entry. To analyze the abundance of proteins, the protein amount in fmols was used as a parameter.

2.8 Statisctical analysis

The data obtained in the proteomic analysis were submitted to the assumptions of multivariate models. After estimating the mean trends and building the ranking graphs (Package: ggplot2), the mean Euclidean algorithm

was used to construct the distance matrix. It was directed to the UPGMA (Unweighted pair group method using arithmetic averages), with that the dendrogram was built (Package: dendextend), proceeded the main components Biplot (Packages: factoextra, FactoMineR). For linear trends, linear correlation with probability was used by the 5% t-test (Package: metam). The Venn diagrams stratified by generations were made using the VennDiagram package. All analyzes were performed using the R program (R Core Team, 2020).

3. RESULTS

3.1 Proteomics Summary

In the second-generation was identified a more significant total number of proteins in the eggs (1724) than the first-genaration (1599). The eggs obtained from *S. frugiperda* larvae exposed to Dipel WP® showed the highest number of proteins identified in both generations (F1= 616 and F2 =853). In F1, the eggs untreated showed the lowest number of identified proteins (140) followed by the Cry1Ab (154) and Cry1Ac (182) treatment eggs. The eggs obtained from the Cry1Ac treatment showed 507 total proteins.

In F2, Cry1Ab presented the lowest number of proteins identified in eggs among the treatments (2). The total of proteins identified in F2 in the eggs that underwent the untreated was 205, Cry1Aa 107, and Cry1Ac 557.

3.2 Descriptive analysis

The highest protein score obtained was in the untreated (2273) and Dipel WP® (1916) in F1. Cry1Ab had the lowest mean protein score in both F1 (528)

and F2 (362). In F2, the highest protein score mean was for the treatment Dipel WP® (2003) and Cry1Aa (1851). In F2, The untreated had the lowest mean protein score among the treatments (1583), except Cry1Ab (Figure 5).

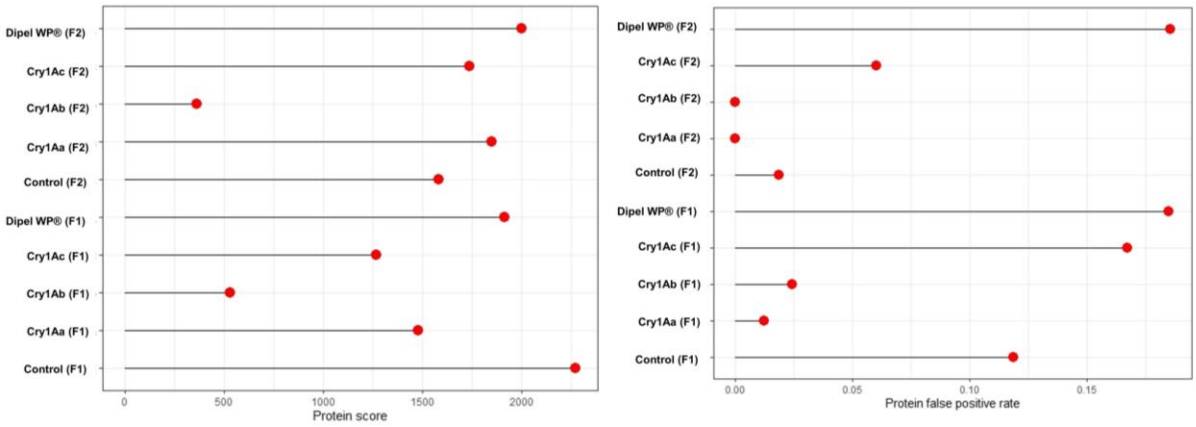

Figure 5. Proteins obtained from samples of *S. frugiperda* eggs exposed to Bt treatments for two generations.

In F1 and F2, the Dipel WP® treatment showed the highest mean false-positive rate (0.1849%). Cry1Ac showed an mean rate of (0.1673), untreated (0.1187%) and Cry1Ab (0.0242%), in F1. The Cry1Aa treatment presented the lowest mean false-positive rate (0.0122%). The mean false positive rate of Cry1A and Cry1Ab was zero and Cry1Ac 0.06% in the second generation. The untreated showed a mean false-positive rate of 0.0186% in F2.

The protein mass means were similar between the treatments in the first generation, with the Cry1Ab treatment with the lowest mean (31705) and Cry1Ac the highest (36511) standing out. In the second generation, the treatment that stood out with the highest mean was Cry1Ab (85454). The other treatments of F2 had mean values very close to each other (Figure 6).

A similar result was found for the protein matched product parameter, the mean between treatments was similar in the first and second generation; however, Cry1Ab in the second generation showed the highest mean (74).

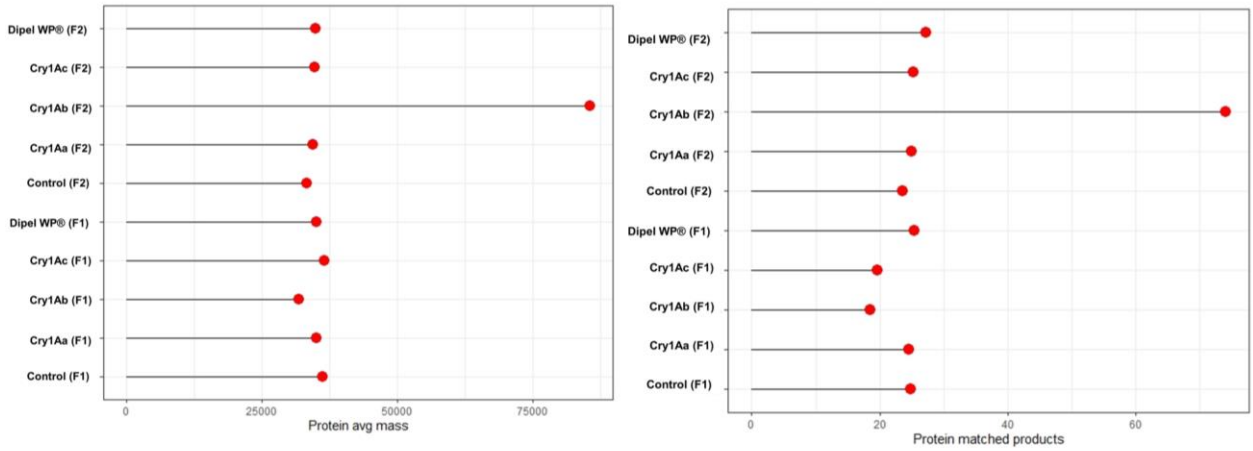

Figure 6. Proteins obtained from samples of *S. frugiperda* eggs exposed to Bt treatments for two generations.

In F1, the mean of protein-matched peptides was lower for the Cry1Ab treatment (4.12) and higher for the Cry1Aa treatment (5.27) and Dipel WP® (5.12). The untreated showed mean of protein-matched peptides (4.98) and Cry1Ac (4.45). In F2, the highest mean of protein matched peptides was for the Cry1Ab treatment (17) and the lowest means were for the Cry1Aa and Untreateds, 4.74 and 4.85, respectively. Cry1Ac had a mean protein matched peptides of 5.13 and Dipel WP® (5.36) (Figure 7).

