ISJ 11: 79-86, 2014 ISSN 1824-307X

RESEARH REPORT

Cytotoxicity in the midgut and fat body of *Anticarsia gemmatalis* (Lepidoptera: Geometridae) larvae exerted by neem seeds extract

GD de Almeida[1], JC Zanuncio[2], S Senthil-Nathan[3], D Pratissoli[4], RA Polanczyk[4], DO Azevedo[1], JE Serrão[1]

[1]*Departamento de Biologia Geral, Universidade Federal de Viçosa, 36570-000, Viçosa, MG, Brazil*
[2]*Departamento de Biologia Animal, Universidade Federal de Viçosa, 36570-000, Viçosa, MG, Brazil*
[3]*Division of Biopesticides and Environmental Toxicology, Sri Paramakalyani Center for Environmental Sciences (SPKCES), Manonmaniam Sundaranar University, Alwarkurichi – 627 412, Tirunelveli, Tamil Nadu, India*
[4]*Departamento de Produção Vegetal, Universidade Federal do Espírito Santo, 29500-000, Alegre, ES, Brazil*

Accepted February 14, 2014

Abstract

Botanical pesticides may be an alternative to the use of synthetic insecticides against agricultural pests and neem extracts have been successfully used against some insect pests. This study evaluated the effect of neem in biological and morpho-physiological parameters of the velvetbean caterpillar *Anticarsia gemmatalis*. The third instar larvae of *A. gemmatalis* were fed on artificial diet containing different concentrations of neem seed kernel extract (NSKE). The biological parameters were adversely affected after ingestion of the diet with neem extracts. Doses of more than 500 ppm of the NSKE in the diet caused 100 % mortality in the larvae of *A. gemmatalis*, whereas lower doses reduced food intake and reproductive capacity, and increased production of pupae with morphological deformities. The cells of the midgut epithelium of *A. gemmatalis* larvae showed swelling, basal membrane detachment and complete disruption after exposure to NSKE. In addition to the cytotoxicity effect observed in the fat body, the treatment reduced lipid and protein reserves. The NSKE negatively affected the physiological and biological parameters of *A. gemmatalis*.

Key Words: neem; velvetbean; food intake; fat body; midgut

Introduction

Botanical pesticides can be an alternative to the use of synthetic pesticides, because they are rapidly degraded in the environment and have low toxicity to natural enemies and mammals (Copping and Menn, 2000). The plant that has shown the highest potential insecticide activity in the world is *Azadirachta indica* A. Juss (Meliacea), through the synthesis of azadirachtin, a tetranortriterpenoid produced as a secondary metabolite. Azadirachtin inhibits the growth, affects survival, cause repellence and feeding deterrence, reduces the fertility of females and causes anatomical abnormalities in several species of insects (Martinez and Emden, 1999; Mordue *et al.,* 2000). In insects, azadirachtin has direct cytotoxic effects on glands (Sayah *et al.,* 2002), reproductive organs (Sayah *et al.*, 1996) and intestine (Nogueira *et al.*, 1997; Correia *et al.,* 2009). In addition, it affects protein metabolism (Huang *et al.,* 2004, 2007) and enzyme synthesis in insects (Lowery and Smirle, 2000).

The velvetbean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) is an important insect defoliator of soybean in North and South America (Nascimento *et al.*, 2003). The frequent use of pesticides to control this insect may be harmful to natural enemies acting in biological control (Vianna *et al.*, 2009), and also to contaminate the environment (Jergentz *et al.*, 2005).

Furthermore, the effect of products derived from *A. indica* has been poorly studied in soybean defoliating caterpillars. Thus, the study evaluated the effects of neem on biological and morpho-physiological parameters of *A. gemmatalis* caterpillars.

Corresponding author:
José Eduardo Serrão
Department of General Biology
Federal University of Viçosa
36570-000, Viçosa
Minas Gerais State, Brazil
E-mail: jeserrao@ufv.br

Material and Methods

Anticarsia gemmatalis

The velvetbean caterpillar was reared with artificial diet (Greene *et al.* 1976) at 25 ± 1 ºC , 70 ±

