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Effect of *Azadirachta indica* (Sapindales: Meliaceae) Oil on the Immune System of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Immatures

Jucelio P. Duarte,[1,3,] **Luiza R. Redaelli,**[1] **Carlos Eugênio Silva,**[2] **and Simone M. Jahnke**[1]

[1]Departamento de Zoologia, Universidade Federal do Rio Grande do Sul, Instituto de Biociências, Av. Bento Gonçalves 9500, Porto Alegre, RS 91501-970, Brazil, [2]Departamento de Microbiologia, Imunologia e Parasitologia, Universidade Federal do Rio Grande do Sul, Instituto de Ciências Básicas da Saúde, R. Sarmento Leite, 500, Porto Alegre, RS 90050-170, Brazil, and [3]Corresponding author, e-mail: juceliod@hotmail.com

Abstract

The insect immune system includes several mechanisms responsible for defending against pathogens, parasites, and parasitoids. Some botanical insecticides, such as *Azadirachta indica* oil, cause changes in the immune system of various insect species. *Spodoptera frugiperda* is an important agricultural pest; thus, knowledge about the effect of neem oil on the immune system of this species can assist in its management. This study aimed to evaluate the effect of *A. indica* oil on the immune system of *S. frugiperda*. Caterpillars (2–3 mg) were placed individually in containers (50 ml) with approximately 10 g of diet, containing 125, 250, and 500 ppm of neem oil with propanone; the control group received only the propanone diet. In four experiments, the total number of hemocytes, the phagocytic activity, the activity of lysozyme-like enzymes, and phenoloxidase activity were measured in caterpillars at the end of the sixth instar. The total number of hemocytes in insects exposed to neem oil was 21% lower than in the control group. The percentage of cells that phagocyted the latex beads was similar among the caterpillars that ingested the different concentrations. The mean diameter of cell lysis halos was reduced only at concentrations of 125 and 250 ppm. Absorbance did not differ between treatments. Knowing that this oil reduces the number of circulation cells and the activity of lysozyme-like enzymes is of great importance to design control strategies, once the neem oil could be added to other biological agents for mortality reducing the chances of this insect surviving in the environment.

Key words: fall armyworm, hemocyte, lysozyme, neem, phenoloxidase

Resumo

O sistema imune nos insetos inclui uma série de mecanismos que são responsáveis por defendê-lo frente a patógenos, parasitos e parasitoides. Alguns inseticidas vegetais, como o óleo de *Azadirachta indica*, causam alterações no sistema imune de várias espécies de insetos. *Spodoptera frugiperda* é uma importante praga agrícola, assim, o conhecimento sobre o efeito do óleo de nim no sistema imune dessa espécie pode auxiliar no manejo deste inseto. Este estudo objetivou avaliar o efeito do óleo de *A. indica* sobre o sistema imune de *S. frugiperda*. Lagartas com 2 a 3 mg foram colocadas, individualmente, em frascos (50 ml) com aproximadamente 10 g de dieta, contendo 125, 250 e 500 ppm de óleo de nim com propanona, além do grupo controle que recebeu a dieta apenas com propanona. Em quatro experimentos foram avaliados, em lagartas no final do sexto ínstar, o número total de hemócitos, a atividade fagocítica, a atividade das enzimas tipo lisozima e da atividade da fenoloxidase. Constatou-se uma redução de 21% do número total de hemócitos naqueles insetos expostos ao óleo de nim em relação ao grupo controle. A porcentagem das células que fagocitaram as esferas de látex foi semelhante entre as lagartas que ingeriram as diferentes concentrações. O diâmetro médio dos halos de lise celular foi reduzido somente nas concentrações de 125 e 250 ppm. A absorbância não diferiu entre os tratamentos. Saber que esse óleo reduz o número de células circulantes e a atividade de enzimas tipo lisozima é de grande importância para traçar estratégias de controle, uma vez que o óleo de nim pode ser adicionado a outros agentes biológicos de mortalidade, reduzindo as chances desse inseto sobreviver no ambiente.