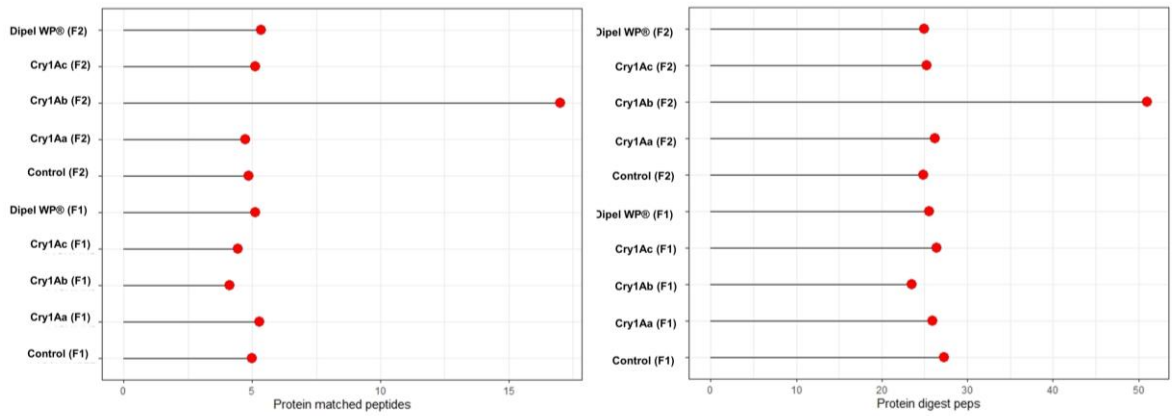

Figure 7. Proteins obtained from samples of *S. frugiperda* eggs exposed to Bt treatments for two generations.

In F1, the mean protein digest peptides was higher for the untreated (27.32) and Cry1Ac (26.45) and lower for the Cry1Ab treatment (23.52). The

treatments Cry1Aa and Dipel WP® presented close mean, 25.91 and 25.58, respectively. In F2, the highest mean of protein digest peptides was used for the Cry1Ab treatment (51) and the lowest in the untreated (24.81) and Dipel WP® (24.98). The treatments Cry1Aa and Cry1Ac showed a mean of 26.20 and 25.21, respectively.

In F1 and F2 the highest mean of protein sequence coverage was from the Dipel WP® treatment 25.16 and 25.34, respectively. In F1, Cry1Aa had a mean of 24.06 and the lowest was for Cry1Ac 20.62. The control and Cry1Ab treatments showedmeans of 22.22 and 21.11, respectively. In F2, Cry1Ab had a mean of 25.20 and Cry1Ac 24.56. The Cry1Aa and untreateds mean 23.67 and 23.46, respectively (Figure 8).

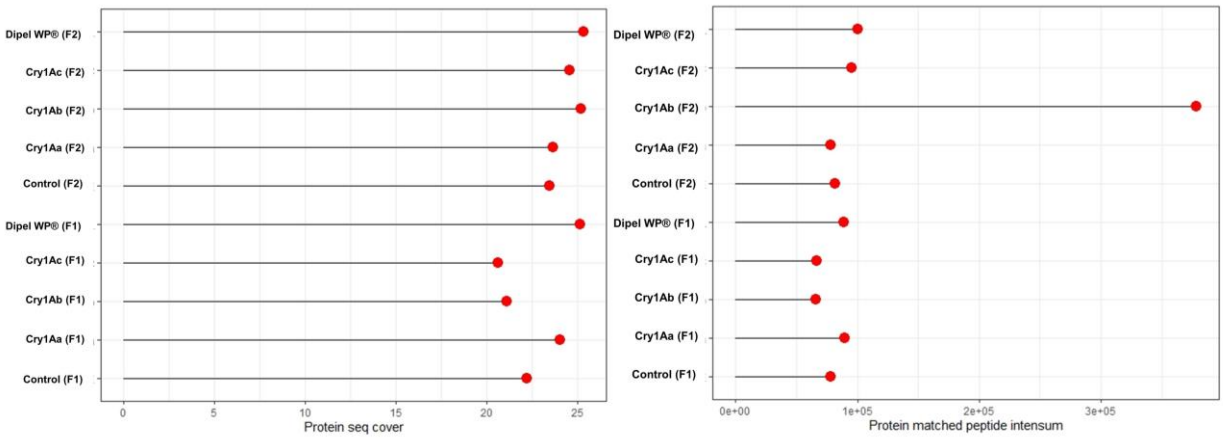

Figure 8. Proteins obtained from samples of *S. frugiperda* eggs exposed to Bt treatments for two generations.

Dipel WP® was the treatment that presented the highest mean of protein matched peptide intensum (88558) in F1 and the lowest in F2 (49455). In F1, the lowest mean treatments were control (33527) and Cry1Aa (35073). Cry1Ac and Cry1Ab and had means of 66586 and 65941, respectively. In F2, the Cry1Ab treatment presented the highest mean of protein matched peptide intensum (378243). Untreated showed a mean of 81454, Cry1Aa 78016 and Cry1Ac 98030.

In F1, the mean of protein top3 matched peptide intensum was lower for the untreated (28673) and higher for the treatments Dipel WP® (44171) and Cry1Aa (42500). The treatments Cry1Ab and Cry1Ac showed a mean of 34711 and 33319, respectively. In F2 the highest mean of protein top3 matched peptide intensum was for the Cry1Ab 139935 treatment and the lowest for Cry1Aa (35453) and control (39670) (Figure 9).

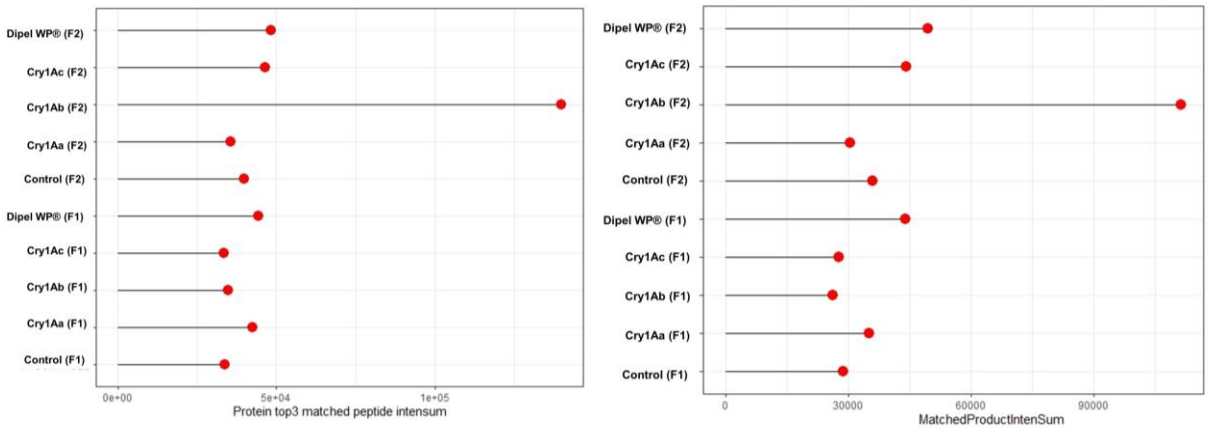

Figure 9. Proteins obtained from samples of *S. frugiperda* eggs exposed to Bt treatments for two generations.