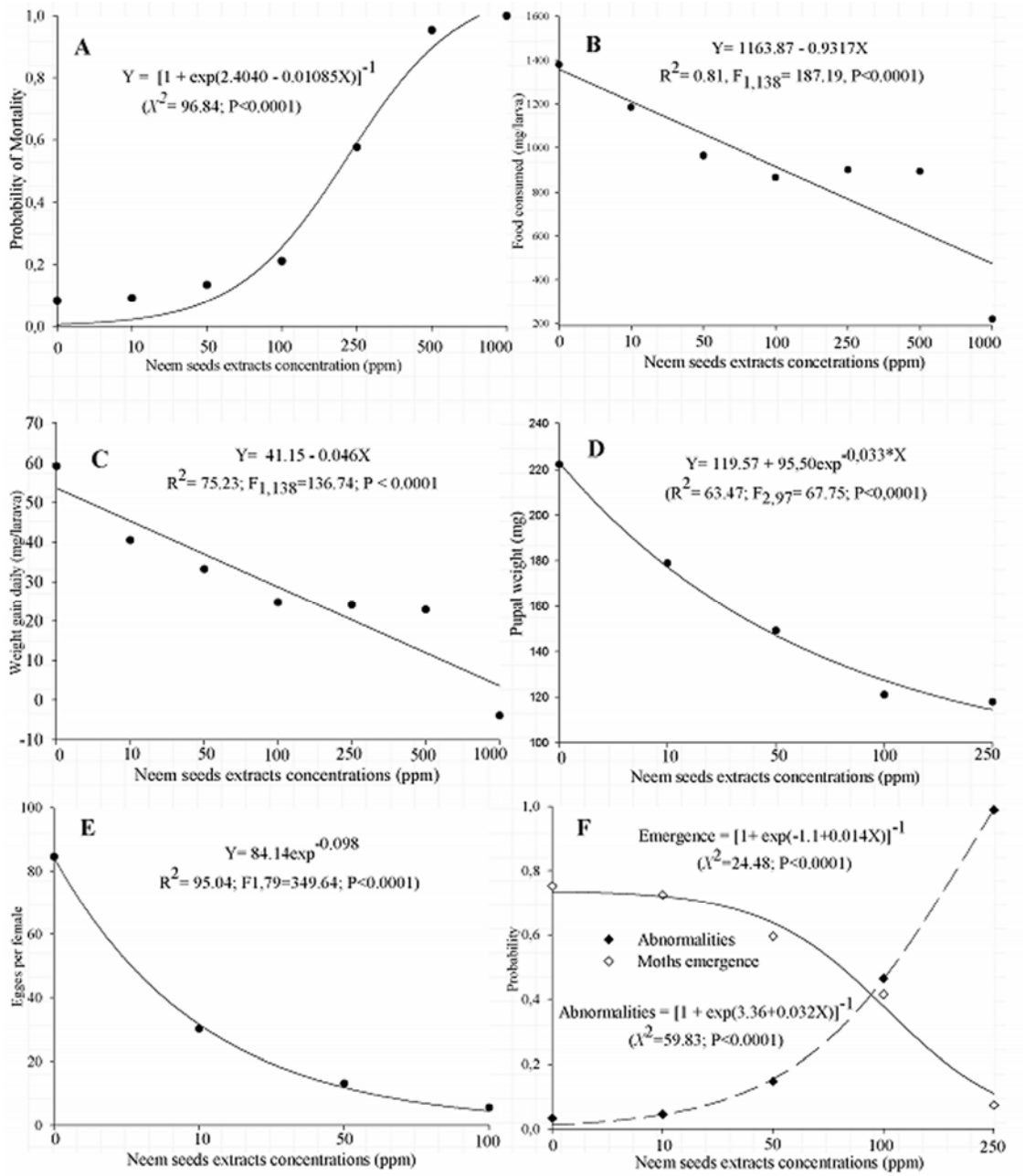

Fig. 1 Biological data of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) after larval feeding for 4 d on artificial diet with neem seed extracts (0.061 g.ml^{-1} of azadirachtin in parent material). (A) Mortality, (B) food consumption, (C) larval weight gain daily, (D) pupal weight, (E) eggs per female and (F) relationship between pupal abnormalities and moths emergence.

10 % relative humidity and photoperiod of 14:10 (L:D) in the Insect's Biological Control Laboratory of Universidade Federal de Viçosa, Viçosa, Minas Gerais State, Brazil.

Preparation of neem seeds extracts

Ripened fruits of *A. indica* were collected from Espírito Santo State, Brazil (18º 39' S and 40º 51' W), pulped in water, air dried and stored at -2 ºC. The obtained seeds (500 g) were macerated in 1.5 L of ethanol and filtered with Wattman-1 filter paper. The solvent was removed from the filtrate by evaporation under vacuum with rotary evaporator at 50 ± 5 ºC for five days. This process was repeated thrice, obtaining 90 mL of a dark solution for preparation of stock solution. The 10 % stock solution was prepared with 10 mL of *A. indica* seed extract diluted in 90 mL of 30 % ethanol.

Fig. 2 Pupal morphological malformations of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) after larval feeding for 4 d with an artificial diet containing a neem seed extracts. A) 0, B) 10, C) 50, D) 100, E) 250, F) 500 and G) 1000 ppm.

