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Toxicity and Residual Activity of a Commercial Formulation of Oil from Neem, *Azadirachta indica* A. Juss. (Meliaceae), in the Embryonic Development of *Diatraea saccharalis* F. (Lepidoptera: Crambidae)

Camila Vieira da Silva[1]*, Larissa Carla Lauer Schneider[2] and Hélio Conte[3]

[1]*Faculty of Apucarana (FAP), Biological Sciences Department, Apucarana PR, Brazil*

[2]*Veterinary Medicine Department, University of Maringa (UEM), Estrada da Paca Bairro São Cristóvão, Umuarama PR, Brazil*

[3]*Biology Cell and Genetics Department, CCB, University of Maringa (UEM), Avenida Colombo, Maringá PR, Brazil*

Abstract

The effects of a commercial formulation of oil from neem, *Azadirachta indica* A. Juss (Meliaceae), on the eggs of sugarcane borer *Diatraea saccharalis* F.,1794 (Lepidoptera: Crambidae), at different stages of its embryonic development were investigated. To evaluate the ovicidal activity of the oil, eggs were sprayed with the product at concentrations of 0.1%, 0.3%, 0.5%, 1.0% and 2.0% at 1, 2, 3, 4, 5 and 6 days after day were laid. Controls were sprayed with distilled water. The neem oil was toxic to eggs, mainly when applied to 2-, 3-, 4- and 5-days-old eggs. The percentage of viability reduction was 31-99%. Higher levels of caterpillar eclosion were obtained in eggs treated with neem oil at older ages. However, the resulting caterpillars had fatal morphological anomalies, except those exposed to 0.1% neem oil. Neem oil is highly toxic when sprayed on *D. saccharalis* eggs, as demonstrated by severely reduced hatching, increased mortality of hatched caterpillars, impaired embryonic development, and residual activity in the production of new individuals. Therefore, neem oil may be a promising agent against the sugarcane borer, during the stage in which penetrates the stalk, causing heavy damage to the sugar-cane crop.

Keywords: Sugarcane borer; Neem oil; Residual activity; Embryonic development; Toxicity

Introduction

Plant species with highly promising insecticidal characteristics belong to the Meliaceae family [1]. This is especially the case for *Azadirachta indica* A. Juss. (Meliaceae), commonly known as, the neem tree [2]. Neem extracts have been extensively studied due to their high content of insecticidal substances, including triterpenoids, azadirachtin and melantriol, which are effective against several pest species [2-6].

The inseticidal substances mentioned above have complex aromatic structures that decrease the development of resistance of insects; they are also biodegradable. Their activity is quickly reduced by light; they are only slightly toxic to mammals, and are compatible with the natural foes of several insects [7-9]. Studies on the effects on insects treated or fed with azadirachtin have reported growth inhibition; death of caterpillars during ecdysis; prolongation of the larval stage; deformation of caterpillars and adults; decrease in longevity, fecundity and fertility [10-13]. Azadirachtin in neem oil has selective effects on insects, mostly with respect, impaired feeding and interrupted growth [14,15].

Evaluations of formulations of neem oil, e.g. AZT (containing 30 mg azadirachtin ml^{-1}), NEEM-AZA (containing 3 mg azadirachtin ml^{-1}), and AZ (pure azadirachtin), on second instar *Plutella xylostella* (Lepidoptera: Plutellidae) caterpillars, revealed a 50 to 90% mortality rate. AZT had also ovicidal activity [16]. Calneem oil has been found to have insecticidal and anti-oviposition effects, as well as ovicidal and repellent properties against caterpillars of *Ephestia cautella* (Lepidoptera: Pyralidae) [12].

Studies of the effect on the embryonic development, e.g. neem oil directly applied to Lepidoptera eggs, are rare. Some studies have documented the effects on oviposition, following treatment of caterpillar adults or pupae [12,13,17,18]. Ovicide effects of neem solution were reported after application to egg masses of different ages in *Diatraea saccharalis* F., 1794 (Lepidoptera: Crambidae), but subsequent effects on the development of caterpillars have not been demonstrated [19]. Other types of extracts with ovicidal effects have been reported in connection with the following, *P. xylostella*, *Heliothis armigera* Hübner, 1805; *Spodoptera frugiperda* (Lepidoptera: Noctuidae); *Bemisia tabaci* (Hemiptera: Aleyrodidae); *Tribolium castaneum* (Coleoptera: Tenebrionidae); *Anopheles stephensi* and *Aedes (Stegomyia) aegypti* (Diptera: Culicidae); *D. saccharalis* [19-25].

