EFFECTS OF BIOPESTICIDES IN *Tetragonisca angustula* LATREILLE (HYMENOPTERA: MELIPONINAE) POLLINATORS

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ABSTRACT: Stingless bees *Tetragonisca angustula* (Latreille) (Hymenoptera: Meliponinae) are pollinators of native and cultivated plants and are therefore in contact with areas contaminated by pesticides. These native bees were evaluated for changes in gene expression of esterase isoenzymes (EST) and peptides after contamination by contact with growth regulators from insecticides Gallaxy® EC 100, Natuneem and Azamax after 48, 120, 168 hours, 30 and 60 days. EST-4 presented an increase in relative activity after contamination with Gallaxy® EC 100 at 6.2×10^{-2} g a.i./mL; Natuneem at 7.5×10^{-5} g a.i./mL; and Azamax at 1.2×10^{-3} g a.i/mL after 60 days, 48 h, and 60 days, respectively. Inhibition of the relative activity of EST-4 was detected after contamination by Natuneem at 1.5×10^{-5} g a.i./mL and Azamax at 1.2×10^{-3} g a.i./mL after 48 h and 30 days, respectively. The insecticide growth regulators promoted changes in protein synthesis of *T. angustula* adult workers resulting in an increase or decrease in the relative intensity of bands, and the appearance of new peptides when compared with controls. Changes in protein synthesis have been identified mainly after long period of contamination, 120 and 168 h with the IGRs Gallaxy® EC 100 (at 0.78 and 1.25 g a.i./mL), Azamax (at 1.2×10^{-3} and 6×10^{-3} g a.i./mL), and Natuneem (at 7.5×10^{-5} and 3×10^{-3} g a.i./mL), and at 60 days with Natuneem (at 1.5×10^{-5} g a.i./mL).
KEYWORDS: Biopesticide growth regulators. Esterases. Peptides. Stingless bees.

EFEITOS DE BIOPESTICIDAS EM POLINIZADORES *Tetragonisca angustula* LATREILLE (HYMENOPTERA: MELIPONINAE)

RESUMO: Abelhas sem ferrão *Tetragonisca angustula* (Latreille) (Hymenoptera: Meliponinae) são polinizadores de plantas nativas e cultivadas e, portanto, estão em contato com áreas contaminadas por biopesticidas. Essas abelhas nativas foram avaliadas quanto a alterações na expressão gênica de isoenzimas esterases (EST) e peptídeos após contaminação por contato com reguladores de crescimento de inseticidas Gallaxy® EC 100, Natuneem e Azamax após 48, 120, 168 horas, 30 e 60 dias. A EST-4 apresentou um aumento na atividade relativa após a contaminação com Gallaxy® 100 EC em $6{,}2 \times 10^{-2}$ g i.a./mL, Natuneem em $7{,}5 \times 10^{-5}$ g i.a./mL e Azamax em $1{,}2 \times 10^{-3}$ g i.a./mL após 60 dias, 48 h e 60 dias, respectivamente. A inibição da atividade relativa de EST-4 foi detectada após contaminação pelo Natuneem a $1{,}5 \times 10^{-5}$ g i.a./mL e Azamax a $1{,}2 \times 10^{-3}$ g i.a./mL após 48 he 30 dias, respectivamente. Os reguladores de crescimento de inseticidas promoveram alterações na síntese protéica de trabalhadores adultos de *T. angustula*, resultando em um aumento ou diminuição da intensidade relativa das bandas e no aparecimento de novos peptídeos em comparação com os controles. Alterações na síntese de proteínas foram identificadas principalmente após um longo período de contaminação, 120 e 168 h com o IGRs Gallaxy® EC 100 (0,78 e 1,25 g i.a./mL), Azamax ($1{,}2 \times 10^{-3}$ e 6×10^{-3} g i.a./mL) e Natuneem ($7{,}5 \times 10^{-5}$ e 3×10^{-3} g i.a./mL) e 60 dias com Natuneem ($1{,}5 \times 10^{-5}$ g i.a./mL).
PALAVRAS-CHAVE: Abelhas sem ferrão. Esterases. Peptídeos. Reguladores de crescimento de biopesticidas.

