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Insecticidal Effect of Cry Toxins Produced by *Bacillus thuringiensis* on *Diceraeus melacanthus* (Dallas, 1851) and *Euschistus heros* (Fabricius, 1798) (Hemiptera: Pentatomidae)

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Abstract

Some species of the Heteroptera (Hemiptera) suborder are of great agricultural importance, mainly as pests, and their control is often necessary. The use of *Bacillus thuringiensis*, an entomopathogenic bacterium normally extracted from the soil and used in biological control, is an alternative to the chemical control of these insects. Mortality tests must be carried out in order to select and determine a viable toxic strain, but currently there is no validated methodology for conducting those tests. In this context, this research aimed to develop and improve a selective bioassay methodology to assess the toxic effect of *B. thuringiensis* Cry toxins on *Diceraeus melacanthus* (green-belly stink bug) and *Euschistus heros* (neotropical brown stink bug) nymphs. A bioassay methodology consisting of tubes and artificial diet was proposed. Bioassays with *D. melacanthus* and *E. heros* nymphs were performed incorporating Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1F, Cry1G, Cry1Ia, Cry2Ab, Cry2A, Cry2Ae, Cry4A, Cry4B, Cry10, and Cry11Aa) into their liquid diet. The artificial feeding system developed in order to carry out the stink bug mortality tests was conducted. Among the toxins tested, we can highlight 2 causing 80-85% nymphal mortality on *D. melacanthus*, and 4 toxins causing 90-100% nymphal mortality for *E. heros* after 7 days of incubation. Both species are susceptible to different Cry toxins, with emphasis on Cry2Ab and Cry4B for *D. melacanthus* and Cry1B, Cry1G, Cry1Ia and Cry2Ab for *E. heros*.

Keywords: biological control; bioassay; Cry toxins; green-belly stink bug; neotropical brown stink bug

1. Introduction

Species in the Heteroptera suborder (Insecta: Hemiptera) are commonly known as true or typical bugs. Most species are phytophagous, but there are predators and hematophagous species as well. They are distributed in practically all regions of the world, and have great agricultural importance as pests of many different crops (Possebom et al., 2020). The main difference between Heteroptera and other insects of the Hemiptera order is the presence of scent glands located in the abdomen as nymphs, and in the abdomen and thorax as adults, with different functions depending on the species (Panizzi et al., 2000; Possebom et al., 2020). Within the Heteroptera suborder, Pentatomidae is the most abundant family of insects found in many crop areas in Brazil, causing significant losses and damage (Panizzi et al., 2012; Sosa-Gómez et al., 2020; Steinhaus et al., 2022).

Euschistus heros (Fabricius, 1798), the neotropical brown stink bug of the Pentatomidae family that feeds mainly on soybean pods (*Glycine max* L., Fabaceae), causing loss of production and reduction in crop quality, when not properly controlled (Panizzi et al., 2012; Sosa-Gómez et al., 2020; Engel et al., 2021). This pentatomid has been reported to feed on other legumes such as peanuts (*Pterogyne nitens* Tul.), Solanaceae, Brassicae, Compositae species (Vivan & Degrande, 2011; Panizzi et al., 2012; Engel et al., 2021), and can be an issue in cotton plants (Soria et al., 2009; Sosa-Gómez et al., 2020). Another pentatomid of agricultural importance is *Diceraeus melacanthus* (Dallas, 1851), the green-belly stink bug, which preferentially feeds on maize (*Zea mays* L., Poaceae) and wheat (*Triticum aestivum* L., Poaceae), particularly in areas where soybean is followed by maize as a second crop (Chocorosqui & Panizzi, 2008; Corrêa-Ferreira et al., 2010; Panizzi et al., 2016; Sosa-Gómez et al., 2020). The occurrence of these stink bugs has been favored by the adoption of cultural practices such as a no-tillage system and intensive field production throughout the year which provides a food supply and causes outbreaks of both species of stink bugs (Cordeiro & Bueno, 2021; Engel et al., 2021; Smaniotto & Panizzi, 2015).

The primary control tactic against these stink bugs almost exclusively involves the use of synthetic insecticides (Bueno et al., 2013; Panizzi, 2013; Gomes et al., 2020; Somavilla et al., 2020; Steinhaus et al., 2022) of broad-spectrum action (pyrethroids, neocotinoids, and organophosphate) (Sosa-Gómez et al., 2020; MAPA, 2021). These compounds are applied indiscriminately and negatively impact agroecosystems, such as reduction in biodiversity, selection of resistant stink bug populations, and emergence of new pest outbreaks (Lundgren et al., 2009; Gomes et al., 2020).