The highest means of protein Matched Product Inten Sum in F1 were Cry1Aa (89353) and Control (77721). And the lowest means were from the treatments Cry1Ab (26054) and Cry1Ac (27674). In F2 Cry1Aa remained with the lowest mean (30445) and the highest were for the treatments Dipel WP® (100415) and Cry1Ab (111289).

3.3 Linear correlation

In the linear correlation analysis description, only strong correlations between the parameters of the proteomic analysis (coefficients greater than 0.8) were used to express the trends between proteomic variables (Figure 10).

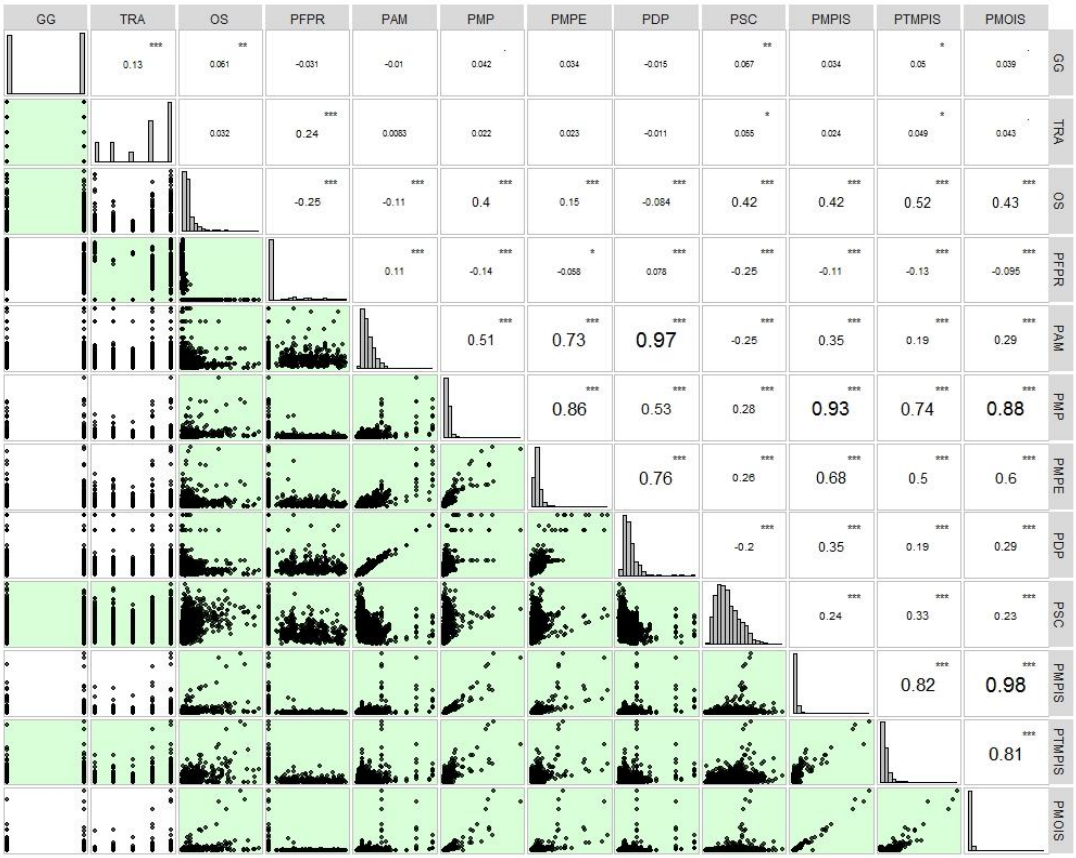

Figure 10. Linear correlation between proteomic analyses parameters. GG= Generation; TRAT= treatment; PTN= protein; OS= protein score; PFPR= protein false Positive Rate; PAM= protein average Mass; PMP= protein matched products; PMPE= protein matched Peptides; PDP= protein digest Peps; PSC= protein.seqCover; PMPIS= protein matched peptide intensum; PTMPIS= protein.top3 matched peptide intenSum; PMOIS=.matched product intensum.

Protein average Mass (PAM) showed a strong and positive linear correlation with protein digest Peps (PDP); therefore, the higher protein average mass, the greater the digestion of peptides. Protein matched products (PMP) had a robust and positive linear correlation with protein matched peptide intensum (PMPIS) and strong correlations with protein matched peptides (PMPE) and matched product intensum (PMOIS); the increase in PMPIS influences the increase of these three parameters.

Protein-matched peptide intensum (PMPIS) showed a strong linear correlation with matched product intensum (PMOIS) and a strong correlation with PTMPIS (protein.top3 matched peptide intenSum). PTMPIS had a strong linear correlation with PMOIS.

3.4 Multivariable trends

In the dendrogram treatment, three large groups were formed according to similarity found between the proteins presented (Figure 11).

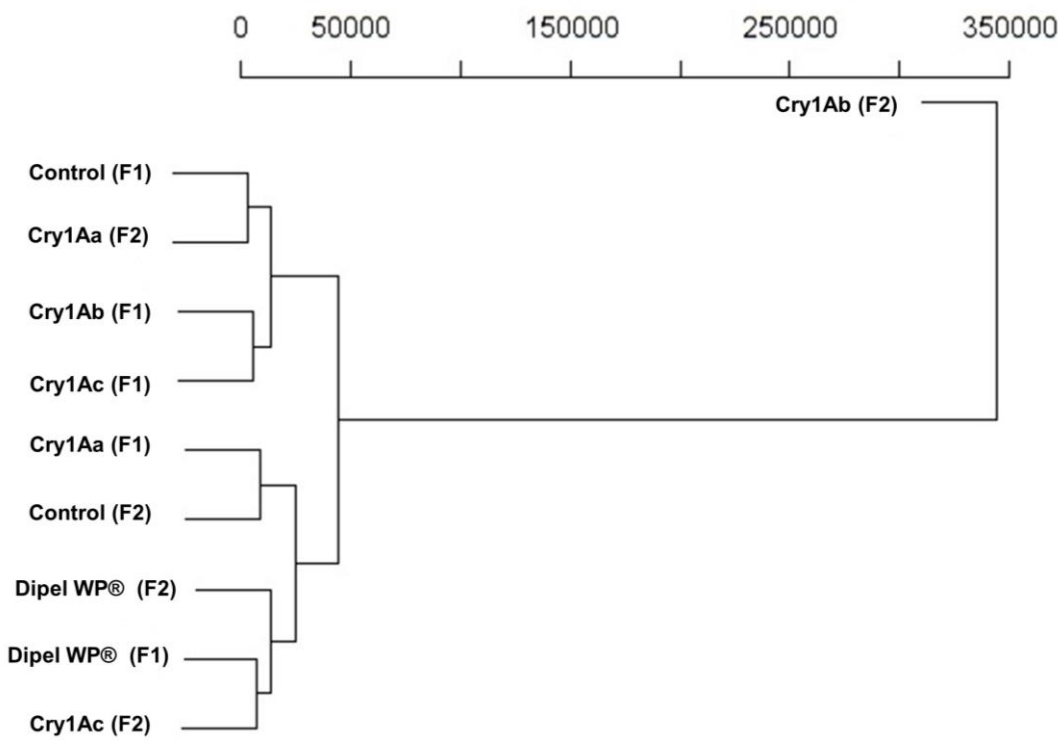

Figure 11. UPGMA dendrogram obtained from cluster analysis of 3323 samples of *Spodoptera frugiperda* egg-proteins using the average euclidean distance measure.