EFECTOS DE LOS BIOPLAGUICIDAS SOBRE LOS POLINIZADORES *Tetragonisca angustula* LATREILLE (HYMENOPTERA: MELIPONINAE)

RESUMEN: Las abejas sin aguijón *Tetragonisca angustula* (Latreille) (Hymenoptera: Meliponinae) son polinizadores de plantas nativas y cultivadas y, por lo tanto, están en contacto con áreas contaminadas por bioplaguicidas. Estas abejas nativas fueron evaluadas para detectar cambios en la expresión génica de isoenzimas esterasa (EST) y péptidos después de la contaminación por contacto con los reguladores del crecimiento insecticidas Gallaxy® EC 100, Natuneem y Azamax después

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de 48, 120, 168 horas, 30 y 60 días. EST-4 mostró un aumento en la actividad relativa después de la contaminación con Gallaxy® 100 EC a 6.2×10^{-2} g i.a./mL, Natuneem a 7.5×10^{-5} g i.a./mL y Azamax a 1.2×10^{-3} g i.a./mL después de 60 días, 48 hy 60 días, respectivamente. La inhibición de la actividad relativa de EST-4 se detectó después de la contaminación por Natuneem a 1.5×10^{-5} g i.a./mL y Azamax a 1.2×10^{-3} g i.a./mL después de 48 hy 30 días. respectivamente. Los insecticidas reguladores del crecimiento promovieron cambios en la síntesis de proteínas de trabajadores adultos de *T. angustula,* resultando en un aumento o disminución de la intensidad relativa de las bandas y en la aparición de nuevos péptidos en relación a los controles. Los cambios en la síntesis de proteínas se identificaron principalmente después de un largo período de contaminación, 120 y 168 h con IGRs Gallaxy® EC 100 (0.78 y 1.25 g i.a./mL), Azamax (1.2×10^{-3} y 6×10^{-3} g i.a./mL) y Natuneem (7.5×10^{-5} y 3×10^{-3} g i.a./mL) y 60 días con Natuneem (1.5×10^{-5} g i.a./mL).
PALABRAS CLAVE: abejas sin aguijón. Esterasas. Péptidos Reguladores de crecimiento para bioplaguicidas.

Introduction

The native *Tetragonisca angustula* (Latrielle) (Hymenoptera: Meliponinae), are stingless known in Brazil as "Jataí" (MALAGODI-BRAGA; KLEINERT, 2004). This is distributed from Mexico to Argentina, except in the Andes, and is widely distributed in Brazil (CAMARGO; PEDRO, 2013). The *T. angustula* diet includes plants from the most diverse groups, as it is a high generalist species (SOUZA; ABREU; NOVAIS, 2019; VIEIRA *et al*., 2020). Through pollination, flowers provide nourishment to bees, and plants benefit from cross-fertilization, consequently, it results, improves crop quality, shelf life, and commercial value dos products (KLATT *et al*., 2014).

A decline in these species or inadequate pollination in some crops can cause losses in the production of 50% or more (KLEIN *et al*., 2007). The loss of fast-moving pollinators has been generated as a consequence of contemporary agriculture due to deforestation for agricultural expansion, agronomic practices with influence on the plant environment fertilizers and, mainly the use of pesticides (EKROOS *et al*., 2020; HALINSKI *et al*., 2020; OLLERTON *et al*., 2014; POWNEY *et al*., 2019; WOODCOCK *et al*., 2016).

The reduction or absence of the use of pesticides would cause a fall in agricultural production, an increase in production costs, and an increase in prices (KNUTSON, 1999). Moreover, the abusive use in crops can compromise the development pollinators (HLADIK; VANDEVER; SMALLING, 2016), affecting the ability to collect food and pollination, as well as honey production (RUVOLO-TAKASUSUKI *et al*., 2015; SILVA; MELO; BLANCO, 2016). Thus, it is recommended conscious use of Integrated Pest and Pollinator Management through the use of selective insecticides, that the same time having a good safety margin for most of the non-target biota (EGAN *et al*., 2020).

The pesticides known as growth regulators, the IGRs (Insect Growth Regulator Pesticides) have target-specific or stage-specific characteristics, have a good safety margin for most non-target biota including, invertebrates, fish, birds, among others. They are relatively safe for humans and pets. IGRs mimic juvenile hormone and / or ecdysone in the process of cuticle formation, inhibiting chitin synthesis and acting on the insect's endocrine system (DESNEUX; DECOURTYE; DELPUECH, 2007).

Among the IGRs, Gallaxy® EC 100 which has as the main chemical novaluron (100 g/L) and biopesticides produced of extracts from plants as *Azadirachta indica* (A. Juss) (Meliaceae) containing the metabolite azadirachtin such as the natuneem (3 g/L) and, the commercial product Azamax (12 g/L) (CHANDLER *et al*., 2011), can be hightly effective, with multiple mecanisms of action on target insects (CAMPOS *et al*., 2019).

