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LEAF-CUTTING ANTS *Acromyrmex niger* SMITH, 1858 (HYMENOPTERA; FORMICIDAE) USED AS BIOINDICATORS OF AGROTOXICS RESIDUES

Utilización de hormigas cortadoras *Acromyrmex niger* Smith, 1858 (Hymenoptera; Formicidae) como bioindicadores de residuos de agrotóxicos

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ABSTRACT

Despite the condition of leaf-cutting ant pests in agroecosystems, it is undeniable the benefits they can bring in certain situations or environments. The leaf-cutting ants of the genus *Acromyrmex* attack mainly leaves of vegetables and fruit trees exposing not only to the agrochemicals used for their control as well as to those used for the control of other pests. Due to the bioindicator potential of environmental quality of the ants and their frequent exposure to agrochemicals such as organophosphates, neonicotinoids and growth regulators insecticide used for pest control, it is necessary to study the sublethal effects that these pesticides may cause. The electrophoresis technique was used to study the activity of esterase isozymes involved in the metabolism of xenobiotics of *A. niger*, combined with changes in the expression of isozymes after contamination by pesticides. *A. niger* showed eight regions of esterase activity, which were called EST-1, EST-2, EST-3, EST-4, EST-5, EST-6, EST-7 and EST-8 according to the electrophoretic mobility. As the specificity to α and β-naphthyl acetate substrates, the Est-7 and Est-8 may be classified as α-esterase and the others as αβ esterases. EST-5 is considered an enzyme of the type cholinesterase II and the others are of the type carboxilesterase. The electrophoretic analysis showed partial inhibition to all esterases subjected to the contact with Malathion organophosforate at the concentrations 1 x 10^{-3} % and 5 x 10^{-3} %, which may be considered as a biomarker for the presence of residues of this insecticide in the environment. The regression analysis for sublethal effects of the tested pesticides demonstrated correlation between dose and mortality only for Thiametoxam neonicotinoid pesticide.

Keywords: *Acromyrmex niger*, esterases, bioindicators.

RESUMEN

A pesar de la condición de plaga de las hormigas cortadoras en agroecosistemas, no se pueden negar los beneficios que ellas pueden traer en determinadas situaciones o en determinados ambientes. Las hormigas cortadoras del género *Acromyrmex* atacan principalmente las hojas de hortalizas y hojas de plantas fructíferas, exponiéndose no solo a los agroquímicos utilizados

para su control sino también a aquellos utilizados en el control de otras plagas. Debido al potencial bioindica-dor de la calidad ambiental de las hormigas y su frecuente exposición a los agrotóxicos como organofosforados, neonicotinoides y reguladores de crecimiento, utilizados en el control de plagas, es necesario el estudio de los efectos subletales que estos agrotóxicos pueden causar. La técnica de electroforesis fue utilizada para evaluar la actividad de las isoenzimas esterasas involucradas en el metabolismo de xenobióticos de *A. niger*, asociada a las alteraciones en la expresión de las isoenzimas después de la contaminación con pesticidas. *Acromyrmex niger* demostró ocho regiones de actividad esterasa, las cuales fueron denominadas EST-1, EST-2, EST-3, EST-4, EST-5, EST-6, EST-7 e EST-8 de acuerdo con la movilidad electroforética. En cuanto a la especificación de los substratos α y β-nafitl acetato, las EST-7 e EST-8 son clasificadas como α-esterasa y las demás αβ esterasas. EST-5 es considerada una enzima del tipo colinesterasa II y las demás son carboxilesterasas. El análisis electroforético presentó inhibición parcial para todas las esterasas sometidas al contacto con malathion en las concentraciones 1 x 10^{-3} % e 5 x 10^{-3} %, que puede ser considerado un biomarcador para la presencia de residuos de este insecticida en el ambiente. El análisis de regresión para el efecto subletal de los pesticidas evaluados demostró correlación entre la dosis y la causa de muerte solo para el pesticida neonicotinoide thiametoxam.

Palabras claves: *Acromyrmex niger*, esterasas, bioindicadores.

INTRODUCTION

Currently, 20 species and nine subspecies of *Acromyrmex* are described (Zanetti, 2002). Their geographical distribution is typical of America and their area of distribution begins in California (United States), going to Mexico and continuing through Central America and all over the countries of South America (except Chile) to Patagonia (Argentina). It also occurs in Cuba and Trinidad (West Indies) and in Brazil they are in all states (Gonçalves, 1961).