The Cry1Ab F2 treatment did not similar to the other treatments, forming an isolated group. The composition of the eggs submitted to Dipel WP® treatmentwas similar between the two generations. Both were similar to the composition of the Cry1Ac (F2) treatment. In this same group, a subgroup was formed between the control (F2) and Cry1Aa (F1) treatments. The third group was formed by the F1 Cry1Ac and Cry1Ab treatments, which were similar to each other, being similar to the subgroup formed by Cry1Aa (F2) and untreated (F1).

In the principal component analysis (PCA), 88.7% of the total proteomic analysis is explained, showing assertive results (≥ 80%) (Figure 12).

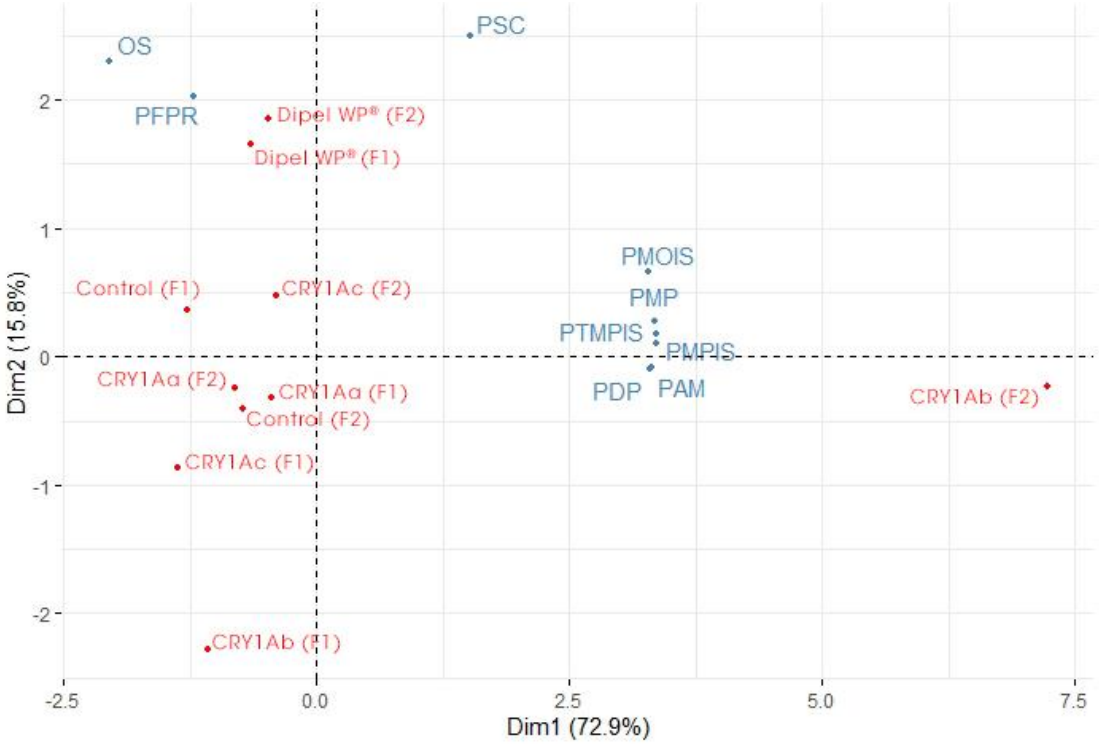

Figure 12. Principal component analysis biplot. Treatments and generations are red and proteomic variables are shown in blue. OS= protein score; PFPR= protein false positive rate; PAM= protein average mass; PMP= protein matched products; PDP= protein digest peptides; PSC= protein seq cover; PMPIS= protein matched peptide intensum; PTMPIS= protein top3 matched peptide intensum; PMOIS=matched product intensum.

In the first quadrant at PCA, there was no treatment; therefore, there are no treatments that had a specific affinity with protein seq cover (PSC), matched product intensum (PMOIS), protein matched products (PMP), protein top3 matched peptide intensum (PTMPIS), protein matched peptide intensum (PMPIS).

In the second quadrant, the treatments Dipel WP® (F1 and F2), Control (F1) and Cry1Ac (F2) occurred. These treatments showed a great affinity with the variables protein score (OS) and protein false positive rate (PFPR). For Dipel WP® regardless of generation, the trend is similar for these proteomic variables.

In the third quadrant, the Cry1Aa treatment of the two generations showed similar composition with each other and with the Control (F2), Cry1Ac (F1) and Cry1Ab (F1) treatments. Therefore, all of these treatments have similar trends. However, they did not show a specific affinity for the proteomic variables.

The Cry1Ab (F2) treatment composition remained different from the other treatments occurring isolated in the fourth quadrant. The variables that had an influence on Cry1Ab (F2) were protein average mass (PAM) and protein digest peptide (DP)

In the heat map, the proteomic variables that most showed variability between treatments were protein score (OS), protein seq cover (PSC), generation (GG), treatment (TRA) and protein false positive rate (PFPR). The other proteomic parameters had low variation between treatments and generations (Figure 13).

Dipel WP® (F1 and F2) was the treatment that showed the greatest variability among the parameters evaluated, varying among the treatments in the parameters OS, PSC, GG, TRA and PFPR. The treatments Cry1Ab (F1) and Cry1Aa (F1) had less variation between the proteomic parameters. Cry1Ab (F2) did not provide enough information to indicate the variability in the parameters PTMPIS, PMOIS, PAM, PMP, PDP, PMPE and PMPIS.

