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Artículos

Soybean protease inhibitors increase *Bacillus thuringiensis* subs. israelensis toxicity against *Hypothenemus hampei* [1]

Inhibidores de proteasas de soya incrementan la toxicidad de *Bacillus thuringiensis* subs. israelensis contra *Hypothenemus hampei*

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Abstract:

Introduction. The coffee berry borer (*Hypothenemus hampei* Ferrari, CBB) is one of the most devastating pests on coffee plantations around the world. Although CBB is susceptible to the effect of δ–endotoxins of *Bacillus thuringiensis* subs. israelensis (Bti) at laboratory level, the efficacy of this control method is poor in the field, presumably due to the inactivation by digestive proteases different to those required for protoxin activation. **Objective.** To study whether the addition of a soybean flour extract enriched with protease inhibitors (PI), mixed with Bti crystals and spores (Bti-sc) in an artificial diet, could improve the toxicity of Bti against CBB. **Materials and methods.** This study was performed in San José, Costa Rica, between 2012 and 2013. A set of adult female CBB insects was exposed to a mixture containing different concentrations of a partially purified soybean meal extract with active PI and lyophilized Bti-sc, and were tested through a bioassay in artificial diet to estimate the sub-lethal concentration (LC50). The mortality results were validated by observing the dissected midgut, whose ultrastructure was analyzed by transmission electron microscopy. **Results.** The soybean extracts partially degraded the Bti-sc complex, it reduced its LC50 by almost four times (from 1.135 to 0.315 $\mu g\ \mu l^{-1}$) and enhanced CBB mortality in a concentration-dependent manner. Histological analyses of the midgut confirmed this synergistic effect, since severe epithelial damage to the intestinal epithelium of CBB exposed to Bti-sc + PI was visualized compared to Bti-sc alone. **Conclusions.** The combination of a soybean extract enriched in PI and Bti-sc enhanced the mortality effect over CBB, which was confirmed by the midgut collapse. Soybean flour is a cost-effective supplement that could increase Bti effectiveness against CBB and delay the appearance of biological resistance.

Keywords: biological control, bacterial toxins, soybean meal, protease inhibitors, synergism.

Resumen:

Introducción. La broca del café (*Hypothenemus hampei*, CBB) es una de las plagas más devastadoras en plantaciones de café alrededor del mundo. Aunque CBB es susceptible al efecto de las δ-endotoxinas del Bacillus thuringiensis subs. israelensis (Bti) a nivel de laboratorio, la eficacia de este método de control es deficiente en campo, posiblemente debido a la inactivación ocasionada por proteasas digestivas diferentes a las requeridas para la activación de las protoxinas. **Objetivo.** Determinar si la incorporación de un extracto de harina de soya con inhibidores de proteasas (PI) mezclado con cristales y esporas de Bti (Bti-sc) en una dieta artifical, podría mejorar la toxicidad de Bti contra CBB. **Materiales y métodos.** Este estudio se realizó en San José, Costa Rica entre 2012 y 2013. Se expuso un conjunto de insectos hembras adultas de CBB a una mezcla que incluyó diferentes concentraciones del extracto de soya parcialmente purificado con PI activo y un liofilizado de Bti-sc, y fueron evaluadas mediante un bioensayo en dieta artificial para estimar la concentración subletal (CL50). Los resultados de mortalidad se validaron mediante observación del intestino medio diseccionado, cuya ultraestructura se analizó mediante microscopía electrónica de transmisión. **Resultados.** El extracto de soya degradó parcialmente el complejo Bti-sc, redujo la CL50 en casi cuatro veces (de 1,135 a 0,315 $\mu g\ \mu l^{-1}$) y potenció la mortalidad de CBB de manera concentración-dependiente. Los análisis histológicos del intestino medio confirmaron este efecto sinérgico, dado que se visualizaron daños severos en el epitelio intestinal de CBB expuestos a Bti-sc + PI comparado con Bti-sc solo. **Conclusiones.** La combinación del extracto de soya enriquecido con PI y Bti-sc potenció la letalidad sobre CBB, que se confirmó por el colapso intestinal. La harina de soya es un suplemento económico que podría aumentar la efectividad de Bti para controlar CBB y retrasar el desarrollo de resistencia biológica.

Palabras clave: control biológico, toxinas bacterianas, harina de soya, inhibidores de proteasas, sinergismo.

Introduction

Coffee (*Coffea* spp.) is one of the main crops of the developing world (Belayneh-Mulaw et al., 2010). It is produced in over 10 million ha in about 80 countries, and roughly 20 million families rely on coffee growing for subsistence (Vega et al., 2009). During 2010-2011, ca. 6,309.861 kg of fresh coffee with an estimated value of US $383 million were marketed in Costa Rica, accounting for almost 10 % of the profits obtained by the local agricultural sector (Rojas, 2012). Nonetheless, during 2018-2019, coffee production reached ca. 1,219,087 kg, the mayor exporters being the United States (48 %), Belgium (15.4 %), Germany (6.5 %) and South Korea (4 %) (González, 2019). In the Major Metropolitan Area of Costa Rica coffee crops cover 18,976 ha, which represents 44 % of its farming area (Wei-Salas and Durán-Quirós, 2015).

Different pests and diseases threaten this commodity, among which the coffee berry borer (*Hypothenemus hampei*, CBB) is currently considered the most devastating and recidivist pest on a global scale (Cárdenas, 2007). This monophagous coleopteran feeds exclusively on coffee endosperm and relies on seeds inside coffee berries as a unique habitat for development, breeding and refuge (Constantino et al., 2011), causing thereby abscission of coffee berries and reductions in seed weight (Cantor et al., 2001) that translate into yearly losses around US $215-358 million only in Brazil (Oliveira et al., 2013).

Based on the alarmingly high infestation levels of H. hampei observed in the last 15 years in Uganda (80 %), Jamaica (58-85 %), Tanzania (90 %), Malaysia (50-90 %), Mexico (60 %), Costa Rica (97 %) (Acuña-González and Betanco-Velázquez, 2007; Jiménez, 2009), and currently in Colombia (17-28 %) (Aristizábal et al., 2015), and Hawaii (10-16 %) (Aristizábal et al., 2017), interest in pesticide development is rising. Highly toxic and environmental persistent synthetic pesticides, including fenithion, fenitrothion, endosulfan, chlorpyrifos, and pirimiphos–methyl, have been misused and abused (Pardey, 2006), raising serious ecological and sanitary concerns. Therefore, current research aims to reduce pest populations through the combination of several strategies such as public education, chemical control, and biological control. This integrated pest management is necessary to identify the pest infestation threshold that requires an insecticide application (Suckling et al., 2014).

The δ-endotoxins produced by *Bacillus thuringiensis* subsp. *israelensis* (Bti) have been shown to kill CBB female adults within nearly 6 days when administered at $ng\ cm^{-1}$ levels in artificial diet (De-la-Rosa et al., 2005), and the mean lethal concentration (LC50) of purified Bti Cry proteins to first-instar larvae of CBB over a period of 6 days is only 0.219 $\mu g\ \mu l^{-1}$ (Méndez-López et al., 2003). Hence, a greener

sustainable alternative for the control of CBB could exploit optimized Bti endotoxins prepared in sprayable bioinsecticides.

Formulations of Cry proteins are biodegradable, safe to vertebrates, fast-acting, and highly specific to their target pests (Schünemann et al., 2014). Besides, their application and production through downstream or upstream processes are easy and affordable (Jouzani et al., 2017). However, measures should be taken to protect their toxic activity as they can be inactivated by endo-proteases, such as serine- and aspartic-proteases that abound in CBB guts (Valencia and Arboleda, 2005).

Protease inhibitors (PI) have been shown to enhance the activity of δ–endotoxins of *B. thuringiensis* subsp. kurstaki against the coleopteran *Leptinotarsa decemlineata* (MacIntosh et al., 1990) and more recently against the lepidopterans *Helicoverpa armigera* (Lomate and Hivrale, 2013) and *Hyphantria cunea* (Zibaee et al., 2010). PI have been isolated in very large amounts from soybean (*Glycine max*), but their addition to Bt-based bioinsecticides is only relevant if they can be purified cost-effectively. In this research, ammonium sulfate precipitation, spectrophotometric assays with a chromogenic substrate, zymograms and transmission electron microscopy (TEM) were used to demonstrate that a partially purified PI extract from soybean increases the toxicity of a mixture of Bti spores and crystals (Bti-sc) against CBB female adults in laboratory diet.

The objective of this work was to study whether the addition of a soybean meal extract enriched in protease inhibitors (PI), to Bti crystals and spores (Bti-sc) on artificial diet, could improve the toxicity of Bti against coffee berry borer (CBB).

Materials and methods

Insect rearing site and diet preparation

Research was performed at the Centro de Investigación en Biologia Celular y Molecular (CIBCM) of the Universidad de Costa Rica, in San Jose, Costa Rica, during 2012 - 2013. Bioassays were carried out employing second generation adult females of the coffee berry borer (*Hypothenemus hampei* Ferrari, CBB) obtained under laboratory conditions and fed with Cenibroca artificial diet (Portilla and Streett, 2006). Briefly, the diet was prepared adding agar, casein, yeast, green coffee, vitamins, salts, and benzoic acid. After a sterilization step (121 °C, 20 min), formaldehyde, ethanol, and Butrol® 9140 as a bactericide and fungicide were added, and the diet was poured on 24 wells plates, dried, sterilized again under UV light for 30 min, and stored at 4 °C (Portilla and Streett, 2006).