However, these insecticides can be detoxified by esterase isoenzymes present high multifunctional hydrolytic activity, catalyze the hydrolysis of many esters (MOREIRA *et al*., 2018). Because the action of these isoenzymes in the metabolism of different compounds, changes in the expression or activity relative has been used to monitor the exposure of insects to xenobiotics (RUVOLO-TAKASUSUKI *et al*., 2015). Given what has been exposed to the importance of meliponinae especially *T. angustula*, and the risks produced by pesticides, this study aimed to analyze the effects of biopesticides Natuneem, Azamax and IGR Gallaxy®100 EC on changes in the isoenzymes esterases expression and peptides SDS-PAGE of contaminated workers with these commercial products.

Materials and methods

Bioassays

From the commercial formulations of the insecticides Natuneem, Azamax® (UPL) and Gallaxy®100 EC, dilutions in water were made and different concentrations obtained and preliminary tests were performed in the laboratory. For this purpose, petri dishes containing filter paper (12.5 ± 0.1 cm) were contaminated with 1 mL of dilute insecticide and T. angustula workers collected at the nest (n = 20) were placed on the plates. The candy (a mixture of honey 40 mL and casting sugar 70 g) was supplied as food for the bees. After 24 h of exposure, concentrations in which the survival rate of the workers was equal to or greater than 50% were selected to perform the bioassays in a semi-field.

Bioassays were performed with four colonies, three colonies treated with an IGR, and a control beehive. The concentrations used were 1.5×10^{-5}, 7.5×10^{-5} and 3×10^{-3} g a.i./mL of the Natuneem, 1.2×10^{-3} and 6×10^{-3} g a.i./mL of the Azamax, and 6.2×10^{-2}, 0.78 and 1.25 g a.i./mL of the Gallaxy® EC 100.

For treatments, 1 mL of each concentration of insecticides was applied on filter paper and introduced into the nests, onto the wall of the beehive. Filter paper with water was used on the control beehive. The collections of contaminated workers and control were taken after 48, 120, 168 h, 30, and 60 days. Samples were placed in labeled containers and kept in a freezer at -20°C.

Extractions of isoenzymes and peptides

PAGE and SDS-PAGE electrophoresis were performed with 20 workers (10 control samples and 10 treated samples). For the esterases electrophoresis, bees were homogenized individually in 90 µL of 0.1% β-mercaptoethanol and 10% glycerol solution, centrifuged at 10.000 g for 10 min. For SDS-PAGE, 30 µL of esterase samples were used and added sample buffer (5% glycerol, 2% Tris-HCl buffer 0.24M pH 6.8, 5% SDS, 0.5% β-mercaptoethanol and 2.5% bromophenol blue). The samples were placed for three minutes on boiling water (± 92°C) and then applied to the gel. For the identification of molecular weights in the electrophoretic profile, we used the molecular pattern of proteins BenchMark™ Protein Ladder (10-220 kDa).

Polyacrylamide gel electrophoresis – page

Polyacrylamide gels at a concentration of 8% followed by stacking gels to 5% were used. The running buffer used was 0.1 M Tris-Glycine pH 8.3. The gels were submitted to electrophoresis at a constant voltage of 200 V for 5 hours at 5°C. After electrophoresis, the gel was incubated for 30 min in 50 mL of sodium phosphate buffer 0.1 M pH 6.2. Then the buffer was discarded and added to the staining solution, 50 mL sodium phosphate buffer 0.1 M pH 6.2, 0.03 g of α-naphthyl acetate, 0.03 g of β-naphthyl acetate; 0.06 g of Fast Blue RR Salt. The esterases were visualized on the gels as brown (α-esterases) or red (β-esterases) bands.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

The system of vertical electrophoresis was performed using polyacrylamide gels at 7% concentration and stacking gel gels at 5%, both containing 10% SDS. The running buffer used was Tris-glycine 0.1 mol/L, pH 8.3, and SDS (10%). The electrophoresis was performed at 90 V for 5h at room temperature. The peptides were visualized with silver nitrate ($AgNO_3$) staining.

Results

Profile of esterase in stingless bees exposed to biopesticide

Electrophoretic analysis of *T. angustula* bees revealed alterations in relative activity of EST-4 in treated individuals, compared to control as shown in Table 1. Natuneem to 1.5×10^{-5} g a.i./mL inhibition of EST-4 in treated individuals were detected, compared to the control after 30 days. The partial increase in the relative activity of EST-4, when the Natuneem 7.5×10^{-5} g a.i./mL was applied, was detected in treated subjects after 48 h. Azamax, when diluted and applied at 1.2×10^{-3} g a.i./mL in the nest, has partially inhibited the relative activity of EST-4 of treated subjects after 48 h. After 60 days, an increase in the relative activity of EST-4 in treated. The Gallaxy® EC 100 this insecticide at 6.2×10^{-2} g a.i./mL partially increased the relative activity of EST-4 of treated subjects compared to the control after 60 days of infection.