Despite the condition of pests of these ants in agroecosystems, requiring the need for chemical control to reduce economic losses, it is undeniable the benefits that ants can bring in certain situations or environments. According to Moutinho *et al.* (1993), the leaf-cutting ants may have positive impacts concerning about the physical and chemical structure of the soil and potentially benefit the vegetation by encouraging its growth, for in areas with nests the soil is less resistant to root penetration and the organic matter in garbage chambers promotes the fertility of the soil.

Pollutants such as pesticides used to control pests in agriculture are composed of several chemicals with different functional groups, each with a particular way of action on the biological targets and harmful action on other organisms and environment (Colborn, 2006). The presence of pesticide residues is usually determined by physical, chemical and biological methods and the simplicity of bioassays contributes to the acceptance of the residual analysis in the field. Theoretically, any organism that is susceptible to an insecticide may be used in these bioassays in any environmental sample serving as a bioindicator for the detection of certain pollutants (Mansour, 1987). According Peakall (1994) can be considered biomarkers any change in biological response of an organism, from molecular level to community structure or even ecosystems as a result of the effect of chemical contamination in the environment.

Van der Oost *et al.* (2003) considerer that the use of multiple biomarkers is important because it may provide answers about the action of environmental chemicals in organisms, and each detected response can be considered a biomarker. In this study, it was used the leaf-cutting ants *A. niger* (Smith, 1858), known popularly as "*quenquén mineira*", which mainly cut leaves of vegetables and also of fruit trees. These ants expose themselves not only to the agrochemicals used for their control as well as to those used for the control of other pests such as organophosphate, neonicotinoid and growth regulator.

Therefore, due to the potential bioindicator of environmental quality of the ants and their frequent exposure to pesticides, was developed a study about sublethal effects that these pesticides may cause. The electrophoresis technique was used to study the activity of esterase isozymes involved in the metabolism of xenobiotics of *A. niger*, combined with changes in the expression of isozymes after contamination by pesticides.

MATERIAL AND METHODS

Biological material

The *A. niger* workers were collected from a single nest at the campus of State University of Maringá (23° 26' of south latitude, 51° 56' of west longitude) in different seasons.

Workers of *A. niger* were collected in the nest and brought alive to the laboratory of Animal Genetics, State University of Maringa, Brazil, sacrificed and subjected to PAGE electrophoresis for identification and biochemical characterization of esterase.

Afterwards we made collections of other *A. niger* workers that were exposed to insecticides (biassays) and maintained in the laboratory for 24 hours at room temperature (± 27° C), relative humidity of 45 % and absence of light.

PAGE electrophoresis for esterase

The *A. niger* workers were sacrificed and homogenized individually in propylene tubes containing 35 µL of the extraction solution prepared with 2-mercaptoethanol and 10 % glycerol. Then, the samples were centrifuged at 56,000 xg for 10 min at 4 °C.

The vertical electrophoreses for esterase were performed using polyacrylamide gels 10 % of concentration and stacking gel with a concentration of 5 %. The running buffer used was

Tris-Glycine 0.1 M pH 8.3. The gels were electrophoresed at a voltage of approximately 200V for 5 hours.

For esterase staining, the gel was incubated at a temperature of ± 37° C for 30 minutes in 50 mL of phosphate buffer solution (0.1 M pH 6.2). After the incubation period, the solution was discarded and added the staining solution prepared with 50 mL of phosphate buffer 0.1 M pH 6.2; 0.03 g of α-naphthyl acetate; 0.02 g of β-naphthyl acetate; 0.06 g of Fast Blue RR Salt dye. The gel was once again incubated until the appearance of esterases bands.

Later, the gels were kept in a preservative solution (acetic acid 75 % and glycerol 10 %, dissolved in distilled water) for at least 24 hours. They were then soaked in gelatin 5 % and placed between two sheets of soaked, stretched and pressed cellophane kept at room temperature until complete drying (Ceron *et al.*, 1992).

Biochemical characterization of esterase

Esterases biochemical characterization was performed by analysis of inhibition as described by Healy *et al.* (1991), after the PAGE electorophoresis.

The homogenized and centrifuged samples were applied in the polyacrylamide gel, and for staining it was cut into two equal parts. One half of the gel was used for the control and the other half for the inhibition test. The inhibitors used were the organophosphate malathion (20 µL), the parachlorine-mercury- benzoate sulfhydryl reagent or ρ-CMB (0.08 g) and eserine sulfate (0.08 g) dissolved in 100 mL of sodium phosphate buffer solution (0.1 M pH 6.2) and tested separately. The half reserved for the control was incubated for 30 min in 50 mL of sodium phosphate buffer solution (0.1 M pH 6.2) and the other half reserved for the inhibition test was incubated in 50 mL of phosphate buffer with the inhibitor to be tested. After incubation with the buffer, the staining occured normally for the control, however, for the test, it was used the remaining 50 mL of the buffer containing the diluted inhibitor. After the visualization of the bands, it was done the comparison between the control and test gels.