CBB in vitro massive rearing

A set of CBB infested berries obtained from different coffee fields located in Cartago and San Jose provinces (Costa Rica), previously disinfected with 2 % (w v^{-1}) benomyl and 0.1 % (w v^{-1}) omite, was dissected to recover CBB adult females that were selected as first generation specimens. These insects were washed with 2 % (w v^{-1}) benzalconium chloride and 1 % (w v^{-1}) benomyl as acaricide and fungicide, respectively, during 1 min and they were disposed on the artificial diet wells in groups of 4 individuals per well. The diets were aseptically sealed and maintained in the following conditions: relative humidity at 75 %, temperature at 28 °C and a photoperiodic regimen of 0:24 h [Light:Darkness] (Portilla and Streett, 2006). After rearing these individuals for a period of 8 weeks, a newly hatched second generation batch of CBB adult females was obtained and employed in the different bioassay described herein.

Purification of soybean PI

A previously published protocol to purify soluble PI from soybeans was modified for this work (Roosta et al., 2011). Briefly, soybean meal was mixed with distilled water for 30 min and separated by centrifugation at 3,000×g for 20 min at room temperature. The pH of the resulting supernatant was adjusted (pH 3.0) with 30 % (v v^{-1}) phosphoric acid, and proteins were precipitated by using 60 % (w v^{-1}) ammonium sulfate. Protein

pellets were resuspended in 20 mM Trizma-HCl (pH 7.5) and dialyzed for 24 h against this buffer into 12-14 kDa cellulose membranes (Spectra/Por®). The resulting suspensions were lyophilized and maintained at 4 °C in air-tight recipients.

Purification of Bti δ-endotoxins

A culture broth of Bti in hydrolysate of casein tryptone (HCT) medium (72 h, 28 °C) (modified from Lecadet et al., 1980), grown under aerobic conditions was mixed with 0.1 mol l^{-1} phosphoric acid solution until the suspension reached pH 4.0. Biomass of this suspension contained in 500 ml was harvested by centrifugation at 10,000×g and 4 °C for 10 min, washed twice with 1 mol l^{-1} NaCl, mixed with sterile distilled water in a 1:18 ratio, and sonicated for 1 min in an ultrasonic bath set to 40 kHz. The derived concentration of spores and parasporal crystals was vigorously agitated into a glass funnel until two phases appeared. The presence of parasporal crystals, which typically predominated in the aqueous phase, was confirmed through microscopic observation of Coomassie blue stained smears and further purified by isopycnic centrifugation at 23,000 rpm (~120,000×g) for 30 min at 15 °C in a discontinuous sucrose gradient. This gradient was set to 67 %, 72 %, 79 %, and 84 % (w v^{-1}) sucrose solutions prepared in 1 % (v v^{-1}) Triton X-100, 100 mM Trizma-HCl (pH 8.0), and 5 mol l^{-1} NaCl. Purified crystals were washed with distilled water twice and lyophilized.

Protein separation and visualization

Soybean meal protein preparations were separated by electrophoresis using 12.5 % (w v^{-1}) polyacrylamide gels and standard procedures. Samples loaded in these gels included trypsin from porcine pancreas (Sigma-Aldrich®) and Kunitz soybean trypsin inhibitor (SKTI, Sigma Aldrich®) as controls, and a pre-stained molecular weight marker (Page Ruler, Thermo Scientific®) to allow size determination of the proteins visualized by Coomassie staining. This technique was also used to examine the ability of the soybean meal extract to prevent proteolytic degradation of Bti proteins, loading the following mixtures onto the gels: Bti δ-endotoxins + porcine trypsin (1:1 ratio), Bti toxins + soybean extract (1:1 ratio), and Bti δ-endotoxins + trypsin + soybean extract (1:1:1).

Zymograms to detect proteolytic activity

As recommended by García-Carreño and Haard (1993), a native 12 % (w v^{-1}) PAGE gels copolymerized with 0.1 % (w v^{-1}) gelatin from bovine skin (Sigma-Aldrich®) was used to corroborate the ability of this PI preparation to inhibit proteolysis. As controls, the proteolytic activity of the trypsin inhibitor SKTI and the soybean meal extract were checked in parallel. After electrophoresis, zymograms were extensively washed in 2.5 % (v v^{-1}) Triton X-100 dissolved in 100 mmol l^{-1} Trizma-HCl (pH 8.0), cooled to 4 °C for 30 min, boiled to 55 °C during 2 h to promote gelatin digestion, and subsequently stained with 2.5 % (w v^{-1}) Coomassie Brilliant Blue R-250. Clear or slightly stained zones on a dark blue background (Pan e tal., 2011) were densitometrically quantified using the Quantity One 4.6.6 software.

Chromogenic detection of proteolytic activity

Proteases with amidase activity cleave the colorless substrate Nα-benzoyl-DL-arginine-4-nitroanilide hydrochloride (Ba*p*NA), giving rise to *p*–nitroaniline, which has a yellowish coloration (Guedidi et al., 2010). To determine the ability of the PI extract to inhibit proteolysis, a 2 mg ml^{-1} Ba*p*NA solution prepared in DMSO was pre-heated to 37 °C and mixed with trypsin or with serial dilutions of the soybean extract. After 10 min, reactions were stopped by addition of 30 % (v v^{-1}) acetic acid and centrifugation. The absorbance of the supernatants at 405 nm was determined by spectrophotometric approaches, and proteolysis was calculated according to the following equation (Eq. (1)):

$$EA\left(\frac{U}{ml}\right) = \frac{(A_{sample} - A_{blank}) \times 1.000 \times final\ volume\ of\ the\ reaction\ mixture(ml)}{Extintion\ coefficient \times time\ reaction\ (min) \times volume\ of\ the\ enzymatic\ solution\ (ml)}$$ [equation]

Enzyme activity (EA) was expressed in enzymatic units per milliliter, Asample and Ablank represent the absorbance of the sample and the blank, respectively. The molar extinction coefficient of p-nitroaniline is 8.800 M^{-1} cm^{-1} (Erlanger et al., 1961). These assays were performed by triplicate, and the results were reported as means±standard deviations (SD). Controls including 500 μg ml^{-1} trypsin from porcine pancreas and mixtures of porcine trypsin with 0.12 mmol l^{-1} SKTI or 0.16 mmol l^{-1} of the irreversible protease inhibitor phenylmethylsulfonyl fluoride were run in parallel.

Bioassays

To estimate the mean lethal concentration of Bti that kills 50 % of CBB (LC50), adult female CBB specimens from a second generation batch were placed on a new Cenibroca artificial diet modified from Portilla and Streett (2006), and exposed to 0.233 μg $μl^{-1}$, 0.389 μg $μl^{-1}$, 0.648 μg $μl^{-1}$ or 1.080 μg $μl^{-1}$ of Bti δ-endotoxin concentrations or a control with no spore-toxin solution. The diets were sealed and maintained following the aforementioned conditions (RH: 75 %, temperature: 28 °C, photoperiod 0:24 h [L:D]). Thereafter, to evaluate the synergistic effect of PI with Bti δ-endotoxins and to determine whether this mixture decreases the Bti LD50, the estimated LC50 of Bti δ-endotoxins with increasing amounts of soybean extract (0.28 μg $μl^{-1}$, 0.57 μg $μl^{-1}$, 1.13 μg $μl^{-1}$, 2.27 μg $μl^{-1}$ or 4.54 μg $μl^{-1}$) were mixed. Controls lacking Bti or both Bti + soybean meal were also assayed by triplicate (Valerio-Oviedo, 2006). CBB mortality was recorded after seven days and data from the experimental treatments were corrected through the Abbot correction calculation for control mortality response (Rosenheim and Hoy, 1989).

Statistical analysis

To compare the enzymatic activities from each soybean meal and CBB midgut raw extracts against their partially purified extracts using triplicated samples (r=3), t-student test ($p<0.05$) were employed. CBB dead insect replication data (r=3) derived from the bioassays, which included 16 experimental units (n=16) per replicate, was subjected to an analysis of variance (one-way ANOVA test, α=5 %) to compare the following treatments: negative control with no addition of Bti toxins or soybean extract, Bti LC50, Bti LC_{50}+1.135 μg $μl^{-1}$ soybean extract, and Bti LC50+3.540 μg $μl^{-1}$ soybean extract. Treatment significant differences were determined through a Fisher post-hoc test. LC50 calculation was performed with Probit regression analysis employing live and dead CBB frequencies, according to the method proposed by Finney and Stevens (1948).

TEM observation of CBB midguts

TEM images of midgut ultrastructure from CBB adult females fed for seven days with 1.135 μg $μl^{-1}$ of Bti δ-endotoxins, 1.135 μg $μl^{-1}$ Bti δ-endotoxins+4.540 μg $μl^{-1}$ PI or artificial diet alone were compared. In this set of experiments, thin histological sections of midguts were fixed in 2.5 % (w v^{-1}) glutaraldehyde, and 2 % (w v^{-1}) paraformaldehyde in 0.1 mol l^{-1} sodium phosphate buffer (pH 7.4) for 12 h at 4 °C and postfixed in 1 % (w v^{-1}) osmium tetraoxide for 2 h. Fixed sections were embedded in Spurr's resin, stained with uranyl acetate/lead solution, and visualized with a transmission electron microscope at an acceleration voltage of 100 kV (McDonald, 2007).