Table 1: Analysis of relative activity for the EST-3 and EST-4 in *Tetragonisca angustula* after contamination with Natuneem, Azamax, and Gallaxy® 100 EC. The concentrations are given in grams of active ingredient per milliliter (g a.i./mL).

	Natuneem				Azamax		Gallaxy® 100 EC			
	1.5×10^{-5}		7.5×10^{-5}		1.2×10^{-3}		6.2×10^{-2}		0.78	
Time	EST-3	EST-4	EST-3	EST-4	EST-3	EST-4	EST-3	EST-4	EST-3	EST-4
48 h	N.C	N.C	N.C	+	N.C	-	N.C	N.C	N.C	N.C
120 h	N.C	N.C	N.E	N.E	N.C	N.C	N.C	N.C	N.E	N.E
168 h	N.E	N.E	N.E	N.E	N.C	N.C	N.E	N.E	N.E	N.E
30 d	N.C	-	N.E	N.E	N.E	N.E	N.C	N.C	N.E	N.E
60 d	N.C	N.C	N.E	N.E	N.C	+	N.C	+	N.E	N.E

N.C: No changes; N.E: No estimated; (-) Inhibition; (+) Increased of relative activity; h: hours; d: days.

Treatment using Natuneem at 1.5×10^{-5} g a.i./mL (after 30 days) has presented a stronger inhibition of EST-4 activity than Azamax at 1.2×10^{-3} g a.i./mL (after 48 h). On the other hand, the partial relative increase of EST-4 on the treatment with Gallaxy at 6.2×10^{-2} g a.i./mL and Azamax at 1.2×10^{-3} g a.i./mL was detected after 60 days. No change in the EST-3 activity was detected, which can lead to understanding that Azamax, Natuneem, and Gallaxy® EC 100 insecticides do not act on cholinesterases in *T. angustula*.

Observations of *T. angustula* made during foraging in the morning and afternoon showed that after 48 hours of the application of the insecticide Azamax at 6×10^{-3} g a.i./mL in the nest, the number of bees at the entrance of the nest and foraging decreased dramatically over the days, which hampered the collection of material for analysis. However, no dead bees were found in the vicinity of the nest, which may be due to the repellent action that azadirachtin exerts on bees.

Alterations on the soluble proteins after exposure to pesticide

The peptides were identified according to the molecular weight given in kDa. The number of peptides presents in *T. angustula* extracts has changed after contamination with Gallaxy® EC 100 at 0.78 and 1.25 g a.i./mL; Natuneem at 1.5×10^{-5}, 7.5×10^{-5} and 3×10^{-3} g a.i./mL; Azamax at 1.2×10^{-3} and 6×10^{-3} g a.i./mL, the alterations were observed in SDS-PAGE profiles of *T. angustula* workers after different treatments with IGRs tested (Table 2).

Table 2: Changes in the expression of soluble peptides present in *T. angustula* extracts after treatment with growth regulators insecticides over time (48 h to 60 days).

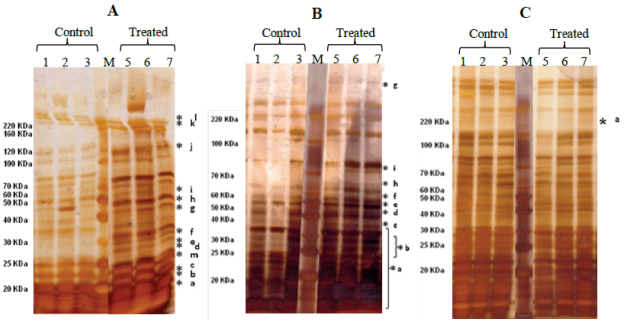
	Gallaxy ® 100 EC			Natuneem		Azamax	
MW	0.78	1.25	1.5×10^{-5}	7.5×10^{-5}	3×10^{-3}	1.2×10^{-3}	6×10^{-3}
20	+120h, 168h	+ 120h, 168h				+168h	
25	+*120h, 168h	+120h, 168h		-120h, +168h		+168h	
30	+*120h, 168h	+120h, 168h		-120h, +168h			
40	+120h, 168h	+120h, 168h		-120h, +168h			
50	+120h, 168h	+120h, 168h		-120h, +168h			
60	+120h, *168h	+120h, 168h		-120h, +168h	+120h		
70	+*168h	+168h		-120h, +168h	+168h		+168h
90					*168h		
100	+*168h	+168h		+*168h			
120				+120h, +*168h			
220	+120h, 168h	+120h, 168h	+ 60d	+48h, +*168h			

(+) Increase in intensity of the band; (-) Decrease of intensity of the band; * = New detection peptide, peptide # = disappearance of detection; MW = molecular weight (kDa).