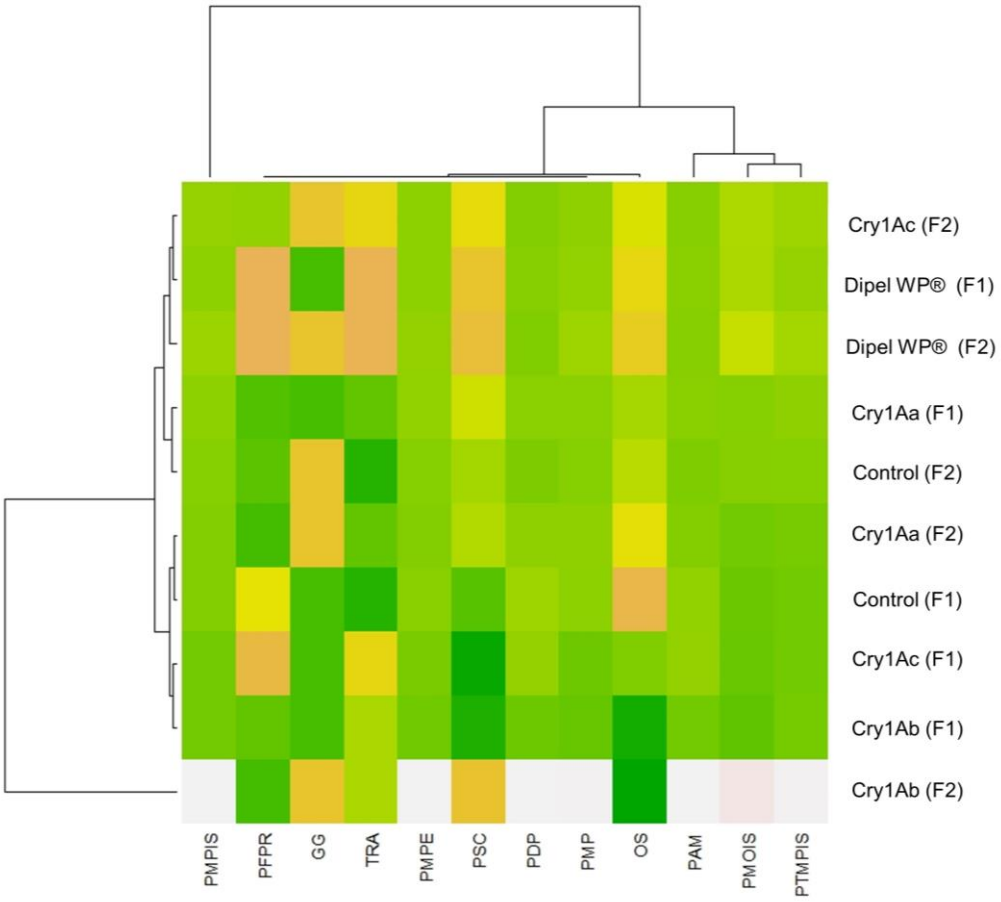

Figure 13: Heat map among treatments, generations and proteomic variables. GG= generation; TRA= treatment; OS= protein score; PFPR= protein false Positive Rate; PAM= protein avg Mass; PMP= protein Matched Products; PMPE= protein matched Peptides; PDP= protein digest Peps; PSC= protein seq cover; PMPIS= protein Matched Peptide IntenSum; PTMPIS= protein.top3 Matched Peptide IntenSum; PMOIS=.Matched Product Inten Sum.

3.5 Venn diagram

In F1, a total of 952 non-redundant proteins were identified (Table 1). In F1 Dipel WP®, the treatment presented the largest amount of exclusive proteins (74) and shared with Cry1Ac (64). Cry1Ac presented 32 exclusive proteins. Cry1Aa and control showed a number of similar exclusive proteins, 3, including 6 shared proteins. Cry1Ab presented four exclusive proteins.

Among the treatments Cry1Aa, Cry1Ab, Cry1Ac and Dipel WP® 22 proteins were shared. Cry1Aa, Cry1Ac Dipel WP® and untreated shared 15 proteins (Figure 14).

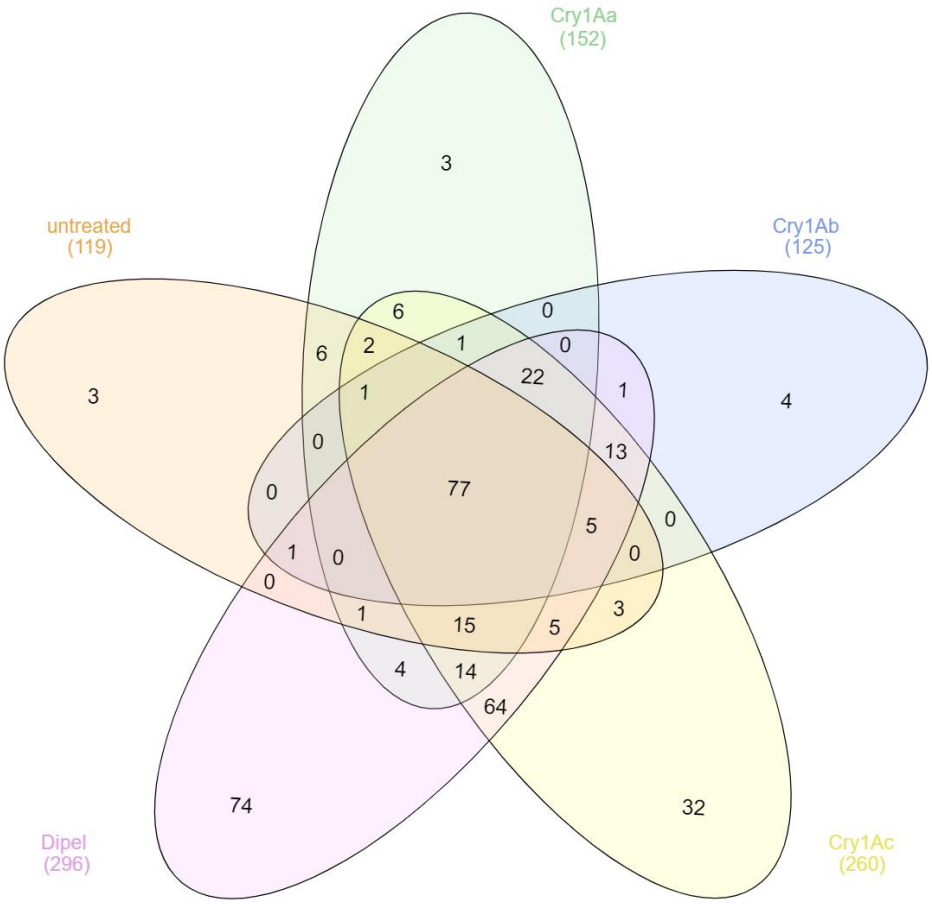

Figure 14: Venn Diagram with non-redundant protein data found in eggs of *S. frufiperda* (F1).

In F2, a total of 989 non-redundant proteins were identified (Table 1). Like F1, in F2, Dipel WP® was the treatment that showed the greatest number of unique proteins (210) and a larger number shared with Cry1Ac protein (126). Cry1Ac presented 19 proteins, the untreated five and Cry1Aa 1, exclusive proteins. Only one protein was shared among all treatments. Among the

untreateds, Cry1Aa, Cry1Ac and Dipel WP®, 81 proteins were shared (Figure 15).