Results

The ammonium sulfate precipitation and dialysis procedures performed resulted in a fold purification factor of the soybean meal extract containing PI of 1.776 and a yield of 64 %, while a CBB midgut extract resulted in a value of 2.130 and a yield of 62 %; compared to their basal raw extracts (Table 1).

TABLE 1
Summary of the proteolytic activity and purification results obtained from the coffee berry borer (CBB, *Hypothenemus hampei* Ferrari) midgut and soybean meal. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Sample	Sample volume (ml)	Total proteolytic activity (U)[a]	Specific proteolytic activity (U ml^{-1} ± SD)[b]	Fold of purification factor[c]	Yield of protein per step (%)[d]
Soybean meal raw extract (*G. max*)	1800	1170.0	0.250 ± 0.001	1.000	100
Soybean meal extract precipitated with 60 % (w v^{-1}) $NH_4(SO_4)_2$, and dialyzed	1620	720.0	0.444 ± 0.005*	1.776	62
CBB raw midgut extract	150	130.3	0.328 ± 0.002	1.000	100
CBB midgut extract precipitated with 70 % (w v^{-1}) $NH_4(SO_4)_2$, and dialyzed	116	81.1	0.699 ± 0.003*	2.130	62

[a] Proteolytic activity was determined by confronting the samples against the chromogenic substrate DL-BApNA for 10 min, 37 ºC (pH 9.0). The enzymatic units are expressed in µmol of *p*-nitroaniline released in the medium per minute. The proteolytic activity of each CBB midgut and soybean meal extract samples was analyzed under optimal pH and temperature. Values are presented for triplicate experiments, expressed as mean ± standard deviation (SD) / La actividad proteolítica fue determinada al enfrentar las muestras contra el sustrato cromogénico DL-BA*p*NA durante 10 min, 37 ºC, (pH 9,0). Las unidades enzimáticas están expresadas en µmol de *p*-nitroanilina librados en el medio por minuto. La actividad proteolítica de cada muestra de intestino medio de broca y extracto de harina de soya fue analizada bajo pH óptimo y temperatura. Los valores son presentados como experimentos triplicados, expresados como el promedio ± desviación estándar (SD).
[b] Specific proteolytic activity = Total proteolytic activity / Sample volume. Specific activity values represent the 3 replicates ± SD / Actividad proteolítica específica = actividad proteolítica total / volumen de la muestra. Los valores de actividad específica representan las 3 réplicas ± SD.
[c] Purification change factor = Specific proteolytic activity of one sample / Specific proteolytic activity of the raw extract (soybean extract or CBB midgut extract), which represents a 100 % of the initial yield / Factor de cambio de purificación = actividad proteolítica específica de una muestra / actividad proteolítica específica del extracto crudo (extracto de soya o extracto de intestino medio de broca), el cual representa el 100 % del rendimiento incial.
[d] Yield per stage = Total proteolytic activity of the corresponding sample / Total proteolytic activity of the raw extract×100 / Rendimiento por etapa = actividad proteolítica total de la muestra correspondiente / actividad proteolítica total del extracto crudo × 100.
* Statistical differences resulting from t-student tests ($p<0.05$), after comparing both raw soybean meal ($t=63.88$, $p<0.001$) or CBB midgut ($t=-182.60$, $p<0.05$) extracts against their corresponding partially purified extracts / Diferencias estadísticas obtenidas a partir de pruebas de t-student ($p<0,05$), luego de comparar los extractos crudos de harina de soya ($t=63,88$, $p<0,001$) o de intestino medio de broca ($t=-182,60$, $p<0,05$) contra sus correspondientes extractos parcialmente purificados.

Cuadro 1. Resumen de la actividad proteolítica y los resultados de purificación obtenidos de extractos del intestino medio de la broca del café (CBB, *Hypothenemus hampei*, Ferrari) y harina de frijol de soya. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

A SDS-PAGE of the soybean meal preparation showed three bands with apparent molecular weights of 92 kDa, 37 kDa, and 20 kDa (Figure 1; lanes PI1 and PI2), while the Kunitz soybean trypsin inhibitor only presented a unique band with apparent molecular weight of 20 kDa (lane SKTI). Additionally, the CBB midgut extract (lanes MG1 and MG2), as well as trypsin from porcine pancreas (TP) generated only one band with an estimated molecular weight of 24 kDa.

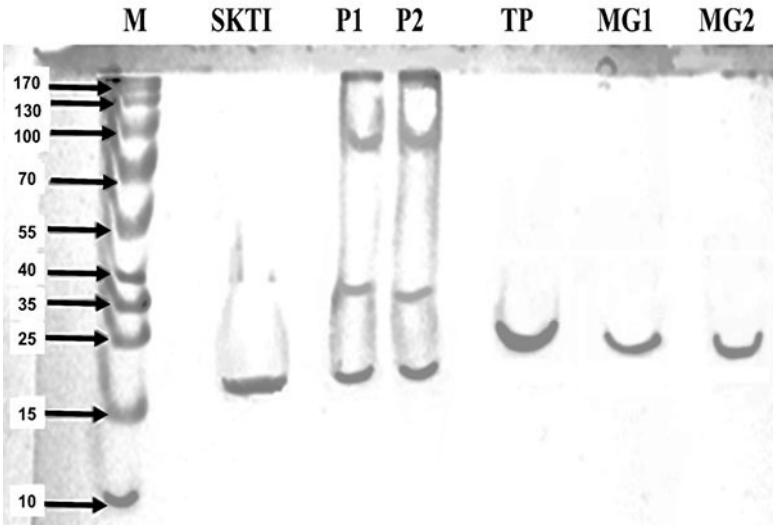

FIGURE 1
Proteins obtained in the extract of soybean meal and a fraction of CBB (*Hypothenemus hampei* Ferrari) midgut undergoing dialysis. SDS-PAGE stained with 2.5 % (w v^{-1}) Coomassie Brilliant Blue and observed under white light. M: Prestained marker for SDS-PAGE (kDa), SKTI: Kunitz soybean trypsin-protease inhibitor, PI1 and PI2: sample of a fraction of the extract of dialyzed soybean meal, TP1 and TP2: trypsin of porcine pancreas, MG1 and MG2: CBB CBB midgut extract. Universidad de Costa Rica, San Jose, Costa Rica. 2012-2013.

Figura 1. Proteínas obtenidas en el extracto de harina de frijol de soya y una fracción de intestinos medios de CBB (*Hypothenemus hampei*, Ferrari) sometidos a diálisis. SDS-PAGE teñido con Azul Brillante Coomassie al 2,5 % (m v^{-1}) y visualizado bajo luz blanca. M: Marcador Preteñido para SDS-PAGE (kDa), SKTI: inhibidor Kunitz de tripsín-proteasas de soya, PI1 y PI2: muestra de una fracción del extracto de harina de frijol de soya dializado, TPI y TP2: tripsina de páncreas porcino, MG1 y MG2: extracto de intestino medio de broca. Universidad de Costa Rica, San José, Costa Rica. 2012-2013.

Employing a gelatin zymogram, the soybean extract (Figure 2; lanes PI1 and PI2) abolished the proteolytic activity of a CBB midgut extract (lanes MG1, MG2, and MG3), which was known to contain a protease with a similar size to porcine trypsin (lane TP).

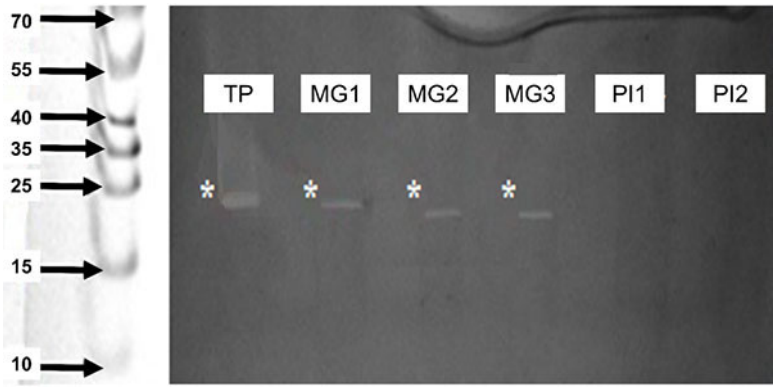

FIGURE 2
Trypsin activity detected in CBB (*Hypothenemus hampei* Ferrari) midgut extracts (MG1-3, kDa) and CBB midgut extract treated with a partially purified soybean meal extract on a 1:1 ratio (PI1-2, kDa). 0.1 % (w v^{-1}) gelatin zymogram. TP: Porcine pancreatic trypsin used as positive control (kDa). White asterisks show the tryptic activities of each sample, if present. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Figura 2. Actividad tríptica detectada en extractos de intestino medio de CBB (*Hypothenemus hampei*, Ferrari) (MG1-3, kDa) y extractos de intestino medio de broca tratados con un extracto parcialmente purificado de harina de frijol de soya en una proporción 1:1 (PI1-2, kDa). Zimograma de gelatina al 0,1 % (m v^{-1}). TP: tripsina de páncreas porcino utilizada como un control positivo (kDa). Los asteriscos color blanco indican las actividades trípticas de cada muestra, en caso de estar presente. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

A SDS-PAGE of Bti-sc revealed two bands of 52 kDa and 24 kDa (Figure 3, lanes Cry1, and Cry2), whose protein quantities were estimated to be approximately 7.1×103 and 7.3×103 ng, respectively. These proteins were partially degraded to bands of 50 and 22 kDa when Bti-sc was mixed with dilutions of trypsin from porcine pancreas (lane TP) and the partially purified soybean extract (lanes CTPI1, CTPI5, and CTPI10), lowering their protein quantities to 7.2×103, 5.9×103, and 3.9×103 ng, respectively.