Palavras-chave: fenoloxidase, hemócitos, lagarta-do-cartucho, lisozima, nim

The insects, unlike vertebrates, do not have an adaptive immunity, which is responsible for producing receptors that recognize specific antigens and allow hosts to develop immunological memory (Jiravanichpaisal et al. 2006). Nevertheless, insects have an innate immunity that is very developed and efficient (Jiravanichpaisal et al. 2006), which is divided into cellular innate and humoral innate responses (Lavine and Strand 2002). Cellular immunity is mediated by hemocytes, which can perform such processes as phagocytosis, nodulation, and encapsulation. Humoral immunity includes several defense mechanisms that are activated without the direct participation of hemocytes, such as the production of antimicrobial peptides and an enzymatic complex that regulates hemolymph coagulation or melanization (Lavine and Strand 2002). These immune processes are important for the insect to defend against pathogens, parasites, and parasitoids.

Some biological insecticides have been reported to cause problems in the insect immune system. Insecticides originated from *Azadirachta indica* A. Juss., also known as neem or Indian lilac, can cause the reduction in the total number of hemocytes and hemolymph volume in *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae) (Sharma et al. 2003), alteration in the proportion of different cell types in *Danaus chrysippus* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) (Pandey et al. 2008), and reduction in the number of nodules and mitotic activity in *Galleria mellonella* (Linnaeus, 1758) (Lepidoptera: Pyralidae) (Er et al. 2017). Reduction of phagocytic (Figueiredo et al. 2006), lysozyme, and prophenoloxidase (proPO) activity in *Rhodnius prolixus* Stål, 1859 (Hemiptera: Reduviidae) (Azambuja et al. 1991b) was also recorded.

The neem tree has more than 100 active biological compounds, mainly terpenoids (Campos et al. 2016), which have insecticidal, repellent, and deterrence properties. Although there are many compounds such as meliantriol, nimbin, nimbidin, nimbinin, nimbolide, salanin, and fatty acids (oleic, stearic, and palmitic), the most important compound present in the oil is azadirachtin (Campos et al. 2016). The effects of azadirachtin on insects can be given through two usually applied ways: 1) topically, i.e., direct contact of the insect with the compound (Er et al. 2017), and 2) by feeding, i.e., exposing the diet to the compound (Azambuja et al. 1991b).

In *Spodoptera frugiperda* (Smith, 1797), it is only known that this oil alters the proportions of different hemocyte types (Correia et al. 2008). This species is an important pest of Poaceae plants (Luginbill 1928, Capinera 2008), such as sorghum, rice, and wheat (Cruz 1995, Busato et al. 2002), but it can attack more than 80 plant species (Pogue 2002, Capinera 2008). Thus, knowledge about the effect of neem oil on the immune system of this species can aid strategies to control this and other pests using bioinsecticides.

Based on the information previously stated, we suppose that *S. frugiperda* immatures that developed on a diet containing *A. indica* oil in different sublethal concentrations have 1) a smaller number of hemocytes; 2) a reduced phagocytic activity; 3) a decreased lysozyme-like enzymes activity; and 4) a lower PO activity. To test these hypotheses, we established four experiments (for each evaluation), in laboratory, with 5-d-old caterpillars in an oil-containing diet and tested when they were in the sixth instar. Therefore, the aim of this study was to evaluate the effect of sublethal concentrations of *A. indica* vegetable oil on the immune system of *S. frugiperda* immatures.

Materials and Methods

Spodoptera frugiperda Rearing
The rearing of *S. frugiperda* began with pupae purchased from Promip. The adults were kept in cages (20 cm high × 15 cm in diameter) made of polyvinyl chloride tubes with holes in the sides. They were fed on a 20% honey and water solution, replenished every 2 d. Internally, the cages were covered with sulfite paper, so that the adults could lay their eggs on the paper.