Bacillus thuringiensis is a Gram-positive aerobic spore-forming bacterium of the Bacillaceae family, of cosmopolitan coverage (Krywunczyk & Fast, 1980; Bravo et al., 2011), and can be found in several substrates such as soil, water, plant surfaces, dead insects, spider webs, and stored grains (Bravo et al., 1998; Valicente, 2019). The entomopathogenic activity of this bacterium is due to proteinaceous inclusions produced during the sporulation phase, which are crystals composed of proteins called endoproteins or crystal proteins (Monnerat & Bravo, 2000; Bravo et al., 2017; Chen et al., 2021). Also known as Cry toxins or Cry proteins, delta-endotoxins participate in the formation of protein crystals, linked to bacterial sporulation, formed from the phase following sporulation, and released when cells are lysed (Bravo et al., 2013). These proteins are the most used for insect biocontrol, they have an action spectrum usually restricted to a specific order of insects (Palma et al., 2014) and have an extremely toxic action (Bravo et al., 2013; Adang et al., 2014; Jurat-Fuentes & Crickmore, 2017).

The use of *B. thuringiensis* in insect pest biocontrol has many advantages such as specificity to target insects, a non-polluting effect on the environment, innocuousness to mammals and vertebrates, and non-toxicity to plants, which allows its direct application (Whiteley & Schnepf, 1986; Schünemann et al., 2014; Jurat-Fuentes & Crickmore, 2017). *Bacillus thuringiensis* has merited researchers' attention for controlling insect larvae/worms/nymphs of the Lepidoptera, Diptera, Coleoptera, Orthoptera, and Hemiptera orders, in addition to other organisms such as mites, nematodes, and protozoa (Palma et al., 2014).

Few studies have been carried out to evaluate the toxicity of Cry toxins with regard to insect mortality in the Heteroptera suborder. Therefore, this research aimed to develop and improve on a selective bioassay methodology to assess the toxic effect of individual Cry toxins on *D. melacanthus* and *E. heros* nymphs, under laboratory conditions.

2. Method

2.1 Insects Used in the Bioassays

This study was performed using *D. melacanthus* and *E. heros* second-instar nymphs obtained at the Insect Rearing Platform of Embrapa Genetic Resources and Biotechnology, Brasilia, Brazil. The insect-rearing conditions were as described by Blassioli-Moraes et al. (2014).

2.2 Artificial Feeding System Initial Setting

The artificial feeding system consisted of a sterile centrifuge tube (50-mL Falcon tube) containing seven nymphs (Figures 1A and 1B) in triplicate. Each tube with nymphs was considered an experimental unit. The tubes were covered with Parafilm® (3 × 3 cm), sterilized under ultraviolet light (UV) and elongated to twice its size. About 100 µL of liquid aphid diet (Dadd & Mitter, 1966) were placed over Parafilm® (Figure 1C) and then 50 µL of the treatment (first aniline blue, then Cry toxins). Soon after, another Parafilm® of the same size was used to cover the droplet in order to produce a sachet containing the mixture (Figure 1D). The liquid diet was previously filtered through a Millipore® membrane (0.22 µm) with the aid of a sterile syringe and stored at -20 °C until the moment of use. The entire procedure took place in a laminar flow cabinet with UV-sterilized materials. The tubes with the feeding system and insects were placed on shelves with the feeding system facing upwards (Figures 1E and 1F)

and kept in a BOD incubator to heat at 26±2 °C, 60±10% relative humidity (RH), and 14:10 light/dark (L:D) photoperiod.