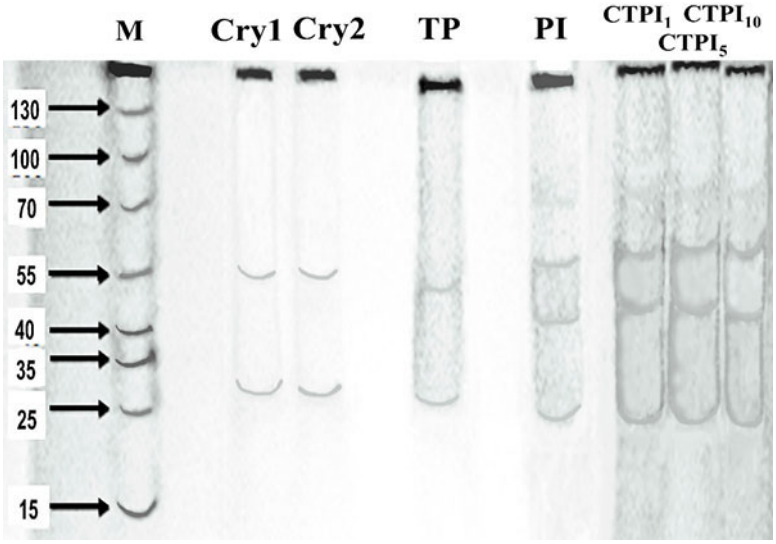

FIGURE 3
Bti mixture of spores and crystals alone or in combination with porcine pancreatic trypsin (TP), soybean extract (PI) or both in different proportions (CTPI1, 1:1, CTPI5 1:5, and CTPI10 1:10). 12 % (w v^{-1}) SDS-PAGE stained with Coomassie Brilliant Blue. M: Prestained marker for SDS-PAGE (kDa), Cry1-2: Bti-sc lyophilized samples. Final volumes were kept constant to avoid dilution effects. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Figura 3. Mezcla de esporas y cristales de Bti solas o en combinación con tripsina de páncreas porcino (TP), extracto de frijol de soya (PI) o ambos en diferentes proporciones (CTPI1, 1:1, CTPI5 1:5, and CTPI10 1:10). SDS-PAGE al 12 % (m v^{-1}) teñido con Azul Brillante Coomasie. M: Marcador Preteñido para SDS-PAGE (kDa), Cry1-2: muestras liofilizadas de Bti-sc. Los volúmenes finales fueron mantenidos constantes para prevenir los efectos de dilución. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

The soybean extract showed 1/3 of the BApNA proteolytic activity measured for trypsin (Figure 4, PI=0.444 U ml^{-1}), and its mean value was similar to that of the Kunitz soybean trypsin inhibitor (SKTI=0.361 U ml^{-1}) but lower than the CBB midgut extract (CBBMG=0.699 U ml^{-1}) and the porcine trypsin (TP=1.218 U ml^{-1}). Furthermore, the soybean extract did not diminish the proteolytic activity of the CBB extract (PI+CBBMG=0.833 U ml^{-1}), while the Kunitz trypsin inhibitor (TP+SKTI=0.210 U ml^{-1}) or a chemical inhibitor (TP+PMSF=0.903 U ml^{-1}) did reduce the proteolysis of porcine trypsin. Altogether, these results confirm that this soybean extract, in addition to its PI activity, contained some level of unexpected protease activity.

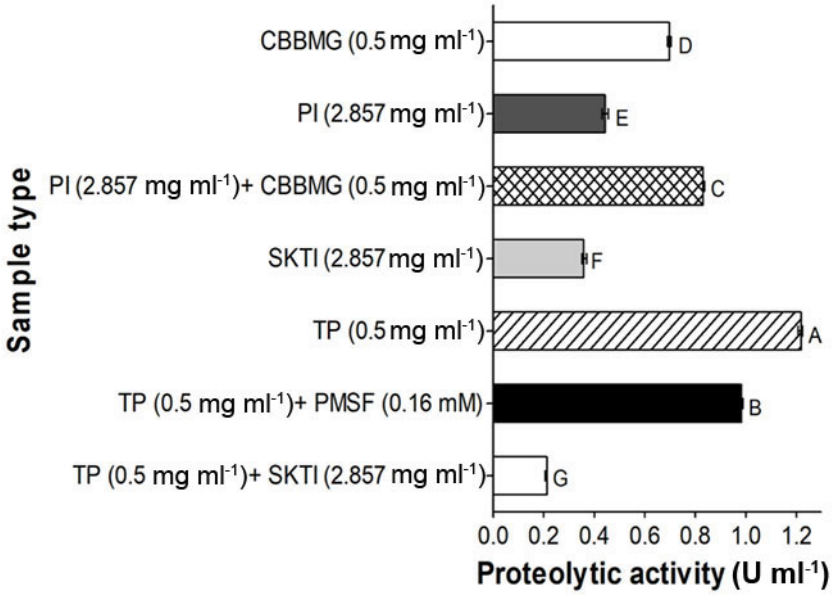

FIGURE 4

Proteolytic activities of partially purified soybean meal, *H. hampei* midgut, and controls as quantified under DL-BApNA hydrolysis by incorporating a soybean meal extract with protease inhibitor (PI) mixed together with crystals and spores of Bti (Bti-sc) in the diet. CBBMG: coffee berry borer midgut extract, PI: *G. max* meal extract, SKTI: soybean Kunitz trypsin inhibitor, TP: trypsin from porcine pancreas, PMSF: phenylmethylsulfonyl fluoride. Results were subjected to a one-way analysis of variance (one-way ANOVA). Capital letters above each bar represent differences among the mean±standard deviation (SD) of each treatment, according to Fisher post-hoc test after applying the one way-ANOVA test (DF=6, F=43140.08, p<0.001). Universidad de Costa Rica, San Jose, Costa Rica. 2012-2013.

Figura 4. Actividades proteolíticas de extractos parcialmente purificados de harina de frijol de soya, intestino medio de *H. hampei*, y controles cuantificados bajo hidrólisis de DL-BApNA al incorporar un extracto de harina de soya con inhibidores de proteasas (PI) mezclado junto con cristales y esporas de Bti (Bti-sc) en la dieta CBBMG: extracto de intestino medio de broca del café, PI: extracto de harina de *G. max*, SKTI: inhibidor Kunitz de tripsín-proteasas de soya, TP: tripsina de páncreas porcino, PMSF: fluoruro de fenilmetilsulfonilo. Los resultados fueron sujetos a una prueba de varianza univariada (ANOVA). Las letras mayúsculas por encima de cada barra representan diferencias entre el promedio ±la desviación estándar (SD) de cada tratamiento, de acuerdo con la prueba post-hoc de Fisher luego de aplicar el análisis de varianza ANOVA de una vía (GL=6, F=43140,08, p<0,001). Universidad de Costa Rica, San José, Costa Rica. 2012-2013.

The corrected CBB mortality percentages obtained in assays with 0.233 µg µl^{-1}, 0.389 µg µl^{-1}, 0.648 µg µl^{-1}, and 1.080 µg µl^{-1} of purified Bti crystals corresponded to 14 %, 59 %, 59 %, and 64 %, respectively; generating a LC50 of 1.135±1.005 µg µl-1 (DF=1; X2=7.8, p=0.005) (Figure 5A). This toxicity increased in a concentration-dependent manner through the addition of PI, as indicated by an experiment in which the LC50 of Bti crystals mentioned above was mixed with increasing amounts of the soybean extract. Here a lower LC50 value, estimated in 0.315±1.008 µg µl-1 (DF=1; X2=7.9, p=0.019) was obtained (Figure 5B). In this experiment the mortality values increased from 50 % in the Bti control without PI to 54 %, 59 %, 62 %, 66 %, and 73 % in wells containing 0.284 µg µl^{-1}, 0.568 µg µl^{-1}, 1.135 µg µl^{-1}, 2.270 µg µl^{-1}, and 4.540 µg µl^{-1} of the soybean extract, respectively (Figure 6).