Every day, the papers were removed from the adult cages, and postures were placed in test tubes and incubated in B.O.D. (25 ± 2°C, 70 ± 10% RH, and 12 h photophase). After the hatch of the caterpillars, they were kept individually in containers (50 ml) with artificial diet adapted from Bowling (1967) (500 g boiled carioca bean, 75 g brewer's yeast, 15 g ascorbic acid, 20 g nipagin, 2.5 g sodium benzoate, 45 g agar, and 200 ml distilled water). All insects, including those used in the bioassays, remained in a climate chamber (27 ± 2°C, 70 ± 10% RH, and 14 h photophase).

Neem Oil and Insect Exposure
Azadirachta indica vegetable oil (pure nonemulsified oil natural neem extracted from neem seed—2,000 ppm azadirachtin), purchased from DogNeem, was incorporated into the diet of caterpillars at concentrations of 125, 250, and 500 ppm (1 ppm = 1 μg oil per gram diet; treatments). These concentrations were established based on previous tests that we have made until we got a concentration that killed less than 80% of the individuals (500 ppm). The incorporation was done by adding propanone in the proportion of 10 ml for each 400 g of diet to emulsify the oil in the diet. In the control group, only propanone was added to the diet.

Five-day-old caterpillars weighing between 2 and 3 mg were individually placed in containers (50 ml) with approximately 10 g of an oil-containing diet or a control diet. The containers remained in a climate chamber until the insects reached the end of the sixth instar, before the prepupa phase.

Hemocyte Count
For each treatment, 40 caterpillars at the end of the sixth instar were mechanically immobilized with tape on an aseptic surface, and the fourth and fifth abdominal segments were sterilized with the aid of a sterile swab with 70% alcohol. The integument between these segments was punctured with an aseptic needle, and 5 μl of spilled hemolymph was collected with an automatic micropipette. The hemolymph was diluted in 25 μl anticoagulant (0.1 M ethylenediaminetetraacetic acid, 0.1 M glucose, 62 mM NaCl, 26 mM citric acid, pH 4.6; Azambuja et al. 1991a) (dilution factor of 6) and placed in a Neubauer chamber to count the total number of hemocytes under a light microscope. The total number of hemocytes in cells per milliliter was estimated by the following equation:

$$N = \frac{(Q1 + Q2 + Q3 + Q4)}{4} \times DF \times 10,000$$

where N = estimated number of cells/ml; Q (1–4) = external quadrants of Neubauer Chamber, and DF = dilution factor.

The data on the total number of hemocytes in each treatment were transformed by $\sqrt{x}$ to make a symmetrical frequency distribution. The total mean number of hemocytes in each treatment was analyzed by analysis of variance (ANOVA) and compared by Tukey's test ($\alpha = 0.05$), both using the IBM SPSS version 23 (IBM 2015) statistical program.

Phagocytic Activity
A solution of latex beads made of carboxylate-modified polystyrene (fluorescent red; mean diameter 0.5 μm; Sigma–Aldrich) and 1× phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl,

10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) was prepared at a ratio of 1:10 as proposed by Ajamhassani et al. (2013). At least 15 caterpillars from each of the treatments were immobilized at the end of the sixth instar. After the antisepsis of the fourth and fifth abdominal segments, 5 µl of the above solution was injected with the aid of an aseptic needle (30 G), being careful not to puncture the insect's gut. Each insect returned to its diet where it remained for 6 h. After this period, each caterpillar was immobilized again, and after antisepsis, 5 µl of hemolymph was collected and diluted as described in the previous section. The material was placed in a Neubauer Chamber, and fluorescence microscopy was employed to count the number of total hemocytes and the number of hemocytes that phagocyted the latex beads. The mean percentages of phagocytic cells were analyzed by ANOVA and compared by Tukey's test ($\alpha = 0.05$), both using the statistical software IBM SPSS version 23 (IBM 2015).