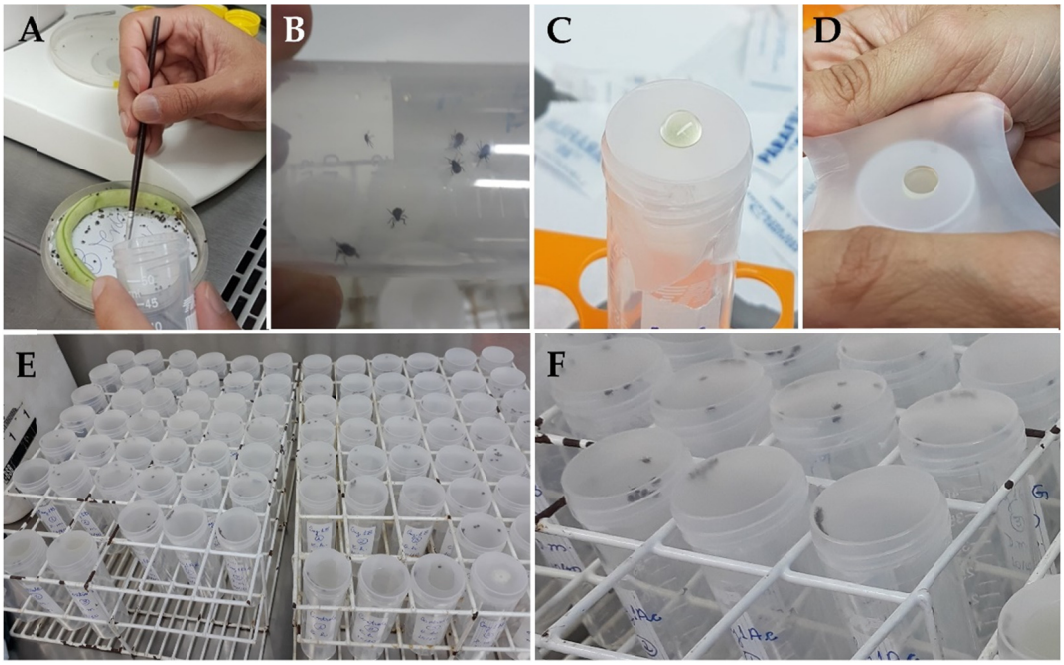

Figure 1. Artificial feeding system setting for *D. melacanthus* and *E. heros* selective trial using recombinant *B. thuringiensis* strains (expressing individual Cry toxins). Collection and verification of nymphs inside tubes (50-mL Falcon tube) (Figures 1A and 1B); liquid diet droplet containing the bacteria covered by Parafilm® paper at the top of the tube (Figures 1C and 1D); arrangement of tubes containing the feeding system and insects on shelves kept in a BOD incubator (Figures 1E and 1F)

2.3 Stink Bug Consumption Ability in an Artificial Feeding System

The entire feeding system was assembled by repeating the processes, in the presence of the blue dye, which signaled the treatment. The 100 µL of liquid diet were placed over Parafilm®, plus 50 µL of aniline blue, and then the feeding system was sealed. The control treatment contained 150 µL of liquid diet without the dye. Tubes were kept in a BOD incubator, then the nymphs' excreta left on the tubes' inner wall was observed to confirm that the diet had been consumed. The entire test took place in a laminar flow cabinet, and only sterile materials were used.

2.4 Cry Toxin Preparation and Purification

The proteins were purified from recombinant *B. thuringiensis* strains that express, individually, each of the following toxins: Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry 1C, Cry1F, Cry1G, Cry1Ia, Cry2Ab, Cry2A, Cry2Ae, Cry4A, Cry4B, Cry10, and Cry11Aa. For this, several constructors were cloned in pHT 315 or pSVP27A plasmids and transformed into acrystalliferous *B. thuringiensis* serovar. *israelensis*, strain 4Q7 (*Bacillus* Genetic Stock Center).

Recombinant strains were multiplied in liquid Embrapa medium (Monnerat et al., 2007) supplemented with chloramphenicol (10 µg mL^{-1}) or erythromycin (10 µg mL^{-1}), according to the expression vector, for 72 h at 30 °C and 200 rpm in a Labconco rotary incubator. After complete sporulation, the spore-crystal mixture was washed three times with PBS (150 mmol L^{-1} NaCl, 2.8 mmol L^{-1} NaH_2PO_4, 4 mmol L^{-1} $NaH_2PO_4 \cdot 7H_2O$, pH 7.2), containing 1 mmol L^{-1} EDTA, 0.1 mmol L^{-1} PMSF, 1 µg L^{-1} pepstatin, and 5 µg mL^{-1} leupeptin.

The crystals were purified by sucrose gradient (Chang et al., 1993), with modifications. The crystals were recovered with a solution of Triton X100 at 1% and 100 mmol L^{-1} PMSF and solubilized in an alkaline solution containing 50 mmol L^{-1} sodium carbonate buffer pH 10.5 with 0.2% (v/v) of β-mercaptoethanol for 1 h at 37 °C under constant agitation at 37 rpm (New Brunswick Innova 2100). The sample was centrifuged for 5 min at 12,000 xg and the soluble protein was quantified with Bradford reagent (Bio Rad) (Bradford, 1976) according to the manufacturer's guidelines.

Purified protein crystals were submitted to 24-h dialysis (twice) against 1 L with a 50 mM sodium phosphate buffer (NaH_2PO_4) containing 300 mM NaCl and pH 8.0. Purity and apparent molecular weight were determined by polyacrylamide gel electrophoresis under denaturant conditions. SDS-PAGE was performed on 10% (v/v) polyacrylamide gels according to stand protocol and using Bio-Rad Mini-Protean. Gels were stained with Coomassie brilliant blue R-250 and bleached with methanol: acetic acid: water (50:10:40 v/v/v). The protein concentration of each tissue was determined using a Bio-Rad protein assay kit and BSA as standard. An aliquot (1 mL) of the proteins was dispensed into an Eppendorf tube (1.5 mL) and stored at -20 °C until it was used in the trials.

2.5 Cry Toxin Bioassays

The *Diceraeus melacanthus* and *Euschistus heros* nymphs' bioassays were carried out by incorporating the Cry toxins (Cry1Aa, Cry1Ab, Cry1Ac, Cry1B, Cry1C, Cry1F, Cry1G, Cry1Ia, Cry2Ab, Cry2A, Cry2Ae, Cry4A, Cry4B, Cry10, and Cry11Aa) into their liquid diet, with a final volume of 150 µL. It was poured into Parafilm® sachets in the proportion of 0.2 µg μL^{-1} of protein. Seven insects were placed in each tube in triplicate for each treatment. The control treatment consisted of only liquid diet, without the toxin.