The azadirachtin present in the seed extract was quantified by high performance liquid chromatograph (HPLC), using ultra violet detector at 217 nm according to the method proposed by Schaaf *et al.* (2000). A total of 20 µL of 20 % *A. indica* seed extract solution were injected into a reserve-phase column (C18), with a flow of 0.6 mL min^{-1}, and column pressure of 97 Kgf. The solvent used was methanol and water (1:1). The presence of azadirachtin in the crude neem seed extract was found at 14.13 min in HPLC, following the same pattern of pure azadirachtin (Sigma-Aldrich - Germany). The azadirachtin was obtained at a concentration of 12.18 µg of azadirachtin per µL of 20 % crude seed extract, corresponding to a stock solution of 6.1 $g.L^{-1}$ of azadirachtin.

Effects on larval and pupal development of Anticarsia gemmatalis

The evaluations of the effects of neem seed kernel extract (NSKE) on the larval and pupal stages of *A. gemmatalis* were adapted from Senthil-Nathan *et al.* (2006a, b). The 6-day-old third instar larvae were starved for four hours, kept individually in Petri dishes (9.5 cm diameter) for four days receiving 1.2 g of artificial diet containing 0 (control), 10, 50, 100, 250, 500 and 1000 ppm of NSKE (0.061 g of azadirachtin per mL) daily. After this period, the caterpillars were fed on diet without neem extracts. Twenty larvae were used for the experiment and it was replicated five times. Larval mortality was assessed daily until the beginning of the pupal stage. Food consumption was determined by subtracting the mass of the diet provided by the mass leftover, and it was evaluated only during the period of exposure of larvae to artificial diets with NSKE. The weight gain daily (WGD) of *A. gemmatalis* larvae was obtained by using the formula WGD = (Wf - Wi) / T, where Wf = weight in the last larval stage, Wi = larval weight at the beginning of the experiment, and T = time in days between the third and last larval stage. The pupal weight and abnormalities were measured 24 h after the molt to the third instar, and the ratio of abnormal pupae and emergence of *A. gemmatalis* moths was also evaluated.

Reproductive parameters

A total of 20 pairs of newly-emerged larvae of *A. gemmatalis* were caged individually in plastic tubes (25 cm diameter) and fed for four days on an artificial diet with different concentrations of NSKE (0, 10, 50 and 100 ppm), followed by evaluation of the number of eggs per female. The eggs were counted between the 3^{th} and 6^{th} days after the emergence, which corresponded to the peak of egg production of *A. gemmatalis* (Greene *et al.,* 1973). The treatments with 500 and 1000 ppm of NSKE were not employed, because no moths were obtained from these treatments.

Histology

The third instar larvae of *A. gemmatalis* were fed on artificial diet containing 0 and 500 ppm of NSKE. Three larvae per concentration were collected after 2, 3 and 4 days of feeding and were transferred to Zamboni fixative solution (Stefanini *et al.,* 1967) for 24 h at 4 ºC. The midgut and fat body were dissected into fixative solution, dehydrated in a graded ethanol series and embedded in historesin JB-4. Subsequently, slices 5 µm thickness were stained with hematoxyline and eosin and analyzed under light microscope.

SDS-PAGE

The analyses of protein profile of haemolymph and fat body of *A. gemmatalis* larvae were adapted from the methods described byHuang *et al.* (2004, 2007). Briefly, the third instar larvae of *A. gemmatalis* were fed on artificial diet with 0, 1 or 500 ppm of NSKE for four days. Samples of hemolymph and fat body of 10 *A. gemmatalis* larvae per concentration were collected and placed in 0.1 % EDTA solution, following homogenization in 125