Activity of Lysozyme-Like Enzymes

To evaluate the activity of lysozyme-like enzymes, the methodology proposed by Mohamed et al. (2013) was adapted. Petri dishes (9.5 cm in diameter) containing 7 ml of agarose (1%) and lyophilized *Micrococcus lysodeikticus* ATCC 4698 (Sigma–Aldrich; 1 mg/ml agarose) were prepared with eight wells (3 mm in diameter each) equidistant. When they reached the end of the sixth instar, 25–40 caterpillars of each treatment were immobilized and 4 µl of hemolymph was removed as in the previous procedures. The aliquot collected from each insect was placed in a single well. The plate was incubated in a B.O.D. at 25°C for 24 h. After this period, the cell lysis halo diameter formed in each well was measured with the aid of a stereomicroscope with a micrometer eyepiece at 60× magnification.

To estimate the concentration of lysozyme-like enzymes in the insect hemolymph, the hemolymph was replaced by a solution of hen egg-white lysozyme (HEWL) and PBS 1× at different concentrations (4, 3, 2, 1, 0.5, 0.25, 0.125, and 0.0625 µg/µl). The halos formed by the action of the HEWL solution were measured after 24 h of incubation. Linear regression was made between the HEWL concentrations and the respective diameters of the halo formed ($R^2 = 0.8256$; slope = 1.0879; intercept = 5.7004; Fig. 1). The diameters of the halo obtained from hemolymph were applied to the regression formula to estimate the concentration of lysozyme-like enzymes present in the insect hemolymph. To compare mean concentrations of lysozyme-like enzymes in hemolymph, the halo mean diameters were analyzed by Kruskal–Wallis test and compared by Dunn's test ($\alpha = 0.05$), both using the statistical software IBM SPSS version 23 (IBM 2015).
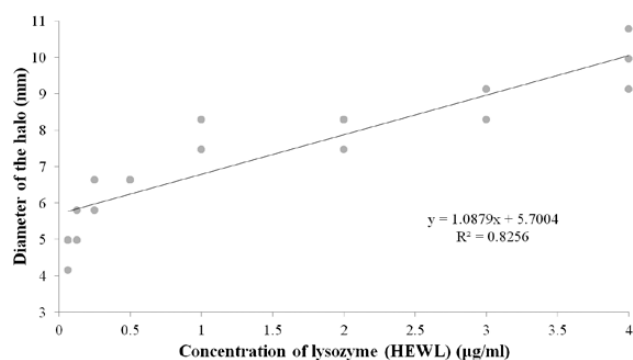
Phenoloxidase Activity

Phenoloxidase (PO) activity was evaluated using the methodology proposed by Cruz et al. (2014). For each treatment, three repetitions with up to eight insects each were conducted. From each of the insects, 15 µl of hemolymph was removed, as in previous procedures, and diluted in 1× PBS at proportions 1:1. A 10 µl aliquot of this hemolymph was placed in a well with 35 µl of 1× PBS from a cell culture plate. After filling all the wells, 15 µl of 3,4-dihydroxy-l-phenylalanine (l-DOPA; Sigma–Aldrich) saturated in 1× PBS (4 mg/ml) was added and then the plate was placed in an absorbance microplate reader with a 492 nm filter and the absorbance (the radiation absorbed by the sample) was recorded after 60 min.

Also, for each reading, four wells were filled with only 45 µl of 1× PBS and 15 µl of l-DOPA, with no hemolymph added (blank space). Absorbance values were subtracted from the mean of blank spaces at each reading to obtain corrected absorbance. The mean corrected absorbance values of each of the treatments were compared by ANOVA and the means compared by Tukey's test ($\alpha = 0.05$), both using the statistical program IBM SPSS version 23 (IBM 2015).

Results

The estimated total mean number of hemocytes in insects that developed on an oil-containing diet was significantly lower than those on the control diet ($F = 7.94$; df = 3, 156; $P < 0.001$; Table 1). This reduction was around 21% at the highest concentration compared with the control group, with a mean of 13×10^6 cells/ml (Table 1).

No differences were observed in the percentage of cells that phagocytized the latex beads when the larvae developed on diets containing different concentrations of neem oil ($F = 1.52$; df = 3, 95; $P = 0.22$; Table 1). In the control group, a mean 44% of the cells phagocyted the beads, while in the group exposed to the highest oil concentration averaged 55% of the cells (Table 1).

