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Squamocin induce histological and ultrastructural changes in the midgut cells of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae)

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ABSTRACT

Annonaceous acetogenins (*Annona squamosa* Linnaeus) comprises of a series of natural products which are extracted from Annonaceae species, squamocin proved to be highly efficient among those agents. Squamocin is mostly referred as a lethal agent for midgut cells of different insects, with toxic effects when tested against larva of some insects. In present study, LC_{50} and LC_{90} of squamocin for *A. gemmatalis* Hübner (Lepidoptera: Noctuidae) were calculated using probit analysis. Morphological changes in midgut cells were analyzed under light, fluorescence and transmission electron microscopes when larvae were treated with LC_{50} and LC_{90} of squamocin for 24, 48 and 72 h. Results revealed that the maximum damage to midgut cells was found under LC_{90} where it showed digestive cells with enlarged basal labyrinth, highly vacuolated cytoplasm, damaged apical surface, cell protrusions to the gut lumen, autophagy and cell death. The midgut goblet cells showed a strong disorganization of their microvilli. Likewise, in insects treated with squamocin, mitochondria were not marked with Mitotracker fluorescent probe, suggesting some molecular damage in these organelles, which was reinforced by decrease in the respiration rate in these insects. These results demonstrate that squamocin has potential to induce enough morphological changes in midgut through epithelial cell damage in *A. gemmatalis*.

1. Introduction

The velvet bean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Noctuidae) is the main defoliating pest of soybean (*Glycine max* L. Merrill, Fabaceae) in Brazil (Sosa-Gomes, 2004; Panizzi, 2013). This species occurs from central region of the United States to Argentina and some Indian islands (Riffel et al., 2012). *Anticarsia gemmatalis* damages other crops also, such as peanuts, alfalfa, bean, pea, rice and wheat (Rahman et al., 2007).

In Brazil, *A. gemmatalis* occurs throughout the year, especially in the vegetative stage of the plants and it is controlled by using synthetic insecticides (Navickiene et al., 2007; Panizzi, 2013). These insecticides may cause side effects such as pest resistance, environmental pollution, toxic waste, emergence of new pests and reduction of beneficial insects (Bourguet et al., 2000; Nicholson, 2007). Hence, alternative methods to control *A. gemmatalis* (De Nardo et al., 2001; Navickiene et al., 2007) need to be developed. The integrated pest and ecological management aim to use safer products than synthetic chemicals for the safety of human health and environment (Matsumura, 2004; Pavela, 2007). The search of natural substances for plant protection, especially in organic agriculture, has increased the interest in botanical insecticides (Martínez et al., 2015; Zanuncio et al., 2016).

Annonaceous acetogenins comprised from a series of natural products extracted from Annonaceae species, among which squamocin proved to be the promising agent. Squamocin also called anonin I, is an acetogenin with 37 carbon atoms, α,β-unsaturated γ-lactone ring and adjacent bis-tetrahydrofuran (bis-THF) ring (Rupprecht et al., 1990). Use of single molecules are important for synthesis of new inseticdes, and computational approaches in designing new insecticide can be really helpful (Speck-Planche et al., 2011), by providing better understanding of physicochemical properties (Aschi et al., 2007), insecticidal activity (Liu et al., 2009; Khajehali et al., 2010; Sparks et al., 2001; Xue et al., 2007), and toxicological profiles (Eldred and Jurs, 1999).

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Acetogenins are cytotoxic (Miao et al., 2016), larvicidal (Costa et al., 2014), antitumor (Chen et al., 2013), neurotoxic (Derbré et al., 2008) and inhibitors of mitochondrial complex I (Duval et al., 2006). These compounds are gut poisons and effective against insect pests such as *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae), *Myzus persicae* Sulzer (Hemiptera: Aphididae) (Ribeiro et al., 2014) and *Aedes aegypti* L. (Diptera: Culicidae) (Costa et al., 2014, 2017). Squamocin is mostly observed as an acting agent on midgut cells of different insects and is previously heavily tested against *A. aegypti* (Costa et al., 2014, 2017).