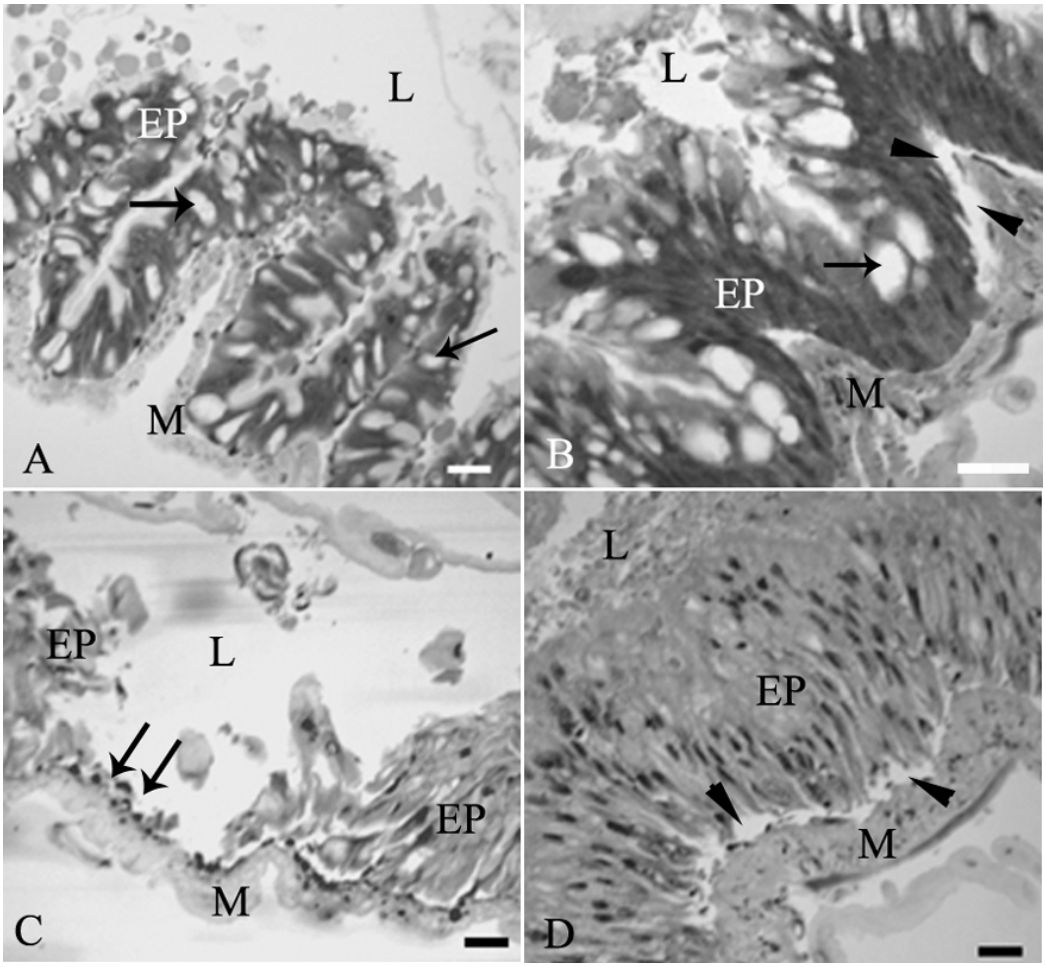

Fig. 3 Morphological changes in the midgut of *Anticarsia gemmatalis* larvae (Lepidoptera: Noctuidae) after feeding with control diet (A), 2 d (B), 3 d (C) and 4 d (D) on diet containing neem seeds extracts. Note epithelium detachment (arrowheads) and disruption (double arrows) in larvae fed on neem extract. Arrows - globet cells, L- lumen, EP- epithelium, M- muscle. Bars = 20 µm.

mM saline solution. These samples were centrifuged at 12000x*g* for 15 min, and the supernatant was collected for total protein quantification according to Braford (1976). The proteins (50 µg) were separated by 12 % SDS-PAGE (Laemmli, 1971), and the gel was stained with Coomassie blue solution.

Statistical Analysis

Food consumption, weight gain, weight of pupae and number of eggs per female were analyzed by regression models. Mortality, pupal abnormalities and adult emergence were subjected to regression analysis of Logit.

Results

The larval mortality of *A. gemmatalis* increased with NSKE concentration in the artificial diet, reaching 100 % at 500 ppm (Fig. 1A). All the concentrations tested affected food consumption, weight gain of larvae and pupae, and fertility of adult females (Figs 1B - E). The quantity of artificial diet consumed by *A. gemmatalis* larvae over four days decreased linearly with increasing concentration of NSKE in the diet ($F_{1,138}$ = 187.19, R^2 = 81.00, p < 0.0001). The larvae fed on control diet (without NSKE) ingested 1400 mg of artificial diet, whereas those fed on NSKE consumed 1193, 1023, 877, 852, 823 and 200 mg of diets containing 10, 50, 100, 250, 500 and 1000 ppm of NSKE, respectively (Fig.1B).

The daily larval weight gain of *A. gemmatalis* was linearly reduced with increasing concentrations of NSKE, reaching negative values with 1000 ppm ($F_{1,138}$ = 136.74, R^2 = 75.23, p < 0.0001) (Fig. 1C). The pupal weight was exponentially reduced with increasing concentration of NSKE in the diet ($F_{2,97}$ = 67.75, R^2 = 63.47, p < 0.0001), with pupae from larvae fed on 250 ppm showing 50 % of weight reduction (Fig. 1D).

There was an inverse relationship between the number of abnormal pupae and the emergence of moths of *A. gemmatalis* with increasing doses of NSKE (Fig. 1F). Furthermore, adults did not emerge from abnormal pupae. The *A. gemmatalis* pupal

abnormalities manifested as small intense malformations in the thorax area to no pupation (Fig. 2). The occurrence of these symptoms was proportional to the increased dosage of NSKE in the diet.