The estimated levels of lysozyme-like enzymes ranged from 0.89 (250 ppm) to 1.77 µg/ml (control group; Table 2). The mean diameter of cell lysis halos was significantly smaller when the larvae developed on the oil-containing diet at concentrations of 125 and 250 ppm, with no difference between the control and highest concentration ($H = 21.57$; df = 3, 144; $P < 0.001$; Table 2).

The corrected absorbance of hemolymph presented no significant difference between the treatments, ranging from 0.19 (control) to 0.23 (250 ppm; $F = 0.41$; df = 3, 6; $P = 0.75$; Table 2). The results indicate a lack of change in the PO activity with the addition of neem oil to the diet.

Fig. 1. Linear regression between the diameter of the cell lysis halo generated after 24 h in medium containing 1% agarose and lyophilized *Micrococcus lysodeikticus* (1 mg/ml agarose) and HEWL (hen egg-white lysozyme) concentrations.

Table 1. Mean number (± SE) of hemocytes in sixth-instar *Spodoptera frugiperda* caterpillars and mean percentage (± SE) of hemocytes that phagocytized latex beads of caterpillars submitted to an artificial diet containing *Azadirachta indica* oil in different concentrations and the control group (propanone only)

	Hemocytes (cells × 10^6/ml; mean ± SE)	Hemocytes that phagocyted (%; mean ± SE)
Control	13.2 ± 0.65 A (40)	44.4 ± 3.15 A (30)
125 ppm	10.3 ± 0.63 B (40)	44.5 ± 3.39 A (21)
250 ppm	9.0 ± 0.50 B (40)	46.6 ± 2.81 A (32)
500 ppm	10.3 ± 0.65 B (40)	55.3 ± 6.06 A (16)

Number in parentheses are the number of individuals evaluated. Means in the same column followed by different letters differ by the Tukey's test ($\alpha = 0.05$).

Table 2. Mean diameter (± SE) of halo cell lysis formed by hemolymph and mean corrected absorbance (A_{492}) of hemolymph (± SE) of sixth-instar larvae of *Spodoptera frugiperda* submitted to an artificial diet containing *Azadirachta indica* oil in different concentrations and the control group (propanone only)

	Halo diameter (mm; mean ± SE)[a]	Corrected absorbance (A_{492}; mean ± SE)[b]
Control	7.63 ± 0.181 [1.77] A (40)	0.19 ± 0.021 A
125 ppm	6.69 ± 0.134 [0.91] B (40)	0.21 ± 0.044 A
250 ppm	6.67 ± 0.142 [0.89] B (40)	0.23 ± 0.027 A
500 ppm	7.19 ± 0.160 [1.37] AB (28)	0.20 ± 0.022 A

Number in square brackets represents the concentration of HEWL (hen egg-white lysozyme) and 1× PBS (µg/ml). Number in parentheses is the number of individuals analyzed.

[a]Means in the column followed by different letters differ by Dunn's test ($\alpha = 0.05$).

[b]Means in the column followed by different letters differ by Tukey's test ($\alpha = 0.05$).

Discussion

This is the first work reporting the effect of sublethal concentrations of *A. indica* oil on the *S. frugiperda* immune system. Reduction in the total number of circulating hemocytes recorded in this work has also been mentioned in other insects exposed to neem seed extract in *S. litura* (Sharma et al. 2003), to neem extract and oil in *D. chrysippus* (Pandey et al. 2008) and *Dysdercus cingulatus* (Fabricius, 1775) (Hemiptera: Pyrrhocoridae) (Pandey and Tiwari 2011), and to azadirachtin in *G. mellonella* (Er et al. 2017). The reduction in the total number of hemocytes, according to Pandey et al. (2008), may be associated with three factors: hemocyte accumulation in a given hemocoel region, the toxic effect of neem compounds, and/or their inhibitory effect on endocrine glands. Azadirachtin inhibits the release of morphogenetic peptides from the brain, which results in blockade of ecdysteroid synthesis and regulation of juvenile hormone (Mordue (Luntz) and Nisbet 2000). Besides, ecdysteroids can regulate hemocyte numbers and hemolymph volume as well as induce cell differentiation in *R. prolixus* (Jones 1967). Thus, in the case of *S. frugiperda*, azadirachtin may have interfered indirectly in the number of circulating hemocytes due to its effect on the weight and size of the individuals through its action on the endocrine system, as observed by Duarte et al. (2019).