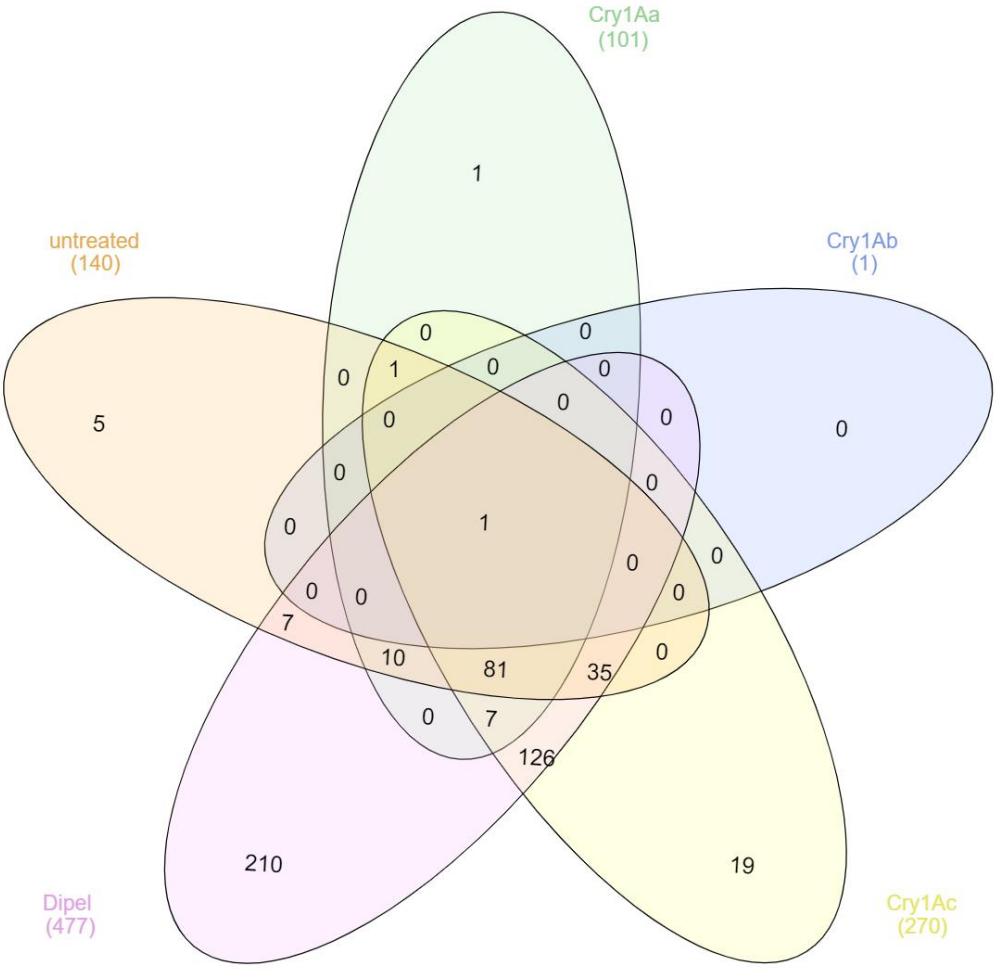

Figure 15: Venn Diagram with non-redundant protein data found in eggs of *S. frugiperda* (F2).

3.6 Group of proteins

In order to compare between treatments and generations, a classification of proteins was carried out in groups. The group of proteins that showed the highest probability were proteins with molecular function (Figure 16 until 20). The proteins that act in the biological processes (stress response) were those that occurred less in all treatments, except to Dipel WP® (F1) did not present this group of protein (Figure 16).

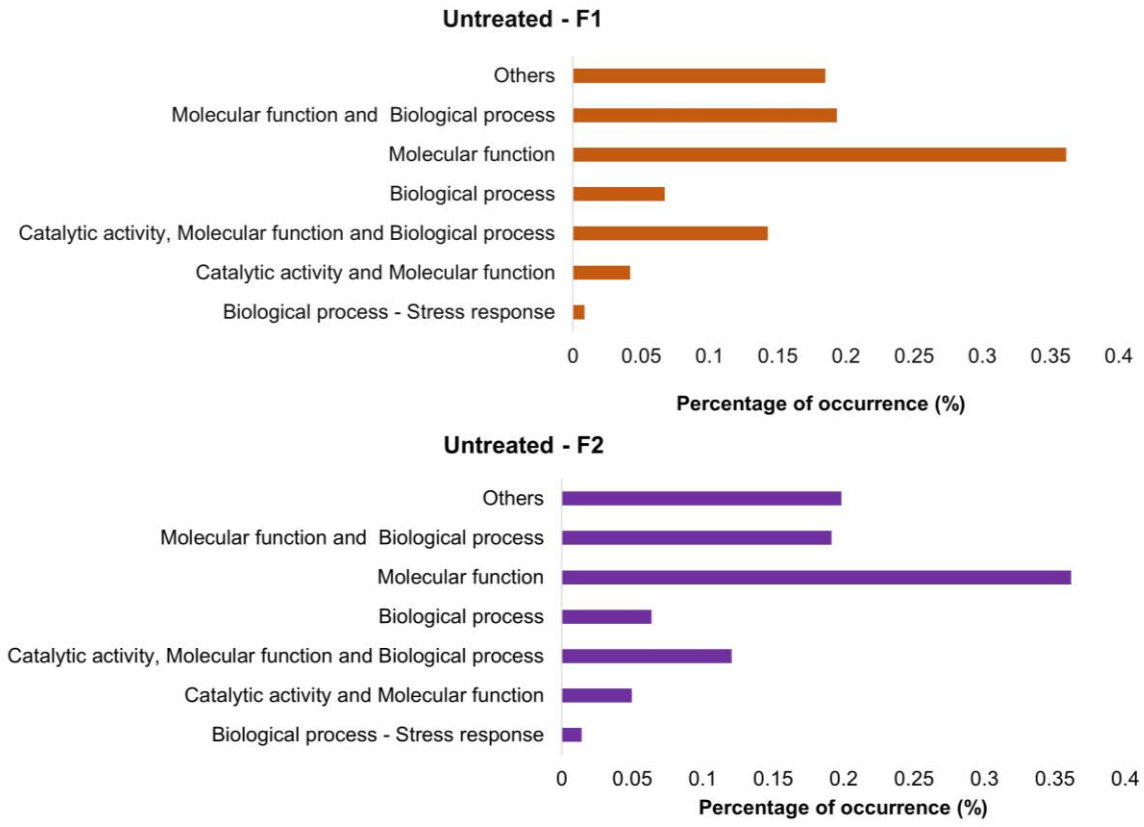

Figure 16. Group of proteins abundant in *S. frugiperda* eggs not exposed to Bt treatments for two generations