Bioassay with insecticide

The ants were collected in plastic bottles and brought to the freezer for about one minute, only to cause stunning, which facilitated the accurate count of subjects per Petri dish (15 cm diameter) for LC_{50} estimate. The bioassay had 4 Petri dishes for each of the three insecticides to be tested. Each dish contained 20 subjects, where one dish was for control and the others relating to the repetitions of the concentrations of the tested insecticides. In the dish for control, the 20 subjects were in contact with filter paper soaked only in water for 24 hours. In the three replicates dishes, the 20 subjects of each dish were in contact with filter paper soaked in 2 ml of the pesticide solution to be tested. These pesticides diluted in water in the following concentrations expressed in percentage: malathion 1; 2; 3; 4; 5; 7; 8 and 10 x 10^{-3} %, thiamethoxam 0,2; 0,5; 1; 2; 3 and 4 x 10^{-3} % and azadirachtin 0.5; 1; 5; 7; 5; 8 and 10 x 10^{-3} %. After 24 hours it was counted how many dead ants there were, and the ones which were alive were sacrificed for electrophoretic analysis. The insecticides used were the organophosphate malathion, the neonicotinoid thiamethoxam and the growth regulator Azadirachtin.

The concentrations of the tested insecticides were obtained from the amount of active ingredient present in the commercial product. Each insecticide was prepared as prescribed by the manufacturer and dilutions were performed from the primary solution to obtain the LC_{50}.

Data analysis

The results were analyzed using the program SPSS v. 13.0 (SPSS Inc. Chicago, Il) for probit analysis of the LC_{50} and for the correlation between the concentration of insecticide and mortality of subjects.

RESULTS

In the electrophoretic analysis of the samples of *A. niger* homogenized individually, 8 regions of esterase activity were detected, which were designated EST-1, EST-2, EST-3, EST-4, EST-5, EST-6, EST-7 and EST-8 according to the electrophoretic mobility (Fig. 1). As to its specificity to substrate α-naphthyl acetate and β- naphthyl acetate, we can categorize EST-7 and EST-8 as α-esterases and the others as αβ-esterases. This study has submitted the samples of *A. niger* to the three groups of inhibitors (organophosphorus, eserine sulfate and sulfhydryl reagents) and revealed that the region EST-5 of *A. niger* should be classified as cholinesterase II and the others as carboxylesterases (Table 1).

For the bioassay with organophosphate Malathion the LC_{50} was 8.7 x 10^{-3} %, $p < 0.001$. The regression analysis that there is no correlation between mortality and the concentration of pesticide, because the value of R^2 0.019, $p = 0.748$ was considered too low.

The neonicotinoid thiamethoxam had high value of R^2 0.916, $p = 0.003$ (Fig. 2). In the regression analysis of the use of this insecticide, it was shown that there is a correlation between mortality and concentration of the product, that is, the higher the concentration, the higher the mortality of the subjects. The value of LC_{50} was 0.3882 %, $p = 0.291$.

The results obtained for the growth regulator Azadirachtin Neen, showed that there is no correlation between the mortality of subjects and the concentration used, for the value of R^2 0.083, $p = 0.579$. The LC_{50} value was 4.9 %, $p < 0.001$, being it the pesticide that caused the lowest mortality.

Electrophoresis analysis demonstrated partial inhibition of all ants esterases that underwent contact with two malathion concentrations, 1 x10^{-3} % e 5 x 10^{-3} % (Table 2). However, there was no alteration in gene expression of esterases in contact with thiametoxan insecticide and growth regulator Neem.