Another hypothesis that may justify the reduction in the number of circulating hemocytes is the toxic effect of neem on cells. Azadirachtin blocks cell division and protein synthesis in tissues (Mordue (Luntz) and Nisbet 2000) and is toxic to *S. frugiperda* cells as it reduces their growth and multiplication (Salehzadeh et al. 2002). In addition, Reed and Majumdar (1998) observed that *S. frugiperda* cells exposed to azadirachtin for 48 h presented a higher number of blebs, holes, and extrusion of cytoplasmic material. Those authors assumed that these factors lead to cell death.

Reduced number of hemocytes can cause several problems in the defense of insects to pathogens and parasitoids because the hemocytes phagocytize small foreign bodies and participate in wound clotting, nodulation (response against bacteria and fungi), and encapsulation (response against agglomeration of microorganisms, parasitoids, and parasites; Lavine and Strand 2002). Some types of hemocytes, such as oenocytoids, are also important because they carry one of the proPOs, an enzyme responsible for catalyzing reactions that culminate in the release of melanin in the hemolymph (Lavine and Strand 2002). Melanin is toxic to parasites, parasitoids,

and pathogens, and plays an important role in hardening the clot during wound healing, preventing other foreign agents from penetrating the hemocoel (Nakhleh et al. 2017). The same authors pointed out that in addition to its participation in the immune system, melanin is also essential for the hardening and darkening of the cuticle after molting.

The fact that no change in the proportion of phagocytic cells was observed in oil treatments indicates that cells do not lose the ability to recognize a foreign body or the ability to attach to it and internalize it. Plasmatocytes and granulocytes together comprise more than 70% of the hemocyte types in Lepidoptera (Gardiner and Strand 2000) and are the major cells involved in phagocytosis, encapsulation, and nodulation (Strand and Pech 1995). *Spodoptera frugiperda* caterpillars that were fed on leaves immersed in neem oil also had no significant effect on the proportion of granulocytes and plasmatocytes compared with the control group at 240 h (longest time tested; Correia et al. 2008). Although the hemocyte profile was not evaluated in the present study, the results suggest that the oil had no effect on the proportion of phagocytic cells, and the reduction in the number of hemocytes found was probably in the prohemocytes. Prohemocytes and plasmatocytes are the only cells produced in the hematopoietic organs during the larval phase of Lepidoptera (Nakahara et al. 2010); thus, azadirachtin might interfere with the production of new prohemocytes due to toxic action on the cells of the hematopoietic organs. This effect on this organ was discussed by Dorrah et al. (2019), who assessed the impact of azadirachtin on larvae of *Sarcophaga argyrostoma* (Robineau-Desvoidy, 1830) (Diptera: Sarcophagidae).

As found in the present study, Pandey et al. (2008) reported that cells with reduced phagocytic capsule numbers were not frequently observed in *D. chrysippus* larvae when topically treated with neem oil. In contrast, when *R. prolixus* was fed in a diet containing azadirachtin, the main compound present in neem oil, its phagocytic activity of yeast reduced in in vitro tests (Figueiredo et al. 2006). The alterations in phagocytic activity observed in the latter case could be due to the method of application, and as a sucking insect, *R. prolixus* has completely different development and feeding habit than *D. chrysippus* larvae.