Tubes containing diet, toxin, and insects were kept in a BOD incubator for seven days. The assessments of the insecticidal capacity of Cry toxins on nymphs were performed, and the number of live insects was recorded every 24 h.

2.6 Statistical Analysis

To test the efficiency of the assessed bacteria, the modified Abbot formula (Abbot, 1925) was used:

$$Efficiency\ (\%) = (LIC - LIT/LIC) \times 100 \quad (1)$$

Where LIC is the number of live insects in the control and LIT is the number of live insects in the treatment. Treatments that achieved at least 80% effectiveness were considered effective (Nakano et al., 1981).

3. Results

3.1 Stink Bug Consumption Ability in an Artificial Feeding System

The insects' feeding ability on the diet provided in the artificial feeding system was verified with the excreta inside the Falcon tubes (Figure 2).

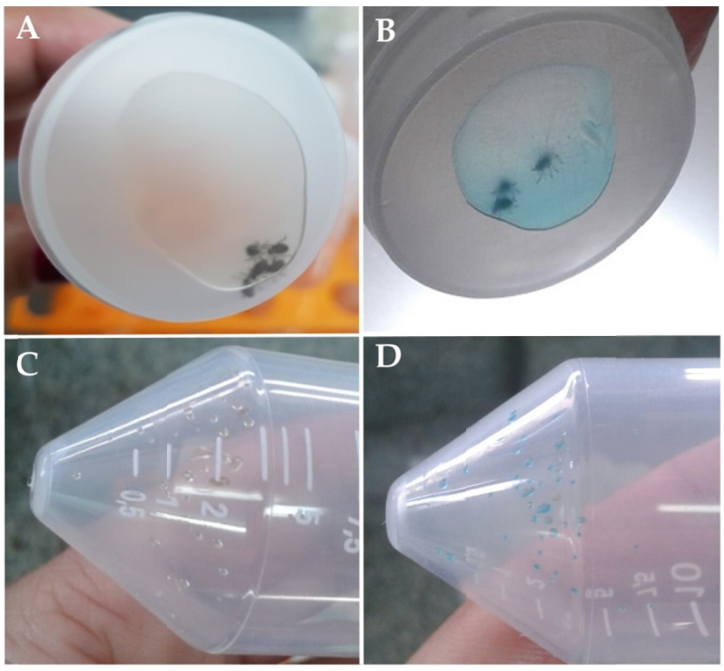

Figure 2. Stink bug nymphs feeding and excretion in the artificial feeding system. A: Top view of the Falcon tube containing only diet and stink bugs feeding on it; B: Stink bugs feeding on the diet-dye mixture; C: Nymph excreta on the tube's inner wall, with an only diet system; D: Excreta of nymphs on the inner wall of the tube, with the dye system in the diet

3.2 Cry Toxins Bioassays

Between the products expressing Cry toxins, Cry2Ab (80.9%) and Cry4B (85.7%) toxins provided > 80% mortality on *D. melacanthus* nymphs 7 days post treatments. The mortality rate for the control treatment was 23.8% (Table 1) (Figure 3). Cry4B passed the 80% threshold on day 6.

Table 1. Mortality (%) of *Diceraeus melacanthus* nymphs fed with Cry toxins for 7 days of incubation

Treatment	Time of exposure to Cry toxins						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	0	0	0	14.3	23.8	23.8	23.8
Cry1Aa	4.8	4.8	4.8	19	28.6	42.8	47.6
Cry1Ab	0	0	4.8	23.8	23.8	38.0	38.0
Cry1Ac	0	4.8	9.6	23.8	28.6	33.3	33.3
Cry1B	0	0	0	4.8	14.3	23.8	28.6
Cry1C	4.8	9.6	23.8	42.8	52.4	52.4	57.2
Cry1F	0	0	9.6	14.3	23.8	23.8	23.8
Cry1G	0	0	4.8	14.3	23.8	28.6	28.6
Cry1Ia	4.8	4.8	4.8	14.3	28.6	38.0	38.0
Cry2Ab	0	4.8	14.3	47.6	71.4	71.4	80.9*
Cry2A	4.8	4.8	14.3	33.4	47.6	71.4	76.2
Cry2Ae	0	4.8	19.0	33.4	57.2	71.4	76.2
Cry4A	0	4.8	9.6	28.6	47.6	42.8	42.8
Cry4B	4.8	23.8	33.4	47.6	57.2	81.0	85.7*
Cry10	0	9.6	23.8	47.6	57.2	61.9	71.4
Cry11Aa	0	14.3	19.0	33.4	52.4	61.9	66.7

Note. *Nymph mortality > 80%.