The digestive tract of insects is divided into three main regions: foregut and hindgut, which are derived from the ectoderm, and the midgut, the main organ where digestion and absorption take places, which has an endodermic origin (Chapman, 2013). Plant feeders usually contain short and enlarged midgut, allowing for a high throughput rate of food which is often abundant in quantity. Main functions of the midgut are digestion and absorption of food, regeneration of cells in case of any damage (Lehane and Billingsley, 2012) and creation of local immune defense by developing physical barrier which prevents dissemination of ingested pathogens in the gut (Silva et al., 2016) or physiological response for detoxification of xenobiotic compounds (Dow, 1987).

There are a variety of plant products that have toxicological properties used to control *A. gemmatalis* (Messiano et al., 2008; Mourão et al., 2014; Ribeiro et al., 2015); however, squamocin from *Annona squamosa* has been reported to be toxic gainst *A. aegypti* larva, but safer to its predators *Culex bigoti* Bellardi (Diptera: Culicidae) and *Toxorhynchites theobaldi* (Dyar & Knab) (Diptera: Culicidae) and to human cells (Costa et al., 2017) and might be an alternative to control *A. gemmatalis* caterpillars. We evaluated the toxic effect, histological and ultrastructural changes in the midgut cells of *A. gemmatalis* exposed to squamocin, in order to contribute for the development of new strategies for controlling this insect pest. Overall histological and ultrastructural changes of squamocin on tissue, cell and organelles under light, transmission and confocal microscopes are reported.

2. Materials and methods

2.1. Insects

Larvae of *A. gemmatalis* were obtained from mass rearing in the "Laboratório de Controle Biológico" of the "Instituto de Biologia Aplicada a Agricultura e Pecuária" (BIOAGRO, Universidade Federal de Viçosa) in Viçosa, Minas Gerais, Brazil. They were maintained at 26 ± 1 °C in 75 ± 5% relative humidity with a 12-h photophase. Larvae were placed in polystyrene boxes (15 × 9 cm) and fed on an artificial diet containing 10 g agar, 15.6 g brewer's yeast, 25 g wheat germ, 25 g soy protein, 31.2 g minced beans, 12.5 g casein, and a 2.5 mL vitamin solution (1.2% ascorbic acid, 0.03% calcium pantothenate, 0.015% niacin, 0.008% riboflavin, 0.004% thiamin and 0.004% HCl) (Greene et al., 1976).

2.2. Squamocin

The squamocin was obtained from the "Laboratório Químico de Produtos Naturais" of the Universidade Federal de Alagoas (Maceió, Alagoas, Brazil). Squamocin (CAS number: 120298-30-8) is a white solid wax obtained from a methanolic extraction of *Annona mucosa* Jacquin (Anonaceae) seeds following by a successive partition with chloroform (85.4 g; 57.3%) using a High Performance Liquid Chromatography (HPLC) (Costa et al., 2014). Squamocin with 97% of purity was pre-solubilized in 1% dimethylsulfoxide, dissolved in distilled water and solubilized in 2% Tween 20 to produce a stock solution of 998.85 mg/L.

2.3. Dose-response bioassay

Squamocin was diluted in 1 mL water to produce a stock solution by adjusting 1 g/L to obtain the required concentrations. Squamocin efficacy was determined by calculating the lethal concentrations (LC_{50} and LC_{90}) values under laboratory conditions. Six concentrations of squamocin besides the control (distilled water) were adjusted in 1 mL stock solution (treatments and distilled water) 31.16, 62.42, 124.85, 249.71, 499.42, 998.85 mg/L. For each treatment, aliquots were taken from the stock solution and mixed with distilled water in 2 mL glass vials. Different concentrations of the treatments were applied in 1 µL in the food of each individual of *A. gemmatalis*. Fourth instar larvae were used per concentration and placed individually in glass vials (2 × 15 cm) covered with a cotton lid and maintained in the dark. The number of dead insects in each vial was counted after squamocin exposure over 72 h. Each treatment was replicated three times in this bioassay.

2.4. Light microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 24, 48 and 72 h. Larvae from both treatment and control were collected, were dissected in saline solution for insects (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4) and the midgut regions divided in anterior, middle and posterior. The samples were transferred to Zamboni's fixative solution (Stefanini et al., 