The reduction in the levels of lysozyme-like enzymes in hemolymph found in *S. frugiperda* may be due to the effect of neem oil on the endocrine system because ecdysteroids are the main regulators of antimicrobial peptide (AMP) synthesis and lysozyme in fat body (Flatt et al. 2008). Moreover, the reduction in hemocyte numbers found in our study may also be associated with the decrease in lysozyme-like enzyme levels in the hemolymph, as circulating cells also participate in the production of these peptides, even secondarily (Lemaitre and Hoffmann 2007). However, Azambuja et al. (1991b) argued that reduction of lysozyme activity by azadirachtin may be more associated with widespread inhibition of insect metabolism rather than the manipulation of the endocrine system. The lack of a significant difference between the highest concentration and the control group may be associated with the lysis of hemocytes and fat body cells. Neem oil causes autophagy of fat body cells, and cell lysis could result in a large release of these enzymes in hemolymph, raising its levels (Schlüter 1985).

Another factor that may be associated with reduced levels of lysozyme-like enzymes is the indirect impact of oil on insect organs due to its deterrent effect on feeding. When the insect cannot eat properly and has low nutrient utilization (Mordue (Luntz) 2004), it may use the nutrients from the fat body, which is responsible for the production of lysozyme-like enzymes, reducing the potential of this organ. The fat body is an important organ especially for

holometabolous insects, as it allows them to survive during metamorphosis and periods of starvation, by mobilizing nutrients for vital functions (Arrese and Soulages 2010).

Lysozymes are enzymes that catalyze the hydrolysis of glycosidic bonds between *N*-acetylmuramic and *N*-acetylglucosamine acids present in bacterial cell wall peptidoglycan. As a result, these proteins have bacteriological and bacteriostatic action on gram-positive bacteria (Yu et al. 2002). Lysozymes can also act synergistically on gram-negative bacteria with other AMPs, such as cecropins (Cytryńska et al. 2001). Thus, the decrease in hemolymph lysozyme-like enzymes may lead to a reduced immune response when the insect is exposed to gram-positive or -negative bacteria.

The lack of difference in the mean corrected absorbance of hemolymph indicates that no change occurred in PO activity because this activity is related to the production of melanin, a protein that provides a darker color at a higher concentration in hemolymph (Vavricka et al. 2010) and consequently absorbs a greater amount of radiation (higher absorbance). Thus, the ingestion of neem oil does not appear to have any effect on immune reactions or on the hemocytes that are involved in PO production and activity. In Lepidoptera, oenocytoids are the hemocytes responsible for synthesizing proPO (Jiang et al. 1997); thus, although we did not evaluate the differential hemocytes count, the oil probably did not interfere with the functioning or proportion of these cells. The product might influence this activity if the insect had been exposed to a foreign agent or injured, since PO, along with other functions, is an important mechanism of the humoral immune response (Jiravanichpaisal et al. 2006), and its levels increase when the insect is exposed to foreign agents. Bali and Kaur (2013) observed that in *S. litura*, after 24 h post-inoculation with 1.0×10^8 per ml of *Beauveria bassiana* (Bals.-Criv.) Vuill., 1912 (Hypocreales: Cordycipitaceae) spores, PO activity increased more than seven-fold compared to the control group. Thus, the neem oil might have suppressed the elevation of these levels if any of these events had happened. If the insect suffers an injury or infection, oenocytoids are immediately lysed, and if it does not, proPO secretion occurs at a low rate to maintain standard hemolymph levels (González-Santoyo and Córdoba-Aguilar 2012). This indicates that the PO levels observed in this work are normal to meet the biological needs of the insect and have not been altered by the presence of this oil in the diet.

In general, the effect of neem oil on the immune system is likely to be due to food deterrence, impact on the endocrine system, and an action toxic to cells. Knowing how this oil affects the immune system of *S. frugiperda* at sublethal concentrations is of great importance to design control strategies. Its effects could be added to other biological agents for mortality and thus reduce the chances of this insect surviving in the environment. However, many laboratory and field studies need to be conducted to assess the potential of the oil for this purpose, including challenge experiments with microorganisms.

Acknowledgments

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