The aim of this study was to identify natural insecticidal alternatives to enable a more efficient control of the sugarcane borer. Therefore, the toxicity of neem oil was evaluated with regard to embryonic development, and the residual activity in larvae that newly emerged from eggs.

Materials and Methods

Experiments were performed at the Laboratory of Morphology and Cytogenetic of Insects, State University of Maringá (Maringá, Paraná, Brazil). Bioassays were conducted at 25°C ± 1°C, a relative humidity of 70% ± 10%, and a 12-h photoperiod. For the acquisition of *D. saccharalis* eggs of 1 to 6 days of age, the adults were placed on posture chambers, and the eggs deposited were maintained in coupling chambers until hatching.

***Corresponding author:** Camila Vieira da Silva, Faculty of Apucarana (FAP), Biological Sciences Department, Apucarana PR, Brazil, Tel: 55-44-3011-4466; Fax: 55-44-3263-7162; E-mail: hconte@uem.br

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Natuneem® (pure neem oil with an azadirachtin rate of over 1,500 ppm) is an organic product, certified by BCS OKO Garantie, Doc. Natur- 9009/09.05/7331-BR, and was used as a bio-insecticide in the experiment. Neem oil was diluted in distilled water to produce concentrations of 0.0% (control), 0.1%, 0.3%, 0.5%, 1.0% and 2.0%. Groups with approximately 50 eggs each were formed. They were maintained in polyethylene plates (6.0 cm diameter and 2.0 cm thick), which were lined with filter paper and filled with cotton; they were wetted daily with distilled water. Plates were then grouped according to concentration and age of the eggs (1, 2, 3, 4, 5 and 6 days after being laid). Four replications were user for each treatment, including the control. Approximately 100 µL of the respective neem oil solution was sprayed on the different egg groups. Data were recorded daily for up to 9 days post-treatment.

The number of hatched caterpillars of the treated eggs was counted daily, so that the possible residual effect of the agent could be analysed. Caterpillars were separated in polyethylene plates and provided with an artificial diet [26]. Plates were kept at the same experimental conditions, as described above. Mortality percentages for eggs of different ages were assessed for each treatment condition, according to Abbott [27]. The frequency (%) and type of abnormalities of hatched caterpillars and frequent changes and abnormal developments in the eggs were photographed by using a digital camera, Cannon 7.0 MG, with a stereomicroscope (Carl Zeiss, Jena, Germany). The viability rate for eggs for each treatment condition age, duration of the embryonic period were analysed using the statistical program PRISM 5.0. Differences among treatments were analysed using the ANOVA and Tukey tests ($p<0.05$).

Results and Discussion

Neem oil was toxic to *D. saccharalis* eggs in all tested concentrations, and the toxicity varied with the dose and the age of the eggs (Table 1). Neem oil has been considered to have an ovicidal action because the agent may obstruct the egg membrane and hinder the embryo´s respiratory exchanges [28]. Similar to the results obtained in previous studies, neem oil blocked the aeropyles of eggs and the body and head of 2 lice species, thus preventing the embryos of both of lice species from accessing oxygen, and from releasing carbon dioxide [29].

No significant differences ($p>0.05$) were found between treatments and control in the development time of eggs (Table 2). Treatment with neem oil at all concentrations resulted in a significant ($p<0.05$) decrease in egg viability, which varied between 38.1 and 98.9% (Table 2). The observed decrease may have occurred because of the activity of an active substance in the formulation, which interfered with the embryonic development. Azadirachtin, the main substance in neem extracts, has a selective effect on insects [30]. In fact, experiments have shown that it may impair mitochondrial functions [31]. Because mitochondria produce energy for cell processes and are required for the embryonic development, they are probably affected.