Two concentrations of IGR Gallaxy® EC 100 were analyzed (0.78 and 1.25 g a.i./mL). Changes in protein synthesis and its consequent alteration in the expression of soluble peptides present in extracts of workers analyzed were observed from 48 h of contamination, in general, there was an increase in the relative intensity of the bands and the appearance of peptides in the treated samples. These effects extended up to 168 h after the application of the insecticide into the bees nest.

Individuals treated with Gallaxy® EC 100 diluted at 0.78 g i.a./mL presented, in general, increased relative intensity of the peptides after 120 (Figure 1A) and 168 h (Figure 1B). After 120 h, a peptide is detected with increased relative intensity in treated individuals, near the region between 20 and 25 kDa (only one peptide), 30 kDa (two peptides), and 220 kDa (two peptides). Between 30 and 40 kDa, between 40 and 50 kDa, in 50 kDa, in 60 kDa, and between 120 and 160 kDa have presented only one peptide with higher intensity compared to the control. Between 25 and 30 kDa, a new peptide was only detected in the treated samples (Figure 1A).

Figure 1: *Tetragonisca angustula* SDS-PAGE total protein gel after exposure to the insecticide Gallaxy EC 100, stained with silver nitrate. A. Control (1 to 3), treated with 0.78 g a.i./mL for 120 h (5 to 7); B. Control (1 to 3), treated with 0.78 g a.i./mL for 168 h (5 to 7); C. Control (1 to 3), treated with 1.25 g a.i./mL for 48 h (5 to 7).

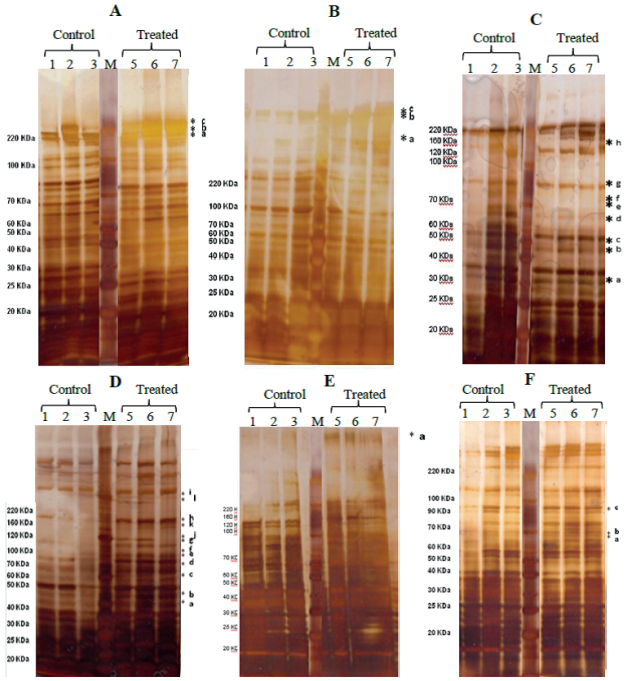


M: molecular weight standard. Asterisks followed by letters horizontally identify the affected peptide regions.

After 168 h, the increased relative intensity of the peptides was detected for regions between 10 and 30 kDa (several peptides), with emphasis on the region between 25 and 30 kDa in the treated samples. The only regions that showed only one stronger peptide, on the treated samples are between 30 and 40 kDa, in 40 kDa, in 50 kDa, between 50 kDa and 60 kDa and above 220 kDa (Figure 1B). The regions between 60 and 70 kDa, as well as, 70 kDa and 100 kDa, emerged more intensely stained than the controls and the emergence of a new band was detected in extracts of workers treated with IGR, in both regions (Figure 1A and B). When Gallaxy® EC 100 was diluted to 1.25 g a.i./mL, reduction of gene expression in only one peptide was detected, with approximately 220 kDa in the treated samples after 48 h (Figure 1C).

When diluted Natuneem at 1.5×10^{-5} g a.i./mL was applied within the *T. angustula* nests, increased protein synthesis of three regions near 220 kDa was detected in treated subjects after 60 days (Figure 2A). With the same IGR at 7.5×10^{-5} g a.i./mL changes in the peptides in the periods of 48, 120, and 168 h were detected. After 48 h, in general, there was almost no peptide modification of treated individuals, but for the region located above 220 kDa there was observed an increased relative intensity of three peptides only in treated individuals (Figure 2B). After 120 h, it was verified partial reduction of peptide relative intensity between 25 and 30 kDa, in 40 kDa, between 40 and 50 kDa, in 60 kDa, between 60 and 70 kDa and 70 kDa. The 120 kDa region showed a more intense peptide in treated individuals, compared to control (Figure 2C).