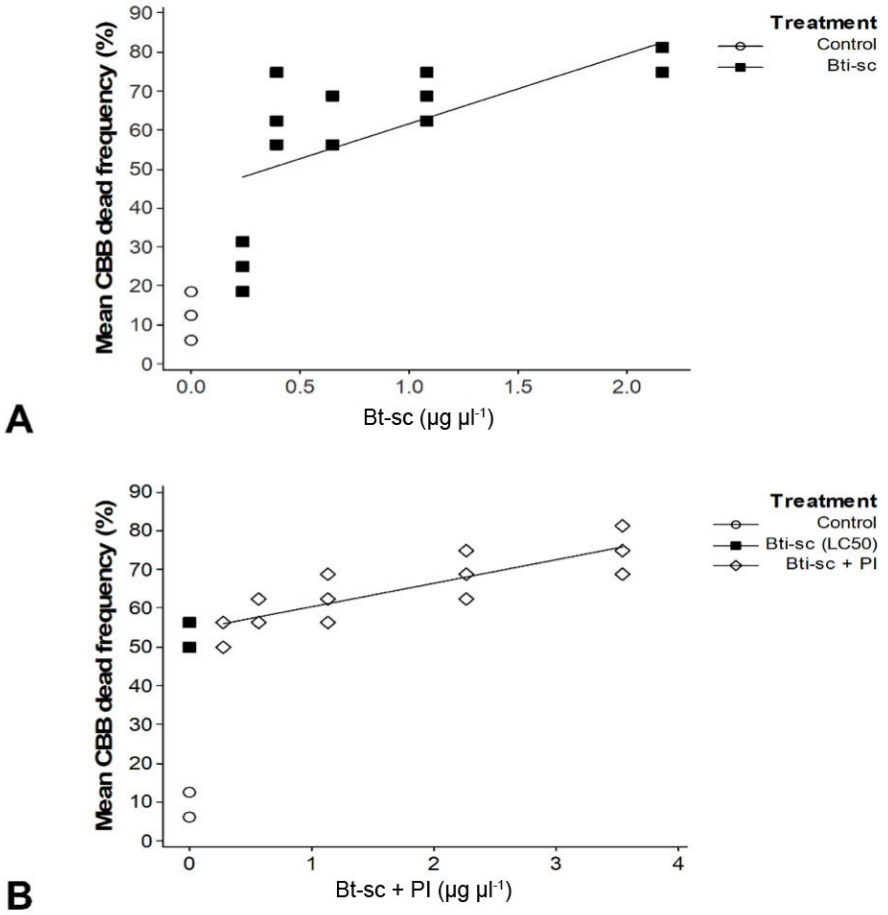

FIGURE 5

Sublethal concentration (LC50) from a mixture of spores and crystals of Bti or the Bti LC50 mixed with increasing concentrations of a partially purified soybean meal extract against coffee berry borer females (CBB). A) Average mortality rate of CBB (*Hypothenemus hampei* Ferrari) exposed to five increasing concentrations of a Bti spore-crystal complex, compared to a baseline control with no treatment, B) Average CBB mortality rate of five samples, including the estimated Bti LC50 + increasing concentrations of soybean meal extract, compared to the Bti LC50 estimated and evaluated individually and negative control. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Figura 5. Concentración subletal (CL50) de una mezcla de esporas y cristales de Bti o la CL50 de Bti combinada con concentraciones crecientes de un extracto parcialmente purificado de harina de frijol de soya, contra hembras de broca (CBB). A) Promedio de la frecuencia de mortalidad de CBB (*Hypothenemus hampei* Ferrari) expuestas a cinco concentraciones crecientes de un extracto de Bti correspondiente al complejo espora-cristal, comparado con un control basal sin tratamiento, B) Frecuencia de mortalidad promedio de CBB de cinco muestras, incluyendo la CL50 estimada de Bti+concentraciones crecientes del extracto de harina de frijol de soya, comparados con la CL50 de Bti estimada y evaluada de manera individual y un control negativo. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

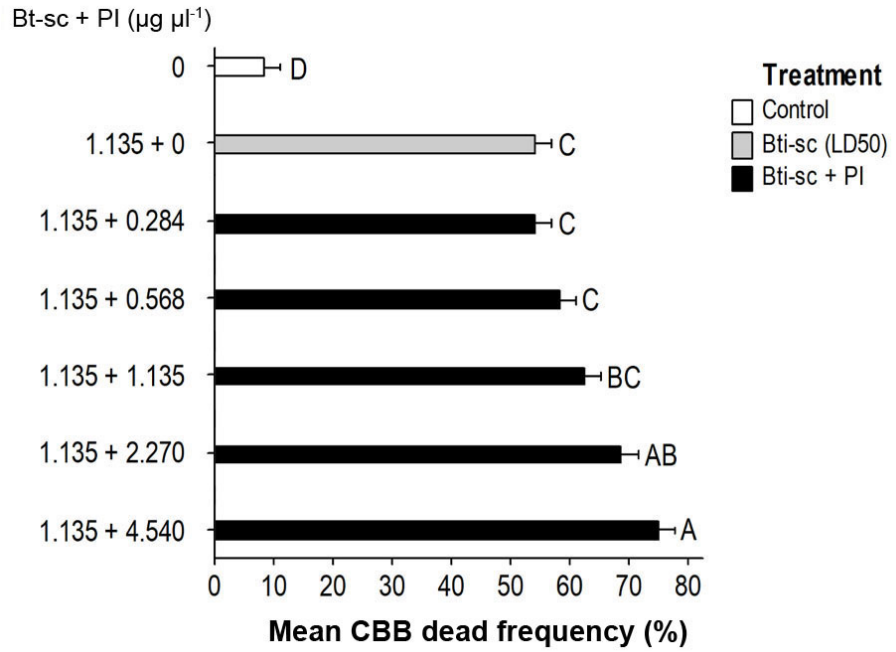

FIGURE 6
Average mortality of females of CBB (*Hypothenemus hampei* Ferrari) evaluated through a bioassay in artificial diet. Different treatments of LC50 Bti-sc extract with increasing concentrations of a partially purified Glycine max extract or a negative control with no addition of Bti-sc nor *G. max* extracts. Mortality records were analyzed through ANOVA test (DF=6, F=58.59, p<0.001) and a Fisher post-hoc test. Data represents the means±standard deviation (SD) of three replicates (16 adult CBB females per replicate for each treatment). Unshared capital letters above the bars indicate significant differences. Bti-sc: B. thuringiensis δ-endotoxin extract mixed with spores, PI: partially purified soybean meal extract. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Figura 6. Mortalidad promedio de hembras de CBB (*Hypothenemus hampei* Ferrari) evaluadas mediante un bioensayo en dieta artificial. Diferentes tratamientos de la LC50 de un extracto de Bti-sc con concentraciones crecientes de un extracto parcialmente purificado de *Glycine max* o un control negativo sin añadir extractos de Bti-sc ni *G. max*. Los registros de mortalidad fueron analizados mediante una prueba ANOVA univariado (GL=6, F=58,59, p<0,001) y una prueba post-hoc de Fisher. Los datos representan los promedios±desviación estándar (SD) de tres réplicas (16 hembras adultas de broca por repetición para cada tratamiento). Las letras capitales encima de cada barra que no están compartidas indican diferencias significativas. Bti-sc: extracto de δ-endotoxinas derivado de *B. thuringiensis* mezclado con esporas, PI: extracto de harina de frijol de soya parcialmente purificado. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

Ultrastructural differences were observed in epithelial midguts from CBB adult females fed with artificial diet (negative control, Figure 5A and 5B) compared to cognates that received 1.135 µg µl^{-1} Bti δ-endotoxins (Figure 7C) or 1.135 µg µl^{-1} Bti δ-endotoxins+4.540 µg µl^{-1} PI (Figure 7D). Furthermore, evidence of programmed cell death events was detected in the treated insects. CBB midguts that only received Bti proteins showed clusters of vacuolated cytoplasm, nuclei with marginalized chromatin, nuclear pyknosis and a rather low level of swelled microvilli. Finally, midguts treated with Bti toxins + soybean PI also showed evident alterations such as lysed and compressed epithelial cells, shortening and more irregular morphology of deteriorated microvilli, and a more dispersed lumen.

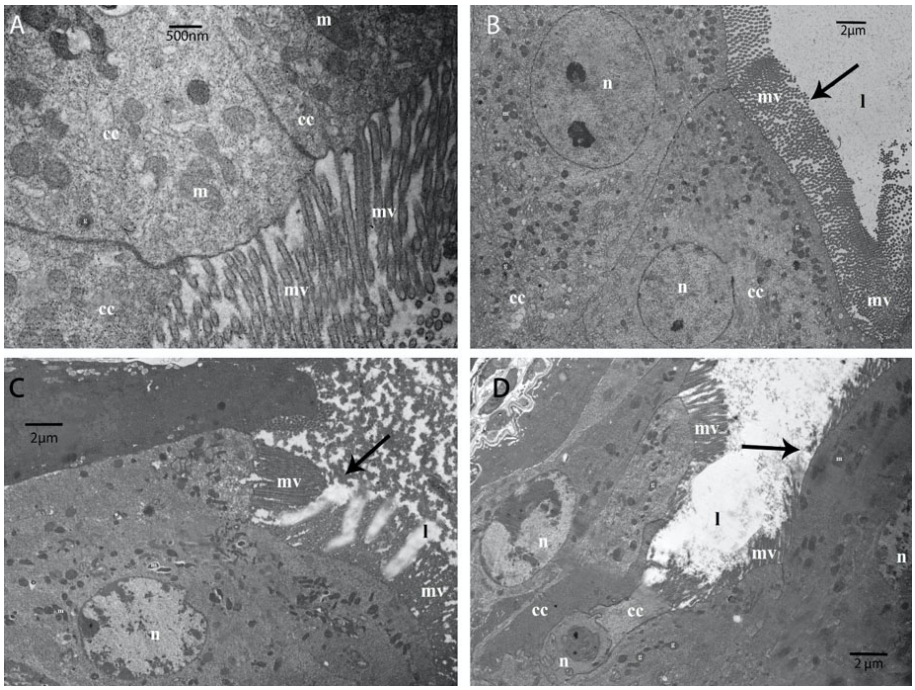

FIGURE 7

Histological effects of Bti toxins evaluated alone or mixed with a partially purified *G. max* extract against CBB (*Hypothenemus hampei* Ferrari). Ultrathin cross–sections of CBB midguts fed with an artificial diet alone (A, B), diet supplemented with 1.135 µg µl^{-1} Bti δ-endotoxins (C), or diet supplemented with 1.135 µg µl^{-1} Bti δ-endotoxins+4.540 µg µl^{-1} soybean extract (D). Black arrows show epithelial microvilli (mv) or remnants of microvilli, which are close to the lumen (l). Columnar cells (cc) composed by nuclei (n), granules (g), and mitochondria (m) appear immediately below those structures. Universidad de Costa Rica, San Jose, Costa Rica, 2012-2013.