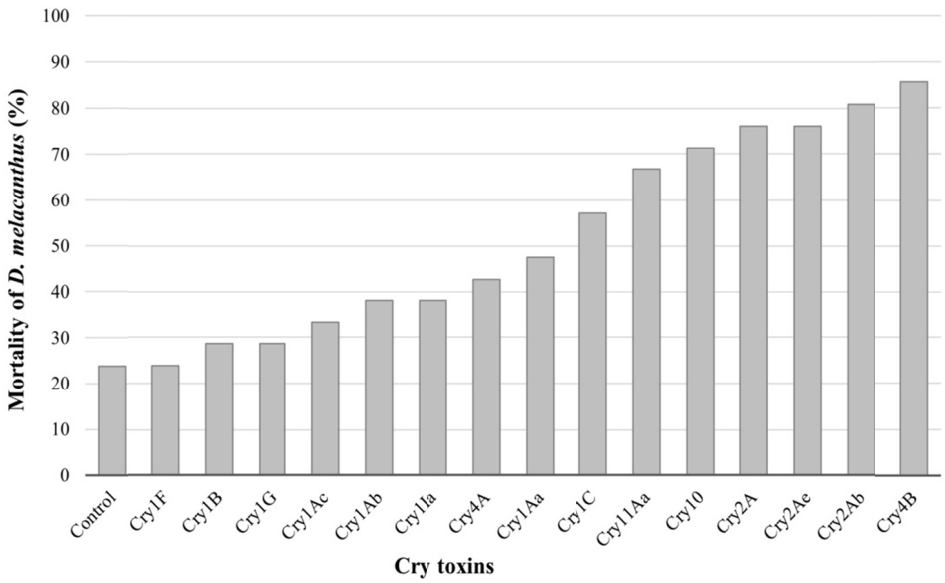

Figure 3. Mortality percentage for *Diceraeus melacanthus* seven days after exposure to Cry toxins from *Bacillus thuringiensis*

The treatments containing Cry toxins Cry2Ab (75.0%) and Cry4B (81.2%) had > 75% efficiency on *D. melacanthus* nymphs 7 days after the trial started. The Cry1F toxin was not efficient. This data was compared to the control treatment (Table 2).

Table 2. Efficiency (%) of Cry toxin treatments on *Diceraeus melacanthus* nymphs for 7 days of incubation

Treatment	Time of exposure to Cry toxins						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control							
Cry1Aa	4.7	4.7	4.7	5.5	6.2	25.0	31.2
Cry1Ab	0	0	4.7	11.1	0	18.7	18.7
Cry1Ac	0	4.7	9.5	11.1	6.2	12.5	12.5
Cry1B	0	0	0	-	-	0	6.2
Cry1C	4.7	9.5	23.8	33.3	37.5	37.5	43.7
Cry1F	0	0	9.5	0	0	0	0
Cry1G	0	0	4.7	0	0	6.2	6.2
Cry1Ia	4.7	4.7	4.7	0	6.2	18.7	18.7
Cry2Ab	0	4.7	14.3	38.9	62.5	62.5	75.0*
Cry2A	4.7	4.7	14.3	22.2	31.2	62.5	68.7
Cry2Ae	0	4.7	19.4	22.2	43.7	62.5	68.7
Cry4A	0	4.7	9.5	16.6	25.0	25.0	31.2
Cry4B	4.7	23.8	33.3	38.9	43.7	75.0	81.25*
Cry10	0	9.5	23.8	38.9	43.7	50.0	62.5
Cry11Aa	0	14.3	19.0	22.2	37.5	50.0	56.2

Note. (-) data not available, the number of live nymphs in the Cry treatment was higher than in the control treatment. * Cry toxins efficiency on nymphs > 75%.

Table 3 shows the trial's findings regarding the average number of dead insects, absolute mortality, and Cry toxins' efficiency on *D. melacanthus* nymphs at the end of 7 days of incubation. For the average number of dead insects, the Cry4B toxin had a 6.0 average compared to 1.6 in the control, with a total of 18 and 5 dead nymphs, respectively, and a sample size of 21 nymphs (n = 21) for each treatment. The Cry1F toxin was not efficient on nymphs. Lowest efficiency indices were observed for Cry1B and Cry1G toxins (6.2%).

Table 3. Mortality and Cry toxins' efficiency on *Diceraeus melacanthus* nymphs after 7 days of incubation

Treatment	Nymph mortality[a] (dead insect average)	Absolut mortality[b] (total dead insect number) (n = 21)	Efficiency (%)	Standard Error
Control	1.6	5	0	0.577
Cry1Aa	3.3	10	31.2	0.577
Cry1Ab	2.6	8	18.7	1.154
Cry1Ac	2.3	7	12.5	0.577
Cry1B	2.0	6	6.2	1.000
Cry1C	3.6	12	43.7	1.000
Cry1F	1.6	5	0	1.154
Cry1G	2.0	6	6.2	1.000
Cry1Ia	2.6	8	18.7	1.154
Cry2Ab	5.6	17	75.0*	2.309
Cry2A	5.3	16	68.7	2.081
Cry2Ae	5.3	16	68.7	1.527
Cry4A	3.3	10	31.2	1.154
Cry4B	6.0	18	81.2*	1.732
Cry10	5.0	15	62.5	2.000
Cry11Aa	4.6	14	56.2	0.577

Note.[a] average number of dead *D. melacanthus* nymphs from three replicates with 7 nymphs each. [b] total number of dead insects in treatments with 21 nymphs for each tested toxin. * treatment efficiency > 75%.