Figure 2: *Tetragonisca angustula* SDS-PAGE total protein gel after exposure to Natuneem, stained with silver nitrate. A. Control (1 to 3), Treated with 1.5 g a.i./mL for 60 days (5 to 7); B. Control (1 to 3), Treated with 7.5 × 10^{-5} g a.i./mL for 48 h (5 to 7); C. Control (1 to 3), Treated with 7.5 × 10^{-5} g a.i./mL for 120 h (5 to 7); D. Control (1 to 3), Treated with 7.5 × 10^{-5} g a.i./mL for 168 h (5 to 7); E. Control (1 to 3), Treated with 3 × 10^{-5} g a.i./mL for 120 h (5 to 7); F. Control (1 to 3), Treated with 3 × 10^{-5} g a.i./mL for 168 h (5 to 7).



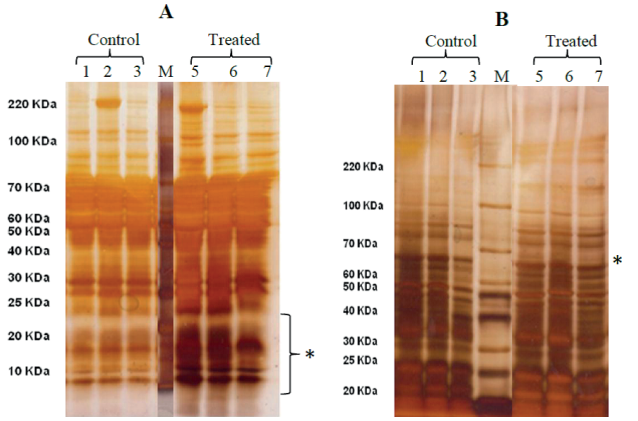
M: molecular weight standard. Asterisks followed by letters horizontally identify the affected peptide regions

At 168 h after the application of Natuneem at 7.5 × 10^{-5} g a.i./mL one can observe an overall increase of the relative intensity of the peptide in the treated samples (Figure 2D). The increase in gene expression in only one peptide was detected in regions 40 kDa, between 40 and 50 kDa, in 50 kDa, in 60 kDa, between 60 and 70 kDa, in 70 kDa, in 100 kDa, in 120 kDa and 220 kDa. The emergence of peptides was also detected in the regions between 100 and 120 kDa, and slightly above 220 kDa. Only one peptide of 220 kDa was observed in the treated samples, when the commercial biopesticide Natuneem has applied at 3 × 10^{-3} g a.i/mL in a *T. angustula* nest after 120 h (Figure 2E). Only the regions between 60 and 70 kDa, which presented two peptides with an increase of relative intensity, and 90 kDa, which arises a new peptide, are altered in treated subjects compared to control after 168 h (Figure 2F).

Natuneem affected protein synthesis of *T. angustula* workers from 48 h of contamination and extended up to 60 days. The region located within or close to 220 kDa emerged with a relative intensity increase of the bands in all gels that showed altered peptide expression (Figure 2). A similar finding was seen in the results for Gallaxy® EC 100 Biopesticide (Figure 1).

For Azamax commercial product, composed of azadirachtin 12 g/L, in general, no changes in protein synthesis compared to controls were observed. With Azamax 1.2 × 10^{-3} g a.i./mL, an increase in relative peptide synthesis in the regions between 10 and 20 kDa was detected after 168 h of contamination (Figure 3A). In the same period, only Azamax at 6 × 10^{-3} g a.i./mL promoted an increase in the intensity of bands present in the region 70 kDa after 168 h (Figure 3B).

Figure 3: *Tetragonisca angustula* SDS-PAGE total protein gel after exposure to Azamax, stained with silver nitrate. A. Control (1 to 3), treated with 1.2 × 10^{-3} g a.i./mL for 168 h (5 to 7); B. Control (1 to 3), treated with 6 × 10^{-3} for 168 h (5 to 7).



M: molecular weight standard. Asterisks followed by letters horizontally identify the affected peptide regions.

Daily observations of the *T. angustula* nests were performed to observe any changes in the population of workers entering/leaving the hive in the morning and afternoon; workers killed around the nest, and behavior change. Regarding the nest contaminated with Gallaxy® EC 100 overall experiments performed, nothing has been found about any change in behavior, death of workers closes to the nest, or reduced activity the nest.

After 168 h of treatment with Azamax at 6 × 10^{-3} g a.i./mL there was dramatically reduced hive activity of workers throughout the day. This fact prevented the collection of samples for periods of 30 and 60 days. A similar situation occurred with Natuneem at 3 × 10^{-3} g a.i./mL after 168 h.