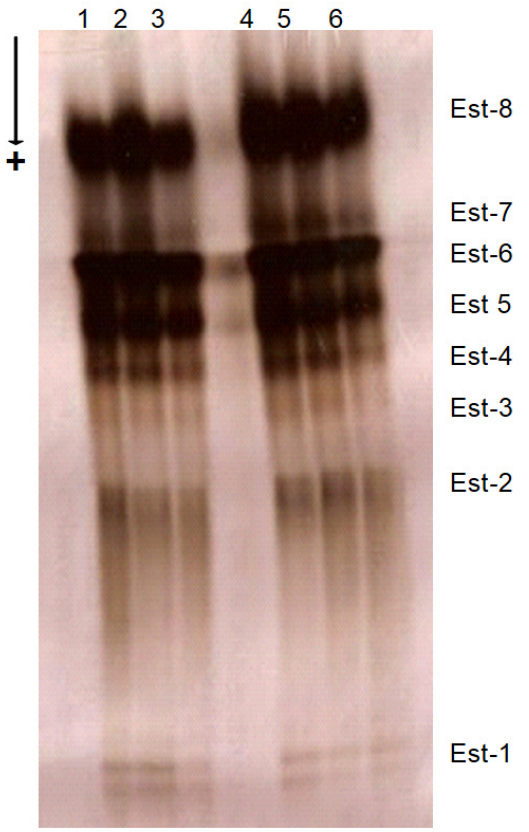

Figure 1. Electrophoretic profile of esterases in extracts of *A. niger* (numbers 1 to 6). Staining with α and β -naphthyl acetate. The arrow indicates the direction of migration.

Table 1. Activity and classification of esterases with the use of inhibitors in . (+) Inhibition, (-) no inhibition.

Esterases	Malathion	P-CMB	Eserine sulfate	Classification
EST-1	+	-	-	Carboxylesterase
EST-2	+	-	-	Carboxylesterase
EST-3	+	-	-	Carboxylesterase
EST-5	+	+	+	Cholinesterase (II)
EST-6	+	-	-	Carboxylesterase
EST-7	+	-	-	Carboxylesterases
EST-8	+	-	-	Carboxylesterases

DISCUSSION

The biochemical characterization of esterase from ants is important to a better understanding of the intermediary metabolism and of xenobiotics. We can highlight that EST-7 and EST-8 might be considered as markers to identify *A. niger*, due to the fact that another important leafcutter ant, *Atta capiguara*, only has six esterase regions (Cantagalli *et al.*, 2009) In the insects, the esterases have been classified into four classes based on sensitivity to organophosphorus inhibitors, to eserine sulfate and to sulfhydryl reagents. According to the classification proposed by Healy *et al.* (1991), the carboxylesterases are inhibited by organophosphates but are insensitive to eserine sulfate; the cholinesterases are sensitive to organophosphates and to eserine sulfate; the arylesterase are not inhibited by organophosphates, but are sensitive to sulfhydryl reagents and the acetylesterase are not inhibited by the three groups of inhibitors.

As Healy *et al.* (1991), the group of cholinesterase can be divided into two subclasses (I and II) according to their pattern of inhibition when used ρ-CMB. Cholinesterase inhibited by ρ-CMB belongs to subclass II, and the one not inhibited belongs to subclass I.

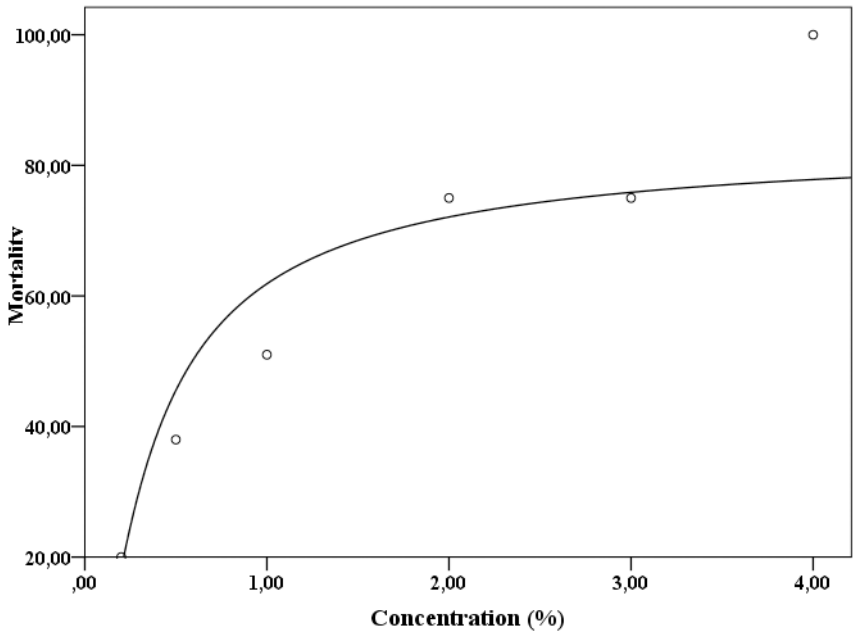

Figure 2. Curve of regression (sigmoid) for the values of mortality of *A. niger* at different concentrations of neonicotinoid thiamethoxam. $R^2 = 0.916$, $p = 0.003$.

Table 2 - Inhibition of esterase activity detected in *A. niger* after contact with malathion, thiamethoxam and growth regulator. (-) No inhibition, (±) partial inhibition.