A significant decrease in the viability was obvious in all treatment groups, when compared to the control group (Table 1). This finding was observed mainly in eggs during the first 3 days of development (≈ 1, 2, and 3 days post-oviposition). The death of the insects depends on the dose and on the duration of exposure to the chemical substance/agent, which may occur some days after application [32]. The emergence of caterpillars in eggs of *Ceratothripoides claratris* (Thysanoptera: Thripidae), treated with neem formulations were significantly affected by the relationship between the age of the eggs and the concentration [33].

A lower decrease in viability has been reported in embryos at more advanced stages (eggs of 4, 5 and 6 days of age), mainly at a concentration of 0.1% (Table 1) [19]. However, the resulting caterpillars presented morphological abnormalities, with consequently high mortality rates (Figures 1E-1H). Two hypotheses could be proposed: either the substance was active in the final development stage or the neonatal caterpillars had physiological modifications, after the contact with residues, which affected the morphological changes. Studies have shown that caterpillars from neem-treated eggs may have a high mortality rate mainly because of the contact of the insects with the chorion until their hatching, or because of consumption of neem present on the chorion [34].

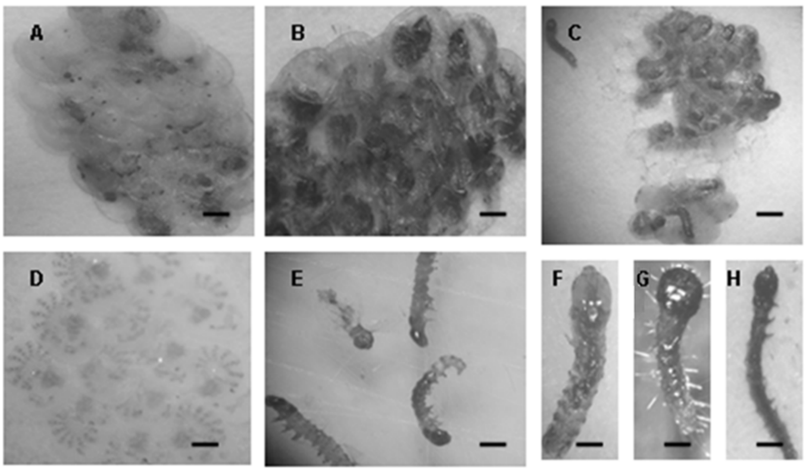

Figure 1: Morphological alterations in *Diatraea saccharalis* (Lepidoptera: Crambidae), caused by neem oil in caterpillars from neem treated eggs.

In A, 1-day-old eggs treated with neem oil (1%) after 9 days; they showed changes in their development. In B-C, 4-day-old eggs treated with neem oil (1%) after 9 days. In D-E, 2-day-old eggs treated with neem oil (2%), with abnormalities in hatched caterpillars, F shows caterpillars with slight melanisation and lack of cephalic capsule. G, shows the reduced length of body, colouring of the darkened body, and a slight increase in the cephalic region. H, shows caterpillars with greater body length and intense melanisation (bar: 3 mm).

Concentrations (%)	Age eggs (days) treated					
	1	2	3	4	5	6
0.0	83.00 ± 1.73a	85.50 ± 4.50a	79.50 ± 2.50[a]	78.00 ± 1.15a	67.50 ± 2.06a	75.50 ± 2.06a
0.1	27.50 ± 1.50b	36.00 ± 1.83b	54.50 ± 2.21b	56.00 ± 2.94b	63.50 ± 3.50a	56.50 ± 4.03b
0.3	1.00 ± 1.00c	3.00 ± 0.58c	9.50 ± 0.96c	37.70 ± 4.17c	32.00 ± 2.16b	35.70 ± 0.85c
0.5	1.50 ± 0.95c	1.00 ± 0.57c	3.75 ± 0.85c	38.50 ± 3.30c	30.75 ± 2.81b	27.00 ± 1.47c
1.0	2.50 ± 1.50c	0.00 ± 0.00c	6.50 ± 0.96c	36.50 ± 1.26c	20.25 ± 1.10c	26.25 ± 1.65c
2.0	0.00 ± 0.00c	0.00 ± 0.00c	5.00 ± 0.71c	0.00 ± 0.00d	0.00 ± 0.00d	7.00 ± 1.29d

Means in each column followed by the same letter are not significantly different by Tukey's test (P<0.05).