Figura 7. Efectos histológicos de las toxinas de Bti evaluadas solas o en combinación con un extracto parcialmente purificado de *G. max* contra CBB (*Hypothenemus hampei* Ferrari). Cortes ultrafinos de secciones de intestinos medios de CBB alimentados con una dieta artificial sola (A, B), dieta suplementada con δ-endotoxinas de Bti a 1,135 µg µl^{-1} (C), o dieta suplementada con δ-endotoxinas de Bti a 1,135 µg µl^{-1}+extracto de frijol de soya a 4,540 µg µl^{-1} (D). Las flechas color negro muestran microvellosidades epiteliales (mv) o restos de microvellosidades, los cuales están cerca del lumen (l). Células columnares (cc) conformadas por núcleos (n), gránulos (g) y mitocondrias (m) aparecen inmediatamente bajo dichas estructuras. Universidad de Costa Rica, San José, Costa Rica, 2012-2013.

Discussion

The partially purified G. max extract enriched in protease inhibitors (PI) increased the activity of B. thuringiensis subs. israelensis (Bti) toxins against *Hypothenemus hampei* (CBB). This toxicity enhancement was detected despite the low purity of the soybean meal extract (1.78), which is slightly lower than the *G. max* fold purification factor (2.63) reported previously, where a soybean meal extract was purified using a 30-60 % $(NH_4)SO_4$ gradient (El-latif, 2015). Hence, complex downstream processes, such as ion exchange chromatography or HPLC, are not required in a Bt-based insecticide supplemented with PI from soybean.

Prior research employing nine *B. thuringiensis* strains, whose spore-crystal complex (Bt-sc) was purified through differential centrifugation with a NaBr gradient, revealed that only the serovars israelensis and thompsoni caused 100 % mortality against first-instar larvae of CBB and reported a sublethal concentration (LC50) of 0.219 µg µl^{-1} for Bti (Méndez-López et al., 2003). Although first-instar larvae are likely more susceptible to Bti endotoxins in the present study, a higher LC50 (1.135 µg µl^{-1}) on adult CBB was recorded, even though there is no literature comparing susceptibility among CBB instars. Similarly, the LC50 recorded

in this research was about six orders of magnitude higher than the values obtained in a prior study, testing fourth-instar *Aedes aegyptii* larvae (2.061×10-6 μg μl^{-1}) (Hernández-Soto et al., 2009), probably because dipterans are more susceptible than coleopterans to Bti Cry proteins. Furthermore, previous research has focused on testing only Bt-sc against CBB larval stages (De-la-Rosa et al., 2005; López-Pazos et al., 2009; Zorzetti et al., 2018). Thus, this is the first evaluation of the susceptibility of *H. hampei* adults to Bt-sc from B. thuringiensis, either alone or mixed with a partially purified plant extract enriched in PI. This finding suggests that Bti-sc could be used as a suitable active ingredient not only for controlling CBB larvae, but also adult insects. This double control effect has been reported for mosquitoes, where the insecticidal activity of Bti-sc kills both larvae and adults, with the adults showing a higher susceptibility to the toxins (Mansour et al., 2012; Zahiri and Mulla, 2005).

In this research, PI was selected from a non-host plant because CBB might have developed adaptations to toxic substances in coffee (Jiménez, 2009; Molina et al., 2011). PI from non-host plants are more effective inhibiting the gut proteases and stopping larval growth of Chilo partellus (Lepidoptera: Pyralidae) than PI from host plants (Panchal and Kachole, 2012).

Soybean contains two major classes of PI, namely the Bowman-Birk (SBBI) and the Kunitz (SKTI) inhibitors (Perić et al., 2009). Besides containing a protein with the apparent molecular weight of SKTI (ca. 18-22 kDa), the G. max extract showed two other bands of approximately 92 kDa and 36 kDa. These polypeptides could correspond to the α-subunit of the β-conglicinine triad (93-89 kDa) and an acidic glicinine (33-40 kDa) (Sriket et al., 2011), storage proteins that can account for up to 70-90 % of the proteins present in G. max seeds (Roosta et al., 2011; Vorlová, 2011). PI from the SKTI family has disulfide bonds, conferring them high stability across different temperatures (Shamim et al., 2011) and pH values (de-Oliveira et al., 2012). These features could simplify downstream processes in the manufacturing of a hybrid Bti-soybean extract formulation and may increase the stability of this formulation when applied onto tropical crops.

The apparent absence of SBBI proteins from the soybean extract obtained could be a consequence of its removal either during the precipitation process with ammonium sulfate and/or the dialysis step. This is possible since SBBI is a low molecular weight protein (~8 kDa) with high solubility in water (Gillman et al., 2015; Gu et al., 2014). Moreover, SBBI is found in lower amounts in soybean meal (<0.2-4.9 mg g^{-1}) compared to SKTI (1.1-19.6 mg g^{-1}) (Anderson and Wolf, 1995). A 3 h extraction under alkaline conditions (pH 8.4-10) should improve SKTI recovery and activity. Other alternatives include working with diluted homogenates or not separating solids through centrifugation (Kakade et al., 1974; Li and Chase, 2010).

The partially purified soybean meal extract unexpectedly processed DL-BApNA, indicating that it contained a measurable level of amidolytic activity (Shamsi et al., 2016). This finding suggests the presence of proteases in the soybean meal preparation, a feature that could be of benefit for the purposes of this work, given that Cry and Cyt proteins digested by insect endo-proteases may exhibit increased insecticidal activity (Vidal-Quist et al., 2010).

While a preliminary study showed rapid pore formation when brush border membrane vesicles of Manduca sexta were exposed to Bt Cry1Aa in conjunction with PMSF or AEBSF chemical serine-protease inhibitors (Kirouac et al., 2006), other reports have documented only hyperplasia and hypertrophy in insect midgut epithelial cells treated with PI (Macedo et al., 2011; Ghodke et al., 2013). These previous results agree with the current findings in this research given the detection of fractures and perforations in the CBB peritrophic membrane of insects exposed to Bti-sc and the soybean meal preparation mixture.

The addition of the soybean extract enriched with PI reduced the LC50 of the Bti-sc preparation nearly 3.5-fold compared to the lethality of Bti-sc alone. Moreover, this Bti-sc extract was more toxic to the CBB midgut when laboratory diet was mixed with *G. max* meal extract. These results are similar to those obtained by Oppert et al. (2011), who ascertained that a combination of *B. thuringiensis* Cry3Aa with a potato carboxypeptidase inhibitor enhanced the mortality of *Rhyzopertha dominica* (Coleoptera) adult insects.

Additionally, the Bt-sc LC50 reached 2-fold reduction and the insect development time increased between 3 to 5 days.

In this study, the effect of PI alone on CBB adults was not tested. However, prior research has found that PI derived from plant sources induce low or modest mortality effects (6-28 %) to larvae or adult target pests (Fabrick et al., 2002; Franco et al., 2004; Gujar et al., 2004; Kuhar et al., 2013; Shamsi et al., 2018; Zhao et al., 2019). Further, PI from plant sources cause a restricted digestion process and nutritional deficiencies over susceptible insects (Zhu-Salzman and Zeng, 2015; Singh et al., 2018), affecting proper insect development, fecundity and survival of different pests (Ruan et al., 2015; Vasudev and Sohal, 2016). Therefore, future experiments should explore the individual effect of the soybean meal enriched in PI over the mortality, development, weight, and fertility of *H. hampei* (Cotabarren et al., 2020).

Conclusions

The addition of a partially purified soybean meal extract enriched in protease inhibitors to a Bti-sc extract reduced the calculated LC50 for Bti from 1.135 $\mu g\ \mu l^{-1}$ to 0.315 $\mu g\ \mu l^{-1}$ and increased the CBB mortality levels, confirmed through the epithelial damages of CBB subjected to this mixture. Altogether, the results shown here provide a new avenue for the development of an eco-friendly biocide for low-income countries, which combines the specificity and toxicity of Bti-sc with a cost-effective and readily available preparation of a soybean meal containing protease inhibitors to prevent toxin degradation. Potential benefits of this synergistic mixture include decreasing the amount of Bti toxin released into the environment, hence delaying biological resistance on CBB and minimizing the potential impact on non-target invertebrates and the surrounding agroecosystem.

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Cited literature

Acuña-González, P., y W. Betanco Velásquez. 2007. Evaluación de la incidencia natural de *Beauveria bassiana* (Bals) Vuill, sobre *Hypothenemus hampei* (Ferrari) y Leucoptera coffeella (Guérin-Méneville) en el cultivo de café en dos zonas cafetaleras de Nicaragua. Tesis Diploma, Universidad Nacional Agraria, Managua, NIC.