The larvae fed on low doses of NSKE (10, 50 and 100 ppm) reached the adult stage but showed exponential reduction in the oviposition rate ($F_{1,79}$ = 349.63, R^2 = 91.04, p < 0.0001). The average number of eggs per moth was 85, 30, 13 and 5 for females from larvae fed on 0, 10, 50 and 100 ppm of NSKE, respectively (Fig. 1E). Furthermore, *A. gemmatalis* fed on diets containing 500 ppm of NSKE showed morphological changes in the midgut when compared with the control larvae, which presented columnar digestive cells with evident striated border (Fig. 3A). In the larvae treated with the neem extract, the midgut cells were swollen and detached from the basal membrane after two days (Fig. 3B), while there was complete destruction of cells in some regions of the midgut epithelium after three days (Fig. 3C) and midgut atrophy, characterized by a narrowed lumen, at four days (Fig. 3D).

The fat body cells of *A. gemmatalis* control larvae showed large granules of lipids and proteins (Fig. 4A). However, the NSKE treated larvae showed lower amounts of lipids and absence of protein accumulation (Fig. 4B). Furthermore, the protein expression pattern in the hemolymph was similar in *A. gemmatalis* larvae fed on diets containing NSKE (Fig. 5). On the other hand, the protein expression in the fat body was inhibited by diets containing 500 ppm of NSKE, whereas, the protein profile of larvae fed a diet with 100 ppm NSKE was similar to that of the control larvae (Fig. 5).

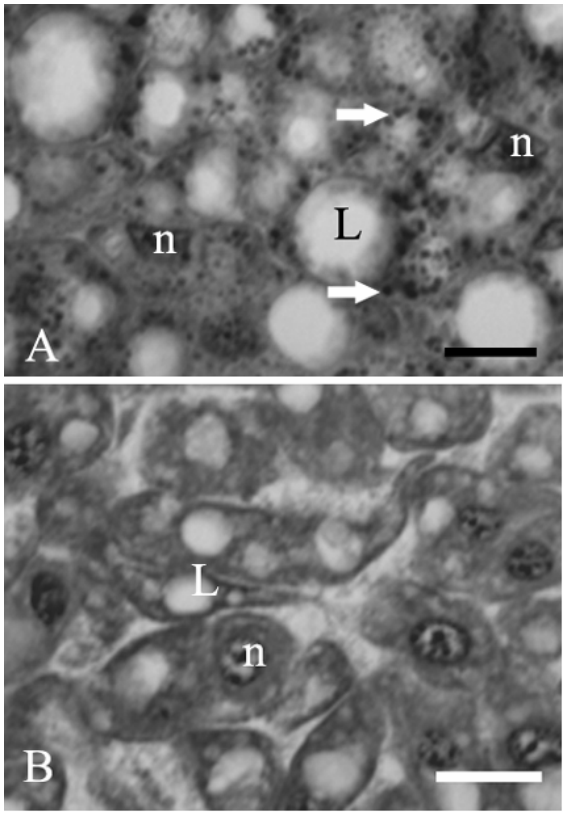

Fig. 4 Fat body of *Anticarsia gemmatalis* larvae (Lepidoptera: Noctuidae) 4 d after feeding on control diet (A) and neem seeds extracts (B). Note protein granule (arrows) control that are lacking in larvae fed on neem extract. L- lipids, n - nucleus. Bars = 10 µm.

Discussion

The NSKE was efficient in controlling *A. gemmatalis* larvae, which exhibited increase in mortality, decrease in food consumption, larval and pupal weight, pupal abnormalities, and lower reproductive capacity after feeding on diet with different NSKE concentrations. Similar effects have also been observed for other caterpillars such as *Spodoptera littoralis* Boisduval (Martinez and Emden, 2001) and *Spodoptera litura* F. (Huang *et al.,* 2004), *Cnaphalocrocis medinalis* Guenée (Senthil-Nathan *et al.,* 2006b), *Plodia interpunctella* Hübner (Rharrabe *et al.,* 2008), *Helicoverpa armigera* Hübner (Kumar *et al.,* 2008), and *Tuta absoluta* (Tome *et al*., 2013) after exposure to neem derivatives.

Azadirachtin and other limonoids present in ethanolic NSKE may be the cause of the antifeeding effects found in *A. gemmatalis.* It has been reported that the addition of 6ß-hidroxigeduim, gedunin, nimbinen, salanin or azadirachtin in artificial diet caused lower nutritional rates in *H. armigera* and *S. litura*, and azadirachtin showed the highest activity at a lower dose (Koul *et al.,* 2003). Therefore, the effects of NSKE found in *A. gemmatalis* larvae may primarily be attributed to the azadirachtin, because the other limonoids have been observed to be effective only at doses higher than those of azadirachtin (Senthil-Nathan *et al.,* 2006b), and the quantities of these substances in the neem seed extract have been found to be generally lower than that of azadirachtin (Caboni *et al.,* 2002).