Discussion

Stingless bees belonging to the genus *Tetragonisca* are common in tropical environments (MACÍAS-MACÍAS *et al*., 2009) and, as pollinators, visit agricultural regions becoming contaminated (WITTER *et al*., 2015). Thus, applying insecticides inside the beehive is a methodology which allows simulating the contamination of bees with pesticides in the environment, and to investigate the possible effects on individuals within the hive. Although scarce for stingless bees, these studies are essential for determining the chronic effects of insecticides, since pollinators remain exposed for a long time to residual doses of insecticides (Botias *et al*., 2015).

In these semi-field tests, environmental changes, half-life, and the persistence of the products used, as well as, the interaction with other compounds, can interfere with the adverse effects observed in bees. However, they simulate the real conditions encountered by bees in the wild, being more

reliable than laboratory tests, where experimental conditions are controlled, the number of individuals and the exposure time is reduced (24 to 72 h) (Moreira *et al*., 2018), as well as, the concentration of insecticides are known (Pisa *et al*., 2017), which may increase the probability of the occurrence and detection of damages after exposure to different insecticides (Romeis *et al*., 2011).

Among them, biopesticides (GRI) as Natuneem, Azamax and Gallaxy® 100 EC has been used in agriculture (CAMPOS *et al*., 2019). The Natuneem, and Azamax have azadirachtin as an active ingredient that may affect survival, cause repellency, feeding disorder, regulate growth, effect antifeeding, locomotory and physiological reduce female fertility, mating behavior, cause anatomical abnormalities, cause detrimental histopathological effects in neural hormones glands, in reproductive tissues and intestine epithelial cells affect protein metabolism in insects (ANDREAZZA *et al*., 2020; BARBOSA *et al*., 2015; BERNARDES *et al*., 2017; BERNARDES *et al*., 2018; FERDENACHE *et al*., 2019; LAI *et al*., 2014; LIMA *et al*., 2015; SHU *et al*., 2018; SILVA *et al*., 2020; SUN *et al*., 2018; TSCHOEKE *et al*., 2019).

In parallel, the commercial Gallaxy® 100 EC, with Novaluron as main active compound, besides acting as a growth regulator in insects, also inhibits the chitin biosynthesis, interfering with cuticle sclerotization during insect molting, moreover, impairs development the silk gland (FARNESI *et al*., 2012; MERZENDORFER, 2013; PITTS-SINGER; BARBOUR, 2017; SANTORUM *et al*., 2020).

The bees that have been contaminated directly or indirectly with insecticides used to control agricultural pests and the exposure of pollinators and other insects to agrochemicals, can be monitored by changes in isoenzyme relative activity, such as the esterases (RUVOLO-TAKASUSUKI *et al*., 2015). Such changes are caused by spontaneous genomic alterations lead to amplification, overexpression, or changes in the sequence of the genetic code of these metabolic genes, as protection mechanisms of the own organisms, against toxic substances such as biopesticides and allelochemicals (CATTEL *et al*., 2019).

In *T. angustula,* esterases EST-3 (colinesterase) e EST-4 (carboxilesterase) previously described by Stuchi *et al.* (2012), were evaluated after exposure to insecticides growth regulators Natuneem, Azamax e Gallaxy®100 EC because they play key roles in detoxification of xenobiotic compounds, participating in insecticide resistance. The EST-3 showed no change in relative activity after exposure to all insecticides and concentrations tested in *T. angustula*. On the other hand, differentially, EST-4 presented increase or inhibition of the relative activity according to tested concentrations and exposure periods in *T. angustula*, which is indicative of your participation in the detoxification of the body of the bee after contamination with IGRs.

However, when bee contamination occurs with chemical pesticides, there is a greater effect on these enzymes. The EST-2 presented effect of detoxification, increasing its activity by 25% in an in vitro test with organophosphate insecticides methylparathion and malathion for 21 days in *A. mellifera* Africanized (ATTENCIA; RUVOLO-TAKASUSUKI; TOLEDO, 2005). Also according to the same authors, different from what was observed in this study, the EST-3, had a 75% and 50% increase in its relative activity in α-naphthyl acetate and α-naphthyl butyrate substrates, respectively, after seven days of 0.05% application methyl parathion. However, when observing the effect of the broad-spectrum neonicotinoid insecticide thiamethoxam, the electrophoretic analyses showed a reduction in the relative activity of the esterases 1, 2, 4, and 5 by contact and by ingestion with *A. mellifera* (HASHIMOTO; RUVOLO-TAKASUSUKI; TOLEDO, 2003).