Treatments containing toxins Cry 1Ia and Cry 2Ab caused 100% mortality in *E. heros* nymphs at the end of 7 days of incubation (Figure 4). The Cry11Aa and Cry1C toxins caused the lowest mortality rates (33.3%). Nymph mortality was above 90% for the Cry1B, Cry1G, Cry1Ia, and Cry2Ab toxins at the end of the trial. Cry2Ab reached 90.4% mortality after 48 hours of exposure, and the 100% mortality level was reached on the 5th day of the trial for Cry2Ab and on the 6th day for Cry1Ia (Table 4).

Table 4. Mortality (%) of *Euschistus heros* nymphs fed with Cry toxin treatments for 7 days of incubation

Treatment	Time of exposure to Cry toxins						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	4.7	19.0	23.8	28.5	33.3	33.3	38.1
Cry1Aa	0	9.5	9.5	19.0	19.0	28.5	37.1
Cry1Ab	0	19.0	47.6	52.4	52.4	52.4	61.9
Cry1Ac	4.7	19.0	38.1	43.0	47.6	52.4	61.9
Cry1B	4.7	28.5	57.1	66.6	80.9	90.4	90.4*
Cry1C	4.7	19.0	23.8	23.8	33.3	33.3	33.3
Cry1F	33.3	57.1	57.1	57.1	57.1	57.1	57.1
Cry1G	33.3	66.6	76.2	76.2	76.2	85.7	95.2*
Cry1Ia	33.3	47.6	47.6	23.8	62.0	100.0	100.0*
Cry2Ab	28.5	90.4	95.2	95.2	100.0	100.0	100.0*
Cry2A	0	38.1	38.1	43.0	43.0	47.6	52.3
Cry2Ae	23.8	33.3	38.1	52.4	71.4	76.2	76.2
Cry4A	19.0	47.6	52.4	52.4	52.4	38.1	38.1
Cry4B	9.5	61.9	66.6	66.6	66.6	71.4	76.2
Cry10	0	9.5	9.5	19.0	38.1	38.1	38.1
Cry11Aa	4.7	19.0	23.8	28.5	28.5	33.3	33.3

Note. * *E. heros* nymph mortality > 90%.

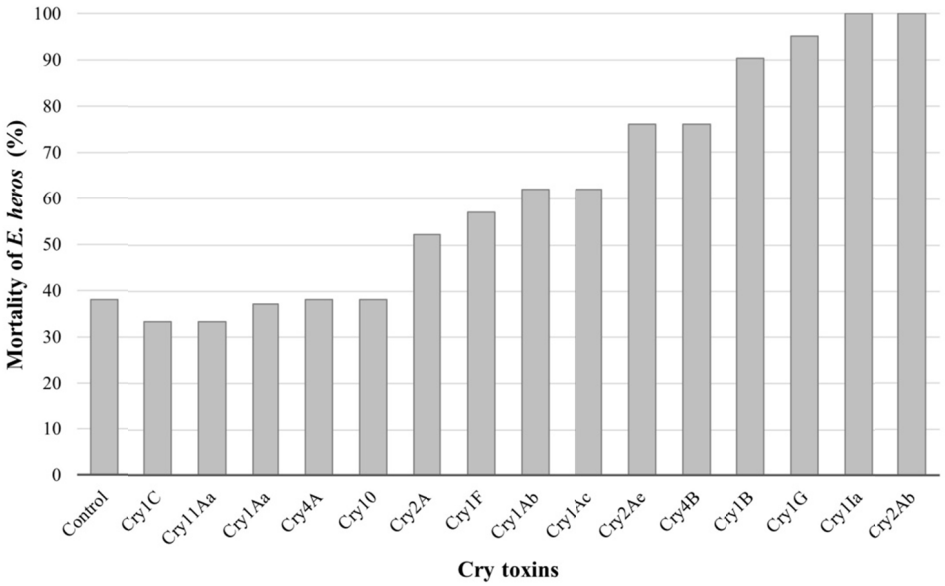

Figure 4. Mortality percentage for *Euschistus heros* seven days after exposure to Cry toxins from *B. thuringiensis*

At the end of 7 days, the control treatment (containing only the diet) had 38% *E. heros* nymph mortality. Among the tested Cry toxins, Cry1B, Cry1G, Cry1Ia, and Cry2Ab had > 80% efficiency on nymphs after 7 days incubation (Table 5). After 48 hours of exposure, the Cry2Ab toxin recorded mortality and efficiency on this insect.