Esterase	Concentrations of malathion (x 10^{-3} %)								Concentrations of thiamethoxam (%)						Concentrations of Growth regulator (%)					
	1	2	3	4	5	7	8	10	0,2	0,5	1	2	3	4	0,5	1	5	7,5	8	10
EST-1	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-2	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-3	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-4	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-5	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-6	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EST-7	±	-	-	-	±	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Among the esterases, the carboxylesterases and cholinesterases are the most common in insects, possibly because they play an important role in the detoxification of xenobiotic compounds, participating in the resistance to insecticides in several groups of insects. Thus, the esterases detected in *A. niger* may be involved in the detoxification of the organism of these insects after contact or ingestion of pesticides.

These enzymes may also be used as biomarkers of pesticide exposure to detect the presence and toxicity of residues of pesticides used in agriculture and as components of environmental monitoring program (Bonacci, *et al.*, 2004).

The insecticides used in this assignment, such as the organophosphate malathion, trigger mechanisms involving the increase in metabolic detoxification by hydrolysis or sequestration of these compounds (Hemingway 2000; Lee and Lees, 2001; Cui *et al.*, 2007) or structural changes in acetylcholinesterase, the primary target for this class of insecticide (Hsu *et al.*, 2006). Neonicotinoids such as thiamethoxam, act on the nicotinic acetylcholine receptors, such as full or partial agonists (Deglise *et al.*, 2002; Tomizawa and Casida, 2003). The growth regulators insecticides such as Azadirachtin, a compound in the neem oil (*Azadirachta indica*), act on the structure of the integument of insects and on the controlling mechanisms of metamorphosis and ecdysis, working primarily in the immature stages of insects (Velloso, 1999).

The organophosphate malathion has high toxicity based on the extremely low value of LC_{50}, which may interfere with the behavior of beneficial insects that are not target of the insecticide, damaging their way of life and reproduction. Ant's esterases have potencial to be used to detect malathion residues, however more studies are needed to understand the primary inhibition response to this organophosphate.

Attencia *et al.* (2005) worked using *Apis mellifera* workers and found that in a concentration of 0.01 % of methyl parathion insecticide, the activity of esterase-1 was reduced by 75 %, 14 and 21 days after the introduction of the insecticide. A 50 %-inhibition was found for esterases 3 and 4, one day after the introduction of the insecticide. The addition of malathion caused a similar effect in altering the activity of esterases 3 and 4, because it reduced the activity by 25 % at the highest concentration used, which was 0.5 %. These results suggested that the esterases 3 and 4 may be used to detect the presence of methyl parathion and malathion residues in the environment.

Despite there was no changes detected in ant's esterases, the regression analysis for the use of thiametoxan insecticide showed a correlation between mortality and concentration of the product, that is, the higher the concentration, the higher the mortality of the subjects, which in fact could influence the mortality of subjects of the populations which live exposed to this insecticide. In this case, after field studies, the mortality of *A. niger* has the potential to be used as a bioindicator of the presence of thiamethoxam in the environment. However, Hashimoto *et al.* (2003) observed changes in the relative activity of esterases 1 and 2 of 50 % of workers of *Apis mellifera* with one and three days of exposure to the insecticide, after the topical application of the insecticide thiamethoxam. The esterase 5 showed an activity reduced in 50 %, 25 % and showed no activity in workers with three, four and five days of exposure, respectively. According to these authors, these regions of esterase activity may be used to detect the presence of insecticide thiamethoxam residues.

The analysis of electrophoresis indicated that there was no change in gene expression of esterases in the presence of growth regulator Neen inseticide.

Thus, in general, *A. niger* may not be considered a good environmental bioindicator of pesticide residues in the environment when used the parameter change in the expression of esterase in the conditions in this work.

Although this study did not evaluate field tests, the low values for LC_{50}, obtained in vitro for the three pesticides used, may suggest that these pesticides in the estimated quantities for agriculture are likely to affect more intensely the viability of these insects which would be exposed during more time to pesticides. Thus, field bioassays need to be developed and the subjects exposed to pesticides taken to a laboratory for the study of esterases. Especially in the case of neonicotinoid thiamethoxam which showed a correlation between dose of neonicotinoid and mortality of *A. niger*. After contamination, in other words, it showed that the evaluation of mortality of these ants has the potential to be used as a parameter for detecting residues of neonicotinoid thiamethoxam.

Therefore, these insecticides are highly toxics to *A. niger*. The mortality rate has potencial to be used to detect thiamethoxam residues and esterases have potencial to be used to detected malathion the in the environment.

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