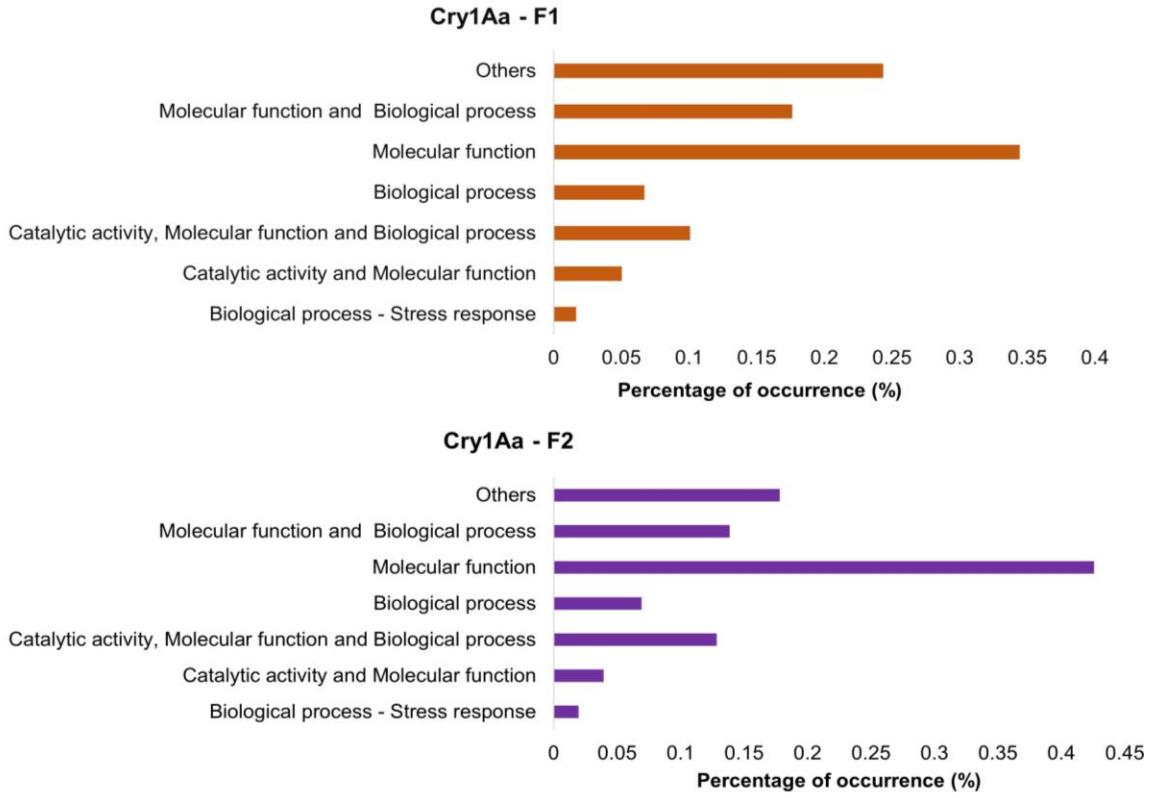

Figure 17. Group of proteins abundant in *S. frugiperda* eggs exposed to Cry1Aa treatment for two generations

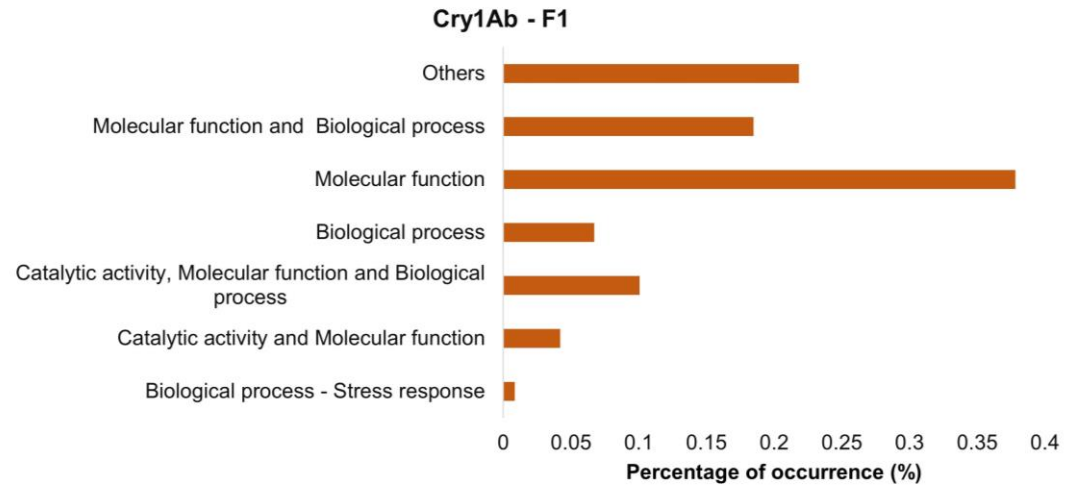

Figure 18. Group of proteins abundant in *S. frugiperda* eggs exposed to Cry1Ab treatment for first generation.

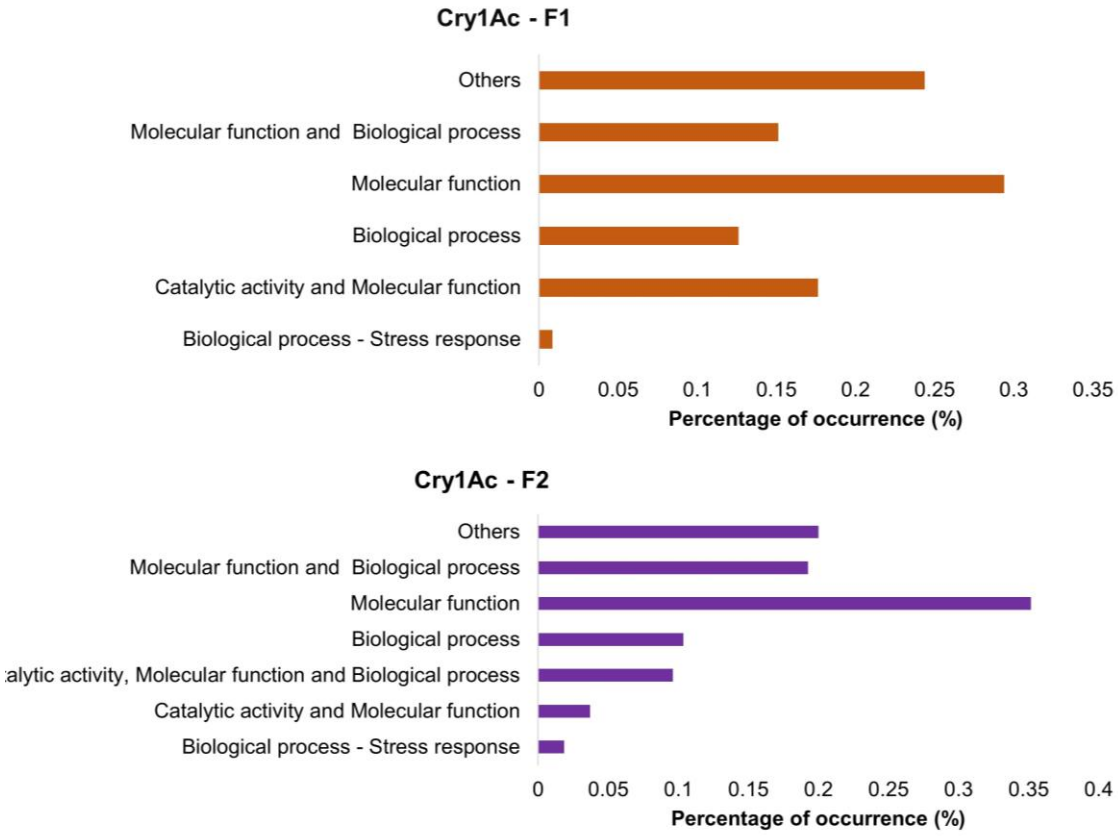

Figure 19. Group of proteins abundant in *S. frugiperda* eggs exposed to Cry1Ac treatment for two generations.