Anderson, R.L., and W.J. Wolf. 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. J. Nutr. 125:581S-588S. doi:10.1093/jn/125.3_Suppl.581S

Aristizábal, L.F., M. Jiménez, A.E. Bustillo, H.I. Trujillo, and S.P. Arthurs. 2015. Monitoring coffee berry borer, *Hypothenemus hampei* (Coleoptera: Curculionidae), populations with alcohol-baited funnel traps in coffee farms in Colombia. FL Entomol. 98:381-383. doi:10.1653/024.098.0165

Aristizábal, L.F., S. Shriner, R. Hollingsworth, and S. Arthurs. 2017. Flight activity and field infestation relationships for coffee berry borer in commercial coffee plantations in Kona and Kau districts, Hawaii. J. Econ. Entomol. 110:2421–2427. doi:10.1093/jee/tox215

Belayneh-Mulaw, T., C.P. Kubicek, and I.S. Druzhinina. 2010. The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia provides a niche for a distinguished diversity of *Trichoderma*. Diversity 2:527-549. doi:10.3390/d2040527

Cantor, F., V.L.R.M. Benassi, e C.J. Fanton. 2001. Broca-do-café, *Hypothenemus hampei* (Coleoptera: Scolytidae). Em: E. Ferrera et al., editores, Histórico e impacto das pragas introduzidas no Brasil. Holos, São Paulo, BRA. p. 99-105.

Cárdenas, S.I. 2007. Caracterización morfológica y agronómica de la colección núcleo de café (*Coffea arabica* L.) del CATIE. Tesis MSc., CATIE, Turrialba, CRI.

Constantino, L.M., L. Navarro, A. Berrio, F.E. Acevedo, D. Rubio, y P. Benavides. 2011. Aspectos biológicos, morfológicos y genéticos de *Hypothenemus obscurus* e *Hypothenemus hampei* (Coleoptera: Curculionidae: Scolytinae). Rev. Colomb. Entomol. 37:173-182.

Cotabarren, J., D. Lufrano, M.G. Parisi, and W.D. Obregón. 2020. Biotechnological, biomedical, and agronomical applications of plant protease inhibitors with high stability: A systematic review. Plant Sci. 292:110398. doi:10.1016/j.plantsci.2019.110398

De-la-Rosa, W., M. Figueroa, and J. Ibarra. 2005. Selection of *Bacillus thuringiensis* strains native to Mexico active against the coffee berry borer *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae: Scolytinae). Vedalia 12:3-9.

de-Oliveira, C.F.R., I.M. Vasconcelos, R. Aparicio, M.D.G.M. Freire, P.A. Baldasso, S. Marangoni, and M.L.R. Macedo. 2012. Purification and biochemical properties of a Kunitz-type trypsin inhibitor from *Entada acaciifolia* (Benth.) seeds. Proc. Biochem. 47:929-935. doi:10.1016/j.procbio.2012.02.022

El-latif, A.O.A. 2015. Protease purification and characterization of a serine protease inhibitor from Egyptian varieties of soybean seeds and its efficacy against *Spodoptera littoralis*. J. Plant Prot. Res. 55:16-25. doi:10.1515/jppr-2015-0003

Erlanger, B.F., N. Kokowsky, and W. Cohen. 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95:271-278. doi:10.1016/0003-9861(61)90145-X

Fabrick, J., C. Behnke, T. Czapla, K. Bala, A.G. Rao, K.J. Kramer, and G.R. Reeck. 2002. Effects of a potato cysteine proteinase inhibitor on midgut proteolytic enzyme activity and growth of the southern corn rootworm, *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae). Insect Biochem. Mol. Biol. 32:405-415. doi:10.1016/S0965-1748(01)00117-5

Finney, D.J., and W.L. Stevens. 1948. A table for the calculation of working probits and weights in probit analysis. Biometrika 35:191-201. doi:10.1093/biomet/35.1-2.191

Franco, O.L., S.C. Dias, C.P. Magalhães, A.C.S. Monteiro, C. Bloch, F.R. Melo, O.B. Oliveira-Neto, R.G. Monnerat, and M.F. Grossi-de-Sá. 2004. Effects of soybean Kunitz trypsin inhibitor on the cotton boll weevil (*Anthonomus grandis*). Phytochemistry 65:81-89. doi:10.1016/j.phytochem.2003.09.010

García-Carreño, F.L., and N.F. Haard. 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17:97-113. doi:10.1111/j.1745-4514.1993.tb00864.x

Ghodke, A.B., S.G. Chavan, B.V. Sonawane, and A.A. Bharose. 2013. Isolation and *in vitro* identification of proteinase inhibitors from soybean seeds inhibiting Helicoverpa gut proteases. J. Plant Interact. 8:170-178. doi:10.1080/17429145.2012.668952

Gillman, J.D., W.S. Kim, and H.B. Krishnan. 2015. Identification of a new soybean Kunitz trypsin inhibitor mutation and its effect on Bowman-Birk protease inhibitor content in soybean seed. J. Agric. Food Chem. 63:1352-1359. doi:10.1021/jf505220p

González, V. 2019. Costa Rica: Coffee annual: Coffee production and trade. Annual report. USDA, USA. https://gain.fas.usda.gov/Recent%20GAIN%20Publications/Coffee%20Annual_San%20Jose_Costa%20Rica_5-15-2019.pdf (accessed Sep. 29, 2019).

Gu, C., X. Song, L. Zhao, S. Pan, and G. Qin. 2014. Purification and characterization of Bowman-Birk trypsin inhibitor from soybean. J. Food Nutr. Res. 2:546-550. doi:10.12691/jfnr-2-9-3

Guedidi, S., Y. Yurekli, A. Deratani, P. Déjardin, C. Innocent, S.A. Altinkaya, S. Roudesli, and A. Yemenicioglu. 2010. Effect of enzyme location on activity and stability of trypsin and urease immobilized on porous

membranes by using layer-by-layer self-assembly of polyelectrolyte. J. Membr. Sci. 365:59-67. doi:10.1016/j.memsci.2010.08.042

Gujar, T., V. Kalia, A. Kumari, and T.V. Prasad. 2004. Potentiation of insecticidal activity of Bacillus thuringiensis subsp. kurstaki HD-1 by proteinase inhibitors in the *American bollworm*, *Helicoverpa armigera* (Hübner). Indian J. Exp. Biol. 42(2):157-163.

Hernández-Soto, A., M.C. Del Rincón-Castro, A.M. Espinoza, and J.E. Ibarra. 2009. Parasporal body formation via overexpression of the Cry10Aa toxin of Bacillus thuringiensis subsp. israelensis, and Cry10Aa-Cyt1Aa synergism. Appl. Environ. Microbiol. 75:4661-4667. doi:10.1128/AEM.00409-09

Jiménez, A.J. 2009. As enzimas presentes no trato digestivo dos insetos: um alvo susceptível de inhibição. Tese Dou., Universidade de Brasília, Brasilia, BRA.

Jouzani, G.S., E. Valijanian, and R. Sharafi. 2017. Bacillus thuringiensis: a successful insecticide with new environmental features and tidings. Appl. Microbiol. Biotechnol. 101:2691-2711. doi:10.1007/s00253-017-8175-y

Kakade, M.L., J.J. Rackis, J.E. McGhee, G. Puski, and R.S. Usda. 1974. Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. Cereal Chem. 51:376-381.

Kirouac, M., V. Vachon, D. Quievy, J.L. Schwartz, and R. Laprade. 2006. Protease inhibitors fail to prevent pore formation by the activated Bacillus thuringiensis toxin Cry1Aa in insect brush border membrane vesicles. Appl. Environ. Microbiol. 72:506-515. doi:10.1128/AEM.72.1.506-515.2006

Jiménez, A.J. 2009. As enzimas presentes no trato digestivo dos insetos: um alvo susceptível de inhibição. Tese Dou., Universidade de Brasília, Brasilia, BRA.

Kuhar, K., R. Kansal, B. Subrahmanyam, K.R. Koundal, K. Miglani, and V.K. Gupta. 2013. A Bowman–Birk protease inhibitor with antifeedant and antifungal activity from Dolichos biflorus. Acta Physiol. Plant. 35:1887-1903. doi:10.1007/s11738-013-1227-8

Lecadet, M.M., M.O. Blondel, and J. Ribier. 1980. Generalized transduction in *Bacillus thuringiensis* var. berliner 1715 using bacteriophage CP-54Ber. Microbiol. 121:203-212. doi:10.1099/00221287-121-1-203

Li, J., and H.A. Chase. 2010. Applications of membrane techniques for purification of natural products. Biotechnol. Lett. 32:601-608. doi:10.1007/s10529-009-0199-7

Lomate, P.R., and V.K. Hivrale. 2013. Effect of Bacillus thuringiensis (Bt) Cry1Ac toxin and protease inhibitor on growth and development of *Helicoverpa armigera* (Hübner). Pest. Biochem. Physiol. 105(2):77-83. doi:10.1016/j.pestbp.2013.01.002

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

Macedo, M.L.R, Md. Freire, O.L. Franco, L. Migliolo, and C.F. de-Oliveira. 2011. Practical and theoretical characterization of Inga laurina Kunitz inhibitor on the control of Homalinotus coriaceus. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 158:164-172. doi:10.1016/j.cbpb.2010.11.005

MacIntosh, S.C., G.M. Kishore, F.J. Perlak, P.G. Marrone, T.B. Stone, S.R. Sims, and R.L. Fuchs. 1990. Potentiation of *Bacillus thuringiensis* insecticidal activity by serine protease inhibitors. J. Agric. Food Chem. 38:1145-1152. doi:10.1021/jf00094a051

Mansour, S.A., M.S. Foda, and A.R. Aly. 2012. Mosquitocidal activity of two *Bacillus* bacterial endotoxins combined with plant oils and conventional insecticides. Ind. Crop Prod. 35:44-52. doi:10.1016/j.indcrop.2011.06.001

McDonald, K. 2007. Cryopreparation methods for electron microscopy of selected model systems. In: J.R. McIntosh, editor, Cellular electron microscopy. Academic Press, Elsiever, San Diego, CA, USA. p. 23-56. doi:10.1016/S0091-679X(06)79002-1

Méndez-López, I., R. Basurto-Ríos, and J.E. Ibarra. 2003. Bacillus thuringiensis serovar israelensis is highly toxic to the coffee berry borer, *Hypothenemus hampei* Ferr. (Coleoptera: Scolytidae). FEMS Microbiol. Lett. 226:73-77. doi:10.1016/S0378-1097(03)00557-3

Molina, D., A. Blanco-Labra, y H. Zamora. 2011. Inhibidores de proteasas de plantas efectivos contra las aspártico proteasas de *Hypothenemus hampei*. Rev. Colomb. Entomol. 37:183-191.