The cellular changes in the midgut epithelium of *A. gemmatalis* larvae are related to the antifeeding action of NSKE, promoting decrease in weight gain in the larvae and pupae of this insect. It has been reported that hypertrophy and displacement of epithelial cells from the basal lamina reduces the digestive capacity (Barbeta *et al.,* 2008; Correia *et al*., 2009). This hypertrophy may be the result of cytoplasm vacuolation, endoplasmic reticulum fragmentation, and microvilli and plasma membrane disruption, as reported in the midgut cells of *Schistocerca gregaria* and *Locusta migratoria* (Orthoptera: Acrididae) (Nasiruddin and Mordue, 1993), *Rhodnius prolixus* (Hemiptera: Reduvidae) (Nogueira *et al*., 1997) and *Aedes aegypti* (Diptera: Culicidae) (Ndione *et al*., 2007) after azadirachtin exposure. These effects may also be related to the decrease in the midgut cells basal membrane folds and associated mitochondria (Nogueira *et al*., 1997)

and ATPase activity (Senthil-Nathan *et al*., 2005), disrupting the ion transport across membranes and promoting excessive water influx. Several studies have shown that pure azadirachtin exerts cytotoxic effect, *in vitro*, on insects (Salehzadeh *et al*., 2003). Furthermore, other compounds synthesized by *A. indica* such as nimbolide and epoxyazadiradione have been noted to cause disruption in plasma membrane and swelling of cells (Cohen *et al*., 1996).

Unlike the midgut epithelial cells, the fat body did not show cellular disorganization, but exhibited reduced lipid droplets and proteins granules. The cytotoxic effects of neem extracts on the midgut epithelium of *A. gemmatalis* larvae may be responsible for the reduction in the amount of lipids and proteins in the fat body, related to the inability of the larvae to metabolize and convert ingested food to reserves of fat body cells. Furthermore, together with alterations in the midgut morphology, azadirachtin has also been observed reduce the activity of the digestive enzymes in Lepidoptera (Timmins and Reynolds, 1992; Senthil-Nathan *et al*., 2005, Rharrabe *et al*., 2008).

The alterations in *A. gemmatalis* fat body protein production may have caused changes in the larval and pupal metamorphosis, as well as reduced oviposition rates in adult moths. Stored proteins are synthesized in the larvae of Lepidoptera, and are used as metamorphosis precursors, in egg production, and nutrient source during the adult life of *A. gemmatalis* (Canavoso *et al*., 2001). Thus, the larvae fed on neem extract that survived the lethal effects of this substance and emerged as adult frequently presented reduced reproductive capacity. In a previous study, topical application of azadirachtin to *Spodoptera exempta* Walker larvae (Lepidoptera: Noctuidae) affected oogenesis and reproductive maturation in adult females of this species owing to reduced protein synthesis in the fat body in the adult (Tanzubi and McCaffery, 1990).

The NSKE caused morphological malformations in *A. gemmatalis* pupae, which prevent adult emergence. These deformities were more extreme with higher doses of this preparation. Similar effects have also been reported in *S. littolarlis* larvae fed on artificial diet containing azadirachtin (Martinez and Emden, 2001). The abnormalities and inhibition of metamorphosis can be attributed to disruptions in the molting hormone synthesis and release under the action of azadirachtin (Mordue and Nisbet, 2000). This effect may explain the larval and pupal mortality of *A. gemmatalis*, because the increase in mortality coincides with the metamorphosis period.

The disruption of *A. gemmatalis* lifecycle at different developmental stages by NSKE can reduce the amount of active ingredient to be applied to control this insect pest. In the present study, *A. gemmatalis* larvae that were fed a diet containing 100 ppm of NSKE showed < 20 % mortality rates, 40 % decrease in food consumption decrease, high rate of larvae with deformities, low number of adult emergence, and low oviposition rates, which limited the population growth in the next generation of this pest. Thus, the use of insecticides based on azadirachtin is important in *A. gemmatalis*