Stuchi (2009), noted that after ingestion of fipronil by *T. fiebrigi* workers, electrophoretic analysis of extracts from head/thorax showed a change of esterases regions EST-1 and EST-4 for the concentration of 0.0012%, but when the insecticide malathion was used, we observed partial inhibition of EST-4 at concentrations of 0.2% and 0.45%. Still, this author, the electrophoretic analysis of *T. angustula* samples presented decreased relative intensity of the EST-3 and EST-4 regions for concentrations of 0.003% and 0.0025% of malathion, respectively, when contaminated by contact. But when infected by ingestion, we observed partial inhibition of EST-3 region at the concentration of 1%, and EST-4 region at 1% and 2%. When thiamethoxam is applied at 0.1%, EST-3 and EST-4 esterases were partially inhibited.

According to Caboni *et al*. (2002), the half-life calculated for the isolated azadirachtin is 13.2 h, while for the commercial formulation was 2.7 h, this fact can be in contradiction with the results obtained with Natuneem because the Biopesticide can somehow still be interfering with the functioning of the body of worker bees for long periods, as detected after 60 days. Another hypothesis is that the other compounds, which are part of this emulsion, should be affecting the body of the insect as reported by Ciociola and Martinez (2002) and Martinez (2002). Similarly, the label of Gallaxy® 100 EC states that this biopesticide is highly persistent in the environment, half-life to of 68-76 days, but the analyses of SDS-PAGE show their effect on peptides up to 168 h after application.

Although studies recommend the use of azadirachtin in systems integrated pest and pollinator management (EGAN *et al*., 2020), and have a moderately toxic effect on bees and provide an increase in productivity in oilseed plants when compared to chemical pesticides (CHALLA; FIRAKE; BEHERE, 2019). Many researches raise concerns about the use of biopesticides in crops concerning pollinators. In that study, there was no death of *T. angustula*, and it did not affect the mortality, flight or breathing of worker bees of *Melipona quadrifasciata* e *Partamona helleri* (BERNARDES *et al*., 2017), the biopesticide azadirachtin reduces the survival of *P. helleri* queens, having an action on the reproductive system and its morphology, which can lead to compromising the maintenance of these stingless bees (BERNARDES *et al*., 2018).

The same behavior is observed with the biopesticide novaluron, as in this study, it does not affect the workers of *T. angustula* and *T. fiebrigi* (FERMINO *et al*., 2011) and *Megachile rotundata* F. (PITTS-SINGER; BARBOUR, 2017). However, low doses of 100 ppb and 100 ppm, respectively, are toxic for the development of *A. mellifera* bees. This dose is even lower when feeding by contact within the colonies of *A. mellifera*, presenting chronic exposure to novaluron at doses of 18.6 ppm, which may result in interruptions in the production of litters, which can last

up to two weeks after exposure (FINE *et al*., 2017). Also, proportions of dead eggs and larvae and lower proportions of live pre-pupae were observed when the bees were exposed to recent spraying of novaluron with *M. rotundata* F. (PITTS-SINGER; BARBOUR, 2017). No mortality was observed in this study, but it shows that in *T. Angustula*, EST-4 (carboxylesterase) plays a role in the detoxification of the bee's body after contamination with IGRs resulting in its survival.

Insecticides growth regulator Gallaxy® EC 100, Natuneem, and Azamax influence the expression of EST-4 isoenzyme of *T. angustula*. The IGRs analyzed promote alterations in *T. angustula* protein synthesis, with increased, decreased synthesis and beginning of new peptide synthesis. Variation of the biopesticide effect on different generations in the treated individuals can also be considered. Because each treatment lasted for 60 days, more than a generation of bees inside the nest was able to be in contact, via spiracles, and/or ingestion of contaminated products of the hive with the insecticide. This may explain the variation of the effect (increase or inhibition) on the relative activity of esterases in the same treatment.

The changes in protein level occurred over a long period, 48 hours to 60 days after contamination by contact. The *T. angustula* bees are sensitive to environmental contamination by IGRs of Gallaxy® EC 100, Natuneem, and Azamax at sublethal doses. The region 220 kDa could be a possible candidate region as an environmental bioindicator of the presence of Gallaxy ® EC 100 and Natuneem in the environment. This region was characterized, in the contaminated samples, by presenting a relative increase in protein synthesis in treated individuals, and even the emergence of peptides that were hitherto absent in control. Indeed, EST-4 also has the potential to be used as a molecular marker for these insecticides.

Conclusion

The growth regulating insecticides of Gallaxy® EC 100, Natuneem, and AzaMax influence the expression of the EST-4 isoenzyme in *T. angustula*. They promoted changes in the synthesis of *T. angustula* proteins, providing an increase in some peptides and a reduction in the synthesis of others, as well as the synthesis of new peptides. Changes in protein levels occurred over a long period of 48 hours to 60 days after contact contamination. *T. angustula* bees were sensitive to environmental contamination by IGRs of Gallaxy EC 100, Natuneem, and Azamax in sublethal doses.

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