Oliveira, C.M., A.M. Auad, S.M. Mendes, and M.R. Frizzas. 2013. Economic impact of exotic insect pests in Brazilian agriculture. J. Appl. Entomol. 137:1-15. doi:10.1111/jen.12018

Oppert, B., T.D. Morgan, and K.J. Kramer. 2011. Efficacy of *Bacillus thuringiensis* Cry3Aa protoxin and protease inhibitors against coleopteran storage pests. Pest Manag. Sci. 67:568-573. doi:10.1002/ps.2099

Pan, D., A.P. Hill, A. Kashou, K.A. Wilson, and A. Tan-Wilson. 2011. Electrophoretic transfer protein zymography. Anal. Biochem. 411:277-283. doi:10.1016/j.ab.2011.01.015

Panchal, B.M., and M. Kachole. 2012. Identification of potent inhibitors of *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) gut proteinase from plant gum PIs. Int. J. Sci. Technol. 1:662-670.

Pardey, A.E.B. 2006. Una revisión sobre la broca del café, *Hypothenemus hampei* (coleoptera: Curculionidae: Scolytinae), en Colombia. Rev. Colomb. Entomol. 32:101-116.

Perić, V., M. Srebrić, L. Jankuloski, M. Jankulovska, S. Žilić, V. Kandić, and S. Mladenović-Drinić. 2009. The effects of nitrogen on protein, oil and trypsin inhibitor content of soybean. Genetika 41:137-144. doi:10.2298/GENSR0902137P

Portilla, R.M., y D. Streett. 2006. Nuevas técnicas de producción masiva automatizada de *Hypothenemus hampei* sobre la dieta artificial Cenibroca modificada. Cenicafé 57(1):37-50.

Rojas, M. 2012. Manejo sostenible de la broca del café (*Hypothenemus hampei*) mediante poda sistemática del cafeto en Costa Rica. Agron. Costarricense 36(2):71-79.

Roosta, H.R., T. Javadi, and F. Nazari. 2011. Isolation and characterization of trypsin inhibitors (Kunitz soybean trypsin inhibitor, Bowman-Birk inhibitor) in soybean. Adv. Environ. Biol. 5:145-153.

Rosenheim, J.A., and M.A. Hoy. 1989. Confidence intervals for the Abbott's formula correction of bioassay data for control response. J. Econ. Entomol. 82:331-335. doi:10.1093/jee/82.2.331

Ruan, J., J. Yan, S. Hou, H. Chen, Q. Wu, and X. Han. 2015. Expression and purification of the trypsin inhibitor from tartary buckwheat in Pichia pastoris and its novel toxic effect on Mamestra brassicae larvae. Mol. Biol. Rep. 42:209-216. doi:10.1007/s11033-014-3760-y

Schünemann, R., N. Knaak, and L. Fiuza. 2014. Mode of action and specificity of *Bacillus thuringiensis* toxins in the control of caterpillars and stink bugs in soybean culture. ISRN Microbiol. 2014:135675. doi:10.1155/2014/135675

Shamim, M., N.A. Khan, and K.N. Singh. 2011. Inhibition of midgut protease of yellow stem borer (*Scirpophaga incertulas*) by cysteine protease-like inhibitor from mature jackfruit (*Artocarpus heterophyllus*) seed. Acta Physiol. Plant. 33:2249-2257. doi:10.1007/s11738-011-0764-2

Shamsi, T.N., R. Parveen, A. Ahmad, R.R. Samal, S. Kumar, and S. Fatima. 2018. Inhibition of gut proteases and development of dengue vector, *Aedes aegypti* by *Allium sativum* protease inhibitor. Acta Ecol. Sin. 38:325-328. doi:10.1016/j.chnaes.2018.01.002

Shamsi, T.N., R. Parveen, M. Amir, M.A. Baig, M.I. Qureshi, S. Ali, and S. Fatima. 2016. Allium sativum protease inhibitor: a novel Kunitz trypsin inhibitor from garlic is a new comrade of the serpin family (EA Permyakov, Ed.). PLoS One 11(11):e0165572. doi:10.1371/journal.pone.0165572

Singh, S., A. Singh, S. Kumar, P. Mittal, and I.K. Singh. 2018. Protease inhibitors: recent advancement in its usage as a potential biocontrol agent for insect pest management. Insect Sci. 27:186-201. doi:10.1111/1744-7917.12641

Sriket, C., S. Benjakul, W. Visessanguan, and K. Hara. 2011. Effect of legume seed extracts on the inhibition of proteolytic activity and muscle degradation of fresh water prawn (*Macrobrachium rosenbergii*). Food Chem. 129:1093-1099. doi:10.1016/j.foodchem.2011.05.080

Suckling, D.M., L.D. Stringer, A.E. Stephens, B. Woods, D.G. Williams, G. Baker, and A.M. El-Sayed. 2014. From integrated pest management to integrated pest eradication: technologies and future needs. Pest Manag. Sci. 70:179-189. doi:10.1002/ps.3670.

Valencia, A., and J. Arboleda. 2005. Digestion of the inhibition αAI by *Hypothenemus hampei* aspartic proteinases. Rev. Colomb. Entomol. 31:117-121.

Valerio-Oviedo, A. 2006. Evaluación de la incorporación de diferentes fungicidas y dosis en dietas artificiales para la reproducción de la broca de café con miras a la multiplicación de sus parasitoides bajo condiciones controladas. Tesis Bach., Instituto Tecnológico de Costa Rica, Cartago, CRI.

Vasudev, A., and S. K. Sohal. 2016. Partially purified Glycine max proteinase inhibitors: potential bioactive compounds against tobacco cutworm, *Spodoptera litura* (Fabricius, 1775) (Lepidoptera: Noctuidae). Turk. J. Zool. 40:379-387. doi:10.3906/zoo-1508-20

Vega, F., J. Jaramillo, A. Castillo, and F. Infante. 2009. The coffee berry borer, *Hypothenemus hampei* (Ferrari) (Coleoptera: Curculionidae): a short review, with recent findings and future research directions. Terr. Arthropod. Rev. 2:129-147. doi:10.1163/187498209X12525675906031

Vidal-Quist, J.C., P. Castañera, and J. González-Cabrera. 2010. Cyt1Aa protein from *Bacillus thuringiensis* (Berliner) serovar israelensis is active against the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). Pest Manag. Sci. 66:949-955. doi:10.1002/ps.1965

Vorlová, L. 2011. Important minor soybens proteins: soybean allergens and enzymes inhibitors. In: H. El-Shemy, editor, Soybean and health. InTech, Brno, CZE. p. 425-432. doi:10.5772/1007

Wei-Salas, S., y A. Durán-Quirós. 2015. Caracterización del uso del suelo en las principales áreas agrícolas de la gran área metropolitana (GAM) de Costa Rica. Agron. Costarricense 39(1):149-160.

Zahiri, N.S., and M.S. Mulla. 2005. Non-larvicidal effects of *Bacillus thuringiensis* israelensis and Bacillus sphaericus on oviposition and adult mortality of *Culex quinquefasciatus* Say (Diptera: Culicidae). J. Vector Ecol. 30:155-162.

Zhao, A., Y. Li, C. Leng, P. Wang, and Y. Li. 2019. Inhibitory effect of protease inhibitors on larval midgut protease activities and the performance of *Plutella xylostella* (Lepidoptera: Plutellidae). Front Physiol. 9:1963. doi:10.3389/fphys.2018.01963

Zhu-Salzman, K., and R. Zeng. 2015. Insect response to plant defensive protease inhibitors. Ann. Rev. Entomol. 60:233-252. doi:10.1146/annurev-ento-010814-020816

Zibaee, I., A.R. Bandani, J.J. Sendi, R. Talaei-Hassanloei, and B. Kouchaki. 2010. Effects of *Bacillus thuringiensis* var. kurstaki and medicinal plants on *Hyphantria cunea* drury (Lepidoptera: Arctiidae). Invertebr. Surviv. J. 7:251-261.

Zorzetti, J., A.P.S. Ricietto, F.A.P. Fazion, A. M. Meneghin, P.M.O.J. Neves, L.A. Vilas-Boas, and G.T. Vilas-Bôas. 2018. Isolation, morphological and molecular characterization of Bacillus thuringiensis strains against Hypothenemus hampei Ferrari (Coleoptera: Curculionidae: Scolytinae). Rev. Bras. Entomol. 62:198-204. doi:10.1016/j.rbe.2018.07.002

Notes

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