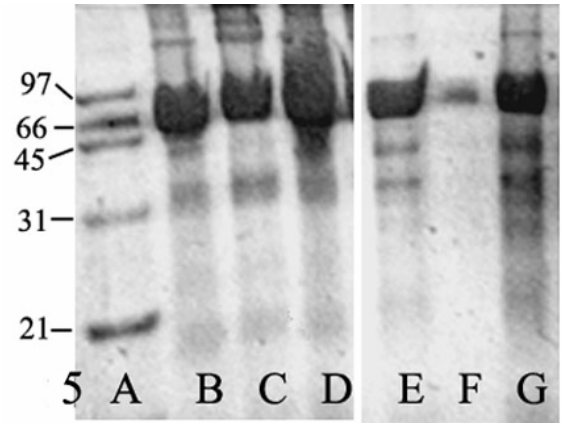

Fig. 5 Protein expression in hemolymph (lanes B, C, D) and fat body (lanes F, G, H) of *Anticarsia gemmatalis* larvae (Lepidoptera: Noctuidae) 4 d after feeding an artificial diet with neem seed extracts. A) molecular weight standard (kDa), B) hemolymph control, C) 500 ppm, D) 100 ppm of neem seeds extract, E) fat body control, F) 500 ppm, G) 100 ppm of neem seeds extracts.

management programs owing to the lethal and residual effects of this compound. Furthermore, the seed extract of *A. indica* ws found to cause mortality, reduce food consumption, decrease larvae and pupae weight gain, inhibit metamorphosis, cause malformations in pupae, reduce fertility in moths, cause morphological changes in the midgut and fat body cells, and change protein production in the fat body of *A. gemmatalis* larvae. In conclusion, the neem extracts had significant effects on *A. gemmatalis* and the adult development and reproduction was affected after the larvae were fed NSKE. Therefore, these extracts may be used as an effective alternative to other synthetic pesticides in the control of velvet bean caterpillar.

Acknowledgements

The authors would like to thank the “Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG)”.

References

Barbeta BL, Marshal AT, Gillon A, Craik DJ, Marlyn AA. Plant cyclotides disrupt epithelial cell in the midgut of Lepidoptera larvae. Proc. Nat. Acad. Sci. USA 105: 1221-1225, 2008.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Caboni P, Cabras M, Angioni A, Russo M, Cabras P. Persistence of azadiractin residues on olives after field treatment. J. Agr. Food Chem. 50: 3491-3494, 2002.

Canavoso LE, Jouni ZE, Karnas KJ, Pennington JE, Wells MA. Fat metabolism in insects. Annu. Rev. Nutr. 21: 23-46, 2001.

Cohen E, Quistad GB, Casida JE. Cytotoxicity of nimbolide, epoxyazariradione and other limonoids from neem insecticide. Life Sci. 58: 1075-1081, 1996.

Copping, LG, Menn, JJ. Biopesticides: a review of their action, applications and efficacy. Pest. Manag. Sci. 56: 651-676, 2000.

Correia AA, Wanderley-Teixeira V, Teixeira AAC, Oliveira JV, Torres JB. Morfologia do canal alimentar de lagartas de *Spodoptera frugiperda* (JE Smith) (Lepidoptera: Noctuidae) alimentadas com folhas tratadas com nim. Neotrop. Ent. 38: 83-91, 2009.

Greene GL, Leppla NC, Dickerson WA. Velvetbean caterpillar: a rearing procedure and artificial medium. J. Econ. Entomol. 69: 487-488, 1976.

Greene GL, Reid JC, Blount VN, Riddle TC. Mating and oviposition of the velvetbean caterpillar in soybeans. Environ. Entomol. 2: 1113-1115, 1973.

Huang Z, Shi P, Chen C, Du J. Protein metabolism in *Spodoptera litura* (F.) is influenced by the botanical insecticide azadirachtin. Pestic. Biochem. Phys. 80: 85-93, 2004.

Huang Z, Shi P, Chen C, Du J. Effects of azadirachtin on hemolymph protein expression in *Ostrina furnacalis* (Lepidoptera: Crambidae). Ann. Ent. Soc. Am. 100: 245-250, 2007.

Jergentz S, Mugni H, Bonetto C, Schulz R. Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. Chemosphere 61: 817-826. 2005.

Koul OK, Multani JS, Singh G, Daniewski WM, Berlonzek S. 6ß-hydroxygedunin from *Azadirachta indica*. Its potentiation effects with some non-azadirachtin limonoids in neem against lepidopteran larvae. J. Agr. Food Chem. 51: 2937-2942, 2003.

Kumar NS, Muragan K, Zhang W. Additive interaction of *Helicoverpa armigera* nucleopolyhedrovirus and azadirachtin. BioControl 53: 869-880, 2008.

Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 237: 680-685, 1971.

Lowery DT, Smirle MJ. Toxicity of insecticides to obliquebanded leafroller, *Choristoneura rosaceana*, larvae and adults exposed previously to neem seed oil. Entomol. Exp. Appl. 95: 201-207, 1992.

Martinez SS, van Emden HF. Sublethal concentrations of azadirachtin affect food intake, conversion efficiency and feeding behaviour of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae). B. Entomol. Res. 89: 65-71, 1999.