Table 5. Efficiency (%) of Cry toxin treatments on *Euschistus heros* nymphs for 7 days of incubation

Treatment	Time of exposure to Cry toxins						
	1 day	2 days	3 days	4 days	5 days	6 days	7 days
Control	-	-	-	-	-	-	-
Cry1Aa	-	-	-	-	-	-	0
Cry1Ab	0	0	31.2	33.3	28.5	28.5	38.4
Cry1Ac	0	0	18.7	20.0	21.4	28.5	38.4
Cry1B	0	11.7	43.7	53.3	71.4	71.4	84.6*
Cry1C	0	0	0	-	0	0	-
Cry1F	30.0	47.0	43.7	40.0	35.7	35.7	30.7
Cry1G	30.0	58.8	68.7	66.6	64.3	78.5	92.3*
Cry1Ia	30.0	35.3	31.2	40.0	42.8	100.0*	100.0
Cry2Ab	25.0	88.2	93.7	93.3	100.0	100.0*	100.0
Cry2A	0	23.5	18.7	20.0	14.3	21.4	23.0
Cry2Ae	20.0	17.6	18.7	33.3	57.1	64.3	61.5
Cry4A	15.0	35.3	37.5	33.3	28.5	42.8	38.4
Cry4B	5.0	52.9	56.2	53.3	50.0	57.1	61.5
Cry10	-	-	-	-	7.1	7.1	0
Cry11Aa	0	0	0	0	-	0	-

Note. (-) data not available, the number of live nymphs in the Cry treatment was higher than in the control treatment. * Cry toxins' efficiency on nymphs observed > 80%.

For the average number of dead insects, the Cry1Ia and Cry2Ab toxins had an average of 7.0 dead nymphs compared to 2.6 in the control (Table 6). The absolute mortality of nymphs exposed to these same toxins was 21 compared to 8 in the control. Therefore, the Cry1Ia and Cry2Ab toxins' efficiency was 100% on *E. heros* nymphs. After seven days of incubation, the Cry1Aa and Cry10 toxins were not efficient, Cry1C and Cry11Aa toxin treatments had more live insects at the end of the trial than the control treatment.

Table 6. Mortality and Cry toxins' efficiency on *Euschistus heros* nymphs after 7 days of incubation

Treatment	Nymph mortality[a] (dead insect average)	Absolut mortality[b] (total dead insect number)	Efficiency (%)	Standard Error
Control	2.6	8	0	1.527
Cry1Aa	2.6	8	0	0.577
Cry1Ab	4.3	13	38.4	1.527
Cry1Ac	4.3	13	38.4	2.516
Cry1B	5.3	19	84.6	1.154
Cry1C	2.3	7	-	3.214
Cry1F	4.0	12	30.7	3.605
Cry1G	6.6	20	92.3	0.577
Cry1Ia	7.0	21	100.0	0
Cry2Ab	7.0	21	100.0	0
Cry2A	3.3	10	23.0	2.516
Cry2Ae	5.3	16	61.5	2.081
Cry4A	4.3	13	38.4	2.309
Cry4B	5.3	16	61.5	2.886
Cry10	2.6	20	0	3.785
Cry11Aa	2.3	17	-	0.577

Note.[a] average number of dead *E. heros* nymphs from three replicates with 7 nymphs each. [b] total number of dead insects in treatments with 21 nymphs for each tested toxin.

4. Discussion

The high damage potential of *Euschistus heros* and *Diceraeus melacanthus* had been previously reported in the literature (Torres et al., 2013; Gomes et al., 2020; Cordeiro & Bueno, 2021; da Silva et al., 2021). Among the options that seek to control insect pests without the use of chemical groups and with high specific toxicity, the use of microorganisms has taken an important position (Schünemann et al., 2014; Sosa-Gómez et al., 2020). *Bacillus thuringiensis* and their toxins are the most manipulated microbial pesticides worldwide and have been accepted by the public as a safe bioinsecticide (Bravo et al., 2011; Ruan et al., 2015).

While studies show that the hemipteran insects are susceptible to Cry protein toxicity (Porcar et al., 2009; Dorta et al., 2010; Melatti et al., 2010; Salazar-Magallon et al., 2015; Torres-Quintero et al., 2016; Schünemann et al., 2018; Torres Cabra et al., 2019; da Costa et al., 2021), brief is the knowledge about the effect of Cry toxins on pentatomids (Schünemann et al., 2014). Research using Cry proteins directly on insects in the Heteroptera suborder are scarce and focused on the effects that genetically-modified (GM) plants (plants expressing insecticidal crystalline proteins derived from *B. thuringiensis*) can also have on non-target organisms and in the third trophic level of the food chain (Wellman-Desbiens & Côté, 2005; da Cunha et al., 2012; Silva et al., 2014).

According to our results, the diet proved to be satisfactory and well accepted by the stink bugs at the tested artificial feeding system, conceivably, the adequate one for bioassays using Cry toxins. This is important to prove that insects were fed with the provided diet and, consequently, ingested bacterial proteins offered in each treatment. An artificial feeding system, like the one used in this research, was efficient for the sucking pest *Myzus persicae* (Sulzer, 1776) (Hemiptera: Aphididae) mortality testing (Paula et al., 2015). Some methodologies for stink bugs have already been established for chemical testing, mainly with 20-mL glass vials (Snodgrass, 1996; Snodgrass et al., 2005; Lopez Jr et al., 2012a, 2012b).