Table 1: Percentage of *Diatraea saccharalis* (Lepidoptera: Crambidae) egg hatching treated with different concentrations of neem oil and compared to control.

Concentrations (%)	Period embryonic	Hatching total (%) Mean±SE	% reduction	Larva mortality (%) Mean±SE
0.0	5.50 ± 0.64	79.17 ± 2.58[a]	---------	---------
0.1	5.75 ± 0.57	49.00 ± 5.70[b]	38.1	21.65 ± 4.04 [a]
0.3	5.87 ± 0.98	19.82 ± 6,91[c]	74.9	89.17 ± 4.90 [b]
0.5	6.21 ± 1.10	17.82 ± 6.82[c]	77.5	97.01 ± 2.29 [b]
1.0	5.60 ± 0.67	15.34 ± 5.97[c]	80.7	95.58 ± 2.36 [b]
2.0	5.28 ± 1.21	0.83 ± 0.83[c]	98.9	97.52 ± 2.56 [b]
F-values	0.4041 [NS]	28.15[***]	97.39[***]	

Means in each column followed by the same letter are not significantly different by Tukey's test (P<0.05); [***] P< 0.0001; [NS] not significant.

Table 2: Toxicity of neem oil on *Diatraea saccharalis* (Lepidoptera: Crambidae) hatching and caterpillars mortality when applied to eggs immediately after being laid.

Caterpillar formation occurred in eggs that were treated for 4 days from the start of development. However, the eggs had a darker colour, and a few caterpillars failed to break the eggs chorion (Figures 1B and 1C). These observations are similar to those obtained for neem-treated insects that completed the embryonic development, but died without rupturing the egg chorion [35]. According to these authors, the neem extract had not interfered in their embryogenesis. On the other hand, it has been reported that there are no changes in development and survival, even when neonatal caterpillars feed on neem extract-treated chorion [36]. Neem treatment of female individuals of *Rhipicephalus sanguineus* (Acari: Ixodidae), resulted in modifications of the chorion after oviposition, impairied maturation, and production of new individuals [18]. In the present study, treatment with neem altered the morphology of the caterpillars, during their formation within the egg (i.e. during the embryonic development), thus, before having contact with the chorion (Figures 1A-1D).

One- or 2-day-old eggs treated with neem oil exhibited a yellowish-orange colour, with small dark spots, which was observed after 9 days of treatment (Table 1, Figure 1D). Control eggs were either mostly hatched, or the caterpillars were fully developed and prepared for hatching (Table 1, Figure 1A). In all treatments, there was a decrease in the viability of the eggs. The strongest decrease occurred in eggs up to 3 days of age. Hatched caterpillars showed had high mortality rates when treated with neem oil, at concentrations ranging 0.3-2.0% that varied between 89.17 ± 4.9% and 97.52 ± 2.56%. No significant differences among treatments have been detected ($p<0.05$; $F=1.44$; Table 2). These results are similar to those obtained by using caterpillars of *Mamestra brassicae* (Lepidoptera: Noctuidae). Treated eggs remained at the first instar stage, whereas caterpillars of control eggs developed towards the second and third instar stages [37].

Sensitivity of neonate caterpillars to neem, i.e. aggressiveness, has been reported when the extract is sprayed immediately after the hatching of the eggs of *Acantholyda erythrocephala* (Hymenoptera: Pamphiilidae) [38]. In the case of *Cycloneda sanguinea* (Coleoptera: Coccinellidae), the authors did not report any changes in the survival and duration of caterpillars that development from neem-treated eggs [36]. After 9 days of treatment, 5 day and 9 day *D. saccharalis* eggs treated with neem oil showed few dead caterpillars, just before completion of hatching. Some died with parts of their body outside the egg. In fact, these results are very similar to those observed in eggs of *Tuta absoluta* (Lepidoptera: Gelechiidae) [39]. Ovicidal effects occurred in eggs at the start of the development (at 1, 2 and 3 days of age), which confirms that older eggs are more resistant to plant extracts due to their higher maturity, and because they hinder the entrance of external products [19,25]. Other studies report that Lepidoptera eggs have a lipid membrane in the internal part of the chorion that impedes ovicidal agents, and thus, makes them insensitive to these compounds [40].