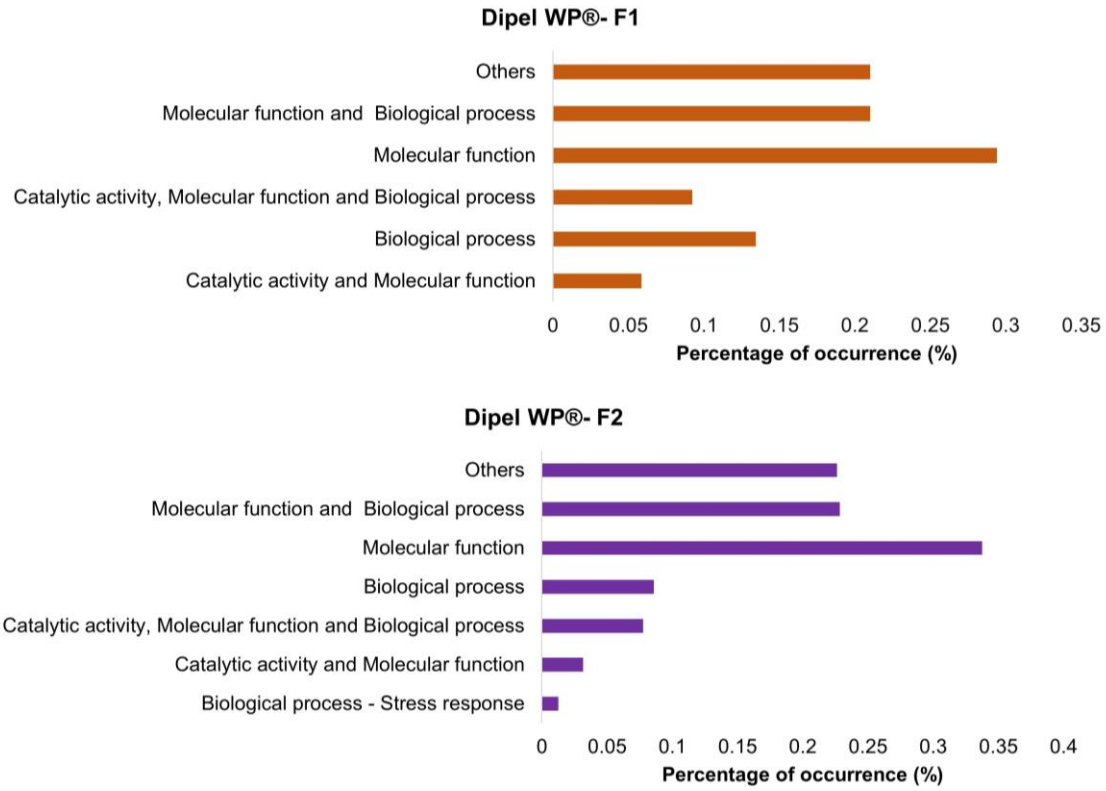

Figure 20. Group of proteins abundant in *S. frugiperda* eggs exposed Dipel WP® treatment for two generations.

4 DISCUSSION

The Cry proteins used in the treatments were not identified in eggs in this work. This is probably due to the low abundance of Cry proteins in eggs, sincethe equipment used to carry out proteomic analysis (Synapt, Waters, Manchester, UK) has a limitation in detecting less abundant proteins. Another factor that possibly interfered in detecting Cry proteins may have been the condition of the eggs in the F2 generation. The eggs used for the analysis presented a drier condition and low viability. As an example, the structural constituent of egg chorion (*SFRICE_024270*) in F1 was found in all treatments. However, in F2 it was found only in the untreated, Dipel WP®, and Cry1Ac.

The proteomics parameters evaluated for the Cry1Ab (F2) treatment and all crosses between treatments with this Cry protein were practically null

because only one protein was found in this treatment and generation (*SFRICE_000878*). A protein related to biological processes.

Most of the genes found in the treatments in F1 and F2 refer to proteins of molecular function, which are involved in ATP, GTP and elongation factor activity. The genes involved with molecular function and biological processes are related to unfolded protein binding, protein folding, calcium ion binding or calcium-mediated signaling. Kun et al., 2017 categorized the gene ontology and proteins into of *Locusta migratoria* eggs at different embryonic stages comparison for diapause and nondiapause regimes in three categories: biological processes, cellular components, and molecular functions. Similar that occurred in all treatments in this work. Fan et al., (2013) evaluated through liquid chromatography-tandem mass spectrometry (LC-Ms/Ms) on the diapause and non-diapause eggs of domesticated *Bombyx mori* and found among proteins eggs-specific protein.

Among the molecular function genes found in *S. fugiperda* eggs are heat shock proteins (Hsps). Rapid increase collectively in these conserved polypeptides helpss insects respond to elevated temperature and various chemical and physical stresses (Zhao and Jones, 2012). Several studies have been evaluated the effects of biotic stress can cause in Hsps (Sonoda et al., 2006, Hu et al., 2018; Yang et al. 2021). Biotic stress mainly refers to the stress that occurs due to damage to plants and animals by other living organisms such as bacteria, viruses, fungi, parasites, beneficial and harmful insects (Zhao and Jones, 2012).

At this moment, there are no studies in the literature that verify the effect of shock proteins on lepidopteran eggs that have undergone treatment with *Bacillus thuringiensis* . However, Rungrassamee et al. 2010, verified the expression levels of Hsps70 and Hsps90 in *Penaeus monodon* after exposure to a bacterium *Vibrio harveyi*. The hsps were significantly increased after a 3-h exposure to the *V. harveyi* bacterium. This evidence suggests putative roles and involvement of the hsp genes as a part of immune response against *V. harveyi* in *P. monodon.*

Yang et al. (2021) related the response mechanisms of *S. frugiperda* to various environmental stressors. The authors found five small heat shock proteins sHsps (SfsHsp21.3, SfsHsp20, SfsHsp20.1, SfsHsp19.3, and

SfsHsp29). The expression levels of all five SfsHsp genes differed among the developmental stages and the different tissues of male and female adults. The different SfsHsp genes of *S. frugiperda* play unique regulatory roles during development and response to various environmental stressors. The authors related that sHsps could help *S. frugiperda* to adapt to diferent environmental.

Among the proteins shared in the Dipel WP® and Cry1Ac treatments in F2, ten heat shock proteins (Hsps, Hsps 70A1, Hsps 70, 10 kDa heat shock protein, mitochondrial, Hsps 70 kDa cognate 4, Hsps 68-like transcript variant 1, Hsps 70 kDa cognate 5, sHsps 20.4, sHsps beta-1, Hsps 27.2) were found with molecular function or biological process – stress response. Three of them were hsp70, as well as used in the work of Rungrassamee et al. 2010 described above.

It is not possible to state that the Hsps shared among these treatments occurred due to the presence of Bt in the F1 diet. Because the hsps react in different ways according to the organism, it may be that some factor related to temperature stress or UV also triggered the action of these proteins. Zhou et al. (2020) investigate the effect of 3rd-instar nymphs on exposure to thiamethoxam, buprofezin, and avermectin at LC_{10} and LC_{25} concentrations and they concluded that this Hsp are not involved in response to insecticides, but *hsp70* genes significantly contribute to the tolerance of *S. furcifera* to temperature and UV-A stress.

5 CONCLUSION

The Cry proteins used in the treatments were not identified in eggs of a susceptible *Spodoptera frugiperda* population in this work.

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