Martinez SS, van Emden HF. Growth disruption, abnormalities and mortality of *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) caused by azadirachtin. Neotrop. Entomol. 30: 113-125, 2001.

Mordue AJL, Nisbet AJ. Azadirachtin from the neem tree *Azadiracta indica* its actions against insects. An. Soc. Entomol. Bras. 29: 616-632, 2000.

Nascimento IR, Murata AT, Bortoli SA, Lopes LMX. Insecticidal activity of chemical constituents from *Aristolochia pubescens* against *Anticarsia gemmatalis* larvae. Pest Manag. Sci. 60: 413-416, 2003.

Nasiruddin M, Mordue AJL. The effect of azadirachtin on the midgut histology of the locust, *Shisctocerca gregaria* and *Locusta migratoria.* Tissue Cell 25: 875-884, 1993.

Ndione RD, Faye O, Ndiaye M, Dieye A, Afoutou JM. Toxic effects of neem products (*Azadirachta indica* A. Juss) on *Aedes aegypti* Linnaeus 1762 larvae. Afr. J. Biotech. 6: 2846-2854

Nogueira NF, Gonzáles M, Garcia EM, Souza W. Effects of azadirachtin A on the fine structure of the midgut of *Rhodnius prolixus*. J. Invertebr. Pathol. 69: 58-63, 1997.

Rharrabe K, Bouayad N, Sayah F. Effects of ingested 20-hydroxyecdysone on development and midgut epithelial cells of *Plodia interpunctella* (Lepidoptera, Pyralidae). Pestic. Biochem. Physiol. 93: 112-119, 2009.

Rharrabe K, Amri H, Bouayad N, Sayah F. Effects of azadirachtin on post-embryonic development, energy reserves and a-amylase activity of *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae). J. Stor. Prod. Res. 44: 290- 294, 2008.

Salehzadeh A, Akhkha A, Cushley W, Adams RLP, Kusel JR, Strang RHC. The antimitotic effect of the neem terpenoid azadirachtin on cultured insect cells. Insect Biochem. Molec. Biol. 33: 681-689, 2003.

Sayah F. Ultrastructural changes in the corpus allatum after azadirachtin and 20-hydroxyecdisone treatment in adults females of *Labidura riparia* (Dermaptera). Tissue Cell 34: 53-62, 2002.

Sayah F, Fayet C, Idaomar M, Karlinsky A. Effects of azadirachtin on vitellogenesis of *Labidura riparia* (Dermaptera). Tissue Cell 28: 741-749, 1996.

Schaaf O, Jarvis AP, van der Esch SA, Giagnacovo G, Oldham NJ. Rapid and sensitive analysis of azadirachtin and related triterpenoids from neem (*Azadirachta indica*) by high-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. J. Chromatogr. 886: 89-97, 2000.

Senthil-Nathan SS, Kalaivani K, Chung PG. The effects of azadiracthin and nucleopolyhedrovirus on midgut enzymatic profile of *Spodoptera litura* Fab. (Lepidoptera: Noctuidae). Pestic. Biochem. Phys. 83: 46-57, 2005.

Senthil-Nathan SS, Kandaswamy K, Chung PG, Murugan K. Effects of neem limonoids on lactate dehydrogenase (LDH) of the rice leaffolder, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). Chemosphere 62: 1388-1393, 2006a.

Senthil-Nathan SS, Kalaivani K, Sehoon K, Muragan K. The toxicity and behavioral effects of limonoids on *Cnaphalocrocis medinalis*

Guenée (Lepidoptera: Pyralidae), the rice leaffolder. Chemosphere 62: 1381-1387, 2006b.

Stefanini M, Demartino C, Zamboni L. Fixation of ejaculated spermatozoa for electron microscopy. Nature 216: 173-174, 1967.

Tanzubil PB, McCaffery AR. Effects of azadirachtin on reproduction in the African armyworm (*Spodoptera exempta*). Entomol. Exp. Appl. 57: 115-121, 1990.

Timmins WA, Reynolds SE. Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* caterpillars: reduced growth due to impaired protein digestion. Entomol. Exp. Appl. 63, 47-54, 1992.

Tome HVV, Martins JC, Correa AS, Galdino TVS, Picanco MC, Guedes RNC. Azadirachtin avoidnace by larvae and adult females of the tomato leafminer *Tuta absoluta*. Crop Prot. 46: 63-69, 2013.

Vianna UR, Pratissoli D, Zanuncio JC, Lima ER, Brunner J, Pereira FF, Serrão JE. Insecticide toxicity to *Trichogramma pretiosum* (Hymenoptera: Trichogrammatidae) females and effects on descendent generation. Ecotoxicol. 18: 180-186, 2009.