Few *D. saccharalis* caterpillars, apparently not affected by neem, hatched and died at 1 or 2 days after completion of hatching. This occurred mainly at neem concentrations of 0.3 and 0.5%, i.e. at the concentrations suggested in the formulation (Table 3). Studies have reported the death of caterpillars, immediately after hatching from the eggs of *Neodiprion abietis* (Hymenoptera: Diprionidae), treated with Neemix 4.5 EC at a concentration of 90 ppm [41]. Since plant extracts are viscose, this effect may be due to impaired locomotion and feeding of caterpillars, especially those at the first instar stage, and thus, reduced viability in this phase [7].

The current study demonstrates that neem oil had the highest effect on hatched caterpillars of treated eggs, because of its interference during the development of the caterpillars. This has caused abnormalities, and the subsequent death of the caterpillars (Table 2, Figures 1E-1H). When eggs were treated with neem oil at a concentration of 2%, formation of caterpillars was reported on the second day of development (2 days after oviposition). However, the caterpillars had a greyish colour; their colour was lighter than that of the control. Hatched caterpillars showed a somewhat soft abdomen, light colour, and in some instances, lack of the cephalic capsule (Figures 1D and 1F). Probably, this concentration of neem oil which has a higher content of azadirachtin, caused the inhibition of ovarian ecdysteroids [42], that are produced by follicular

Age Eggs (days) treated	Treatments									
	0.1		0.3		0.5		1.0		2.0	
	LE	R*	LE	R*	LE	R*	LE	R*	LE	R*
1	27.5 ± 1.5a	67	1.0 ± 1.0a	99	1.5 ± 0.9ª	98	2.5 ± 1.5ª	97	0.0 ± 0.0a	----
2	36.0 ± 1.8b	58	3.0 ± 0.5b	96	1.0 ± 0.5ª	99	0.0 ± 0.0a	----	0.0 ± 0.0ac	----
3	54.5 ± 2.2c	31	9.5 ± 0.9ab	88	3.7 ± 0.8ª	95	6.5 ± 0.9ab	92	5.0 ± 0.7bd	94
4	56.0 ± 2.9c	28	37.7 ± 4.1c	51	38.5 ± 3.3b	50	36.5 ± 1.2c	53	0.0 ± 0.0ac	----
5	63.5 ± 3.5c	15	32 ± 2.1c	57	30.7 ± 2.8b	59	20.2 ± 1.1d	73	0.0 ± 0.0ac	----
6	56.5 ± 4.0c	25	35.7 ± 0.8c	25	27.0 ± 1.4b	64	26.2 ± 1.6e	65	7.0 ± 1.3bd	91
F-values	24.60***		70.31***		67.90***		147.3***		27.69***	

LE- Mean ± SE caterpillars hatched.

R*- % reduction over Abbott [27], mortality of caterpillars hatching.

Means in each column followed by the same letter are not significantly different by Tukey's test (P<0.05);); *** P< 0.0001.

Table 3: % Mean ± SE in the caterpillars of eggs and respective mortality in *Diatraea saccharalis* (Lepidoptera: Crambidae) after one or two days of hatching.

cells of oocytes, and was incorporated into eggs during ovarian development [43]. Embryonic ecdysone signaling is required in eggs for germ band retraction, head involution, and morphogenetic movements that shape the first instar caterpillars [44].

Conclusions

The results show that neem oil is highly toxic to eggs and first instar caterpillars when the extract is sprayed on eggs, as demonstrated by impaired embryonic development and residual activity in the production of new individuals. Thus, neem oil may be an excellent agent for use against the sugarcane borer, during the stage in which the insect penetrates the stalk, causing heavy damage to the sugar cane crop.

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