Treatments with Cry toxins against *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) nymphs in *Citrus sinensis* (L.) Osbeck systemically colonized seedlings (Dorta et al., 2010) showed different toxicities regarding the individual proteins. The highlights in that study were Cry4B, Cry10, Cry11, and Cyt1A, which caused around 65% mortality in *D. citri* nymphs. Interestingly, the results found in the current study for the Cry4B toxin showed a mortality rate above 85% in *D. melacanthus* nymphs, while the Cry10 toxin obtained the lowest rates (38.1%) on *E. heros*. The Cry2Ab protein demonstrated 100% mortality on *E. heros* assured within the first 48 hours (90.4% mortality). In our study, the Cry2Ab protein resulted in 100% mortality results when ingested by *E. heros*, and 80.9% on *D. melacanthus*. It could be seen as a possible interaction between this protein and these insects' intestine. In the interaction assessment of Cry toxins with the intestine of the hemipteran *Lygus hesperus* Knight (Heteroptera: Miridae), it was observed that there was no interaction with Cry1Ac, but Cry2Ab presented a strong extracellular interaction (Brandt et al., 2004).

The mechanism of action of different proteins has been identified as receptors for Cry toxins in the lepidopteran midgut (de Maagd et al., 2012; Bravo et al., 2017), but not for pentatomids, and, when reported, it is related to the third trophic level. Therefore, the insect gut pH, the presence of specific receptors on microvilli of midgut cells, as well as the proteolytic activation of Cry toxins ingested by insects are essential and necessary factors in the interactions for the occurrence of toxicity (Li et al., 2011; Bravo et al., 2013; Javed et al., 2019; Chen et al., 2021). Consequently, those factors may be associated with the differences in mortality obtained between both tested stink bug demonstrates by individual Cry toxins. However, there is little information regarding the digestive physiology of hemipterans associated with Cry toxins, and the literature is insufficient to elucidate about the mechanism of resistance or susceptibility of these insects (Schünemann et al., 2014).

Subsequently, spores and vegetative cells of *B. thuringiensis* were detected in the midgut of the predator *Podisus nigrispinus* (Dallas, 1861) (Hemiptera: Pentatomidae) fed on *Bombyx mori* (Lepidoptera: Bombycidae) treated with *B. thuringiensis kurstaki* (HD1) (Nascimento et al., 1998). Histological sections of the *P. nigrispinus* midgut shows the effects of proteins Cry1F, Cry1A.105, and Cry2Ab2, with histopathological changes, after predating *Spodoptera frugiperda* (J.E. Smith, 1797) (Lepidoptera: Noctuidae) fed on GM Bt-maize but not being lethal to 3rd trophic level (Souza et al., 2021). In additional, other studies showed that the Cry1Ac toxin caused ultrastructural changes in the digestive cells of the predatory stink bug *P. nigrispinus* when it fed on *S. frugiperda* that had consumed GM Bt-cotton expressing the toxin (da Cunha et al., 2012). Nevertheless, mortality or impacts were not observed for *E. heros* fed on GM Bt-soybean plants expressing the Cry1Ac protein (Silva et al., 2014; Schünemann et al., 2018). In the present study, the Cry1Ac protein caused low mortality (33.3%) in *D. melacanthus* and reasonable mortality (61.9%) for *E. heros* in a direct exposure to the toxin. The susceptibility to Cry toxins was evidenced for *E. heros* nymphs exposed in the absence of substrate, but only the combination of toxins (contained Cry2, Cry1, and Cry9 proteins) resulted in the highest mortality and efficiency (> 98%)

(Schünemann et al., 2018). *B. thuringiensis*' spectrum of action may depend on the combination of individual Cry toxins revealing synergisms between them (Estruch et al., 1997; Schünemann et al., 2018).

Euschistus heros and *D. melacanthus* are susceptible to Cry toxins correlating effect directly with the average mortality of these insects' pests. Individual toxins produced by *B. thuringiensis* have insecticidal activity and different degrees of lethality in both phytophagous-sucking insects of the Heteroptera suborder. Nonetheless, assays of protein interaction with the insect gut are suggested and can help with more information regarding the digestive physiology of hemipterans associated with Cry toxins, as well as the verification of interaction and possible synergism between the toxins against these pests. In this way, we can expand knowledge about the pathosystem so that toxins can be most efficiently used, with other strategies to control, against the stink bug pests.

5. Conclusions

The Cry toxin consumption system through artificial diet in Falcon tubes is efficient and can be used for phytophagous stink bug species. The two stink bug species evaluated in this study are susceptible to different Cry toxins, with emphasis on Cry2Ab and Cry4B for *D. melacanthus* and Cry1B, Cry1G, Cry1Ia, and Cry2Ab for *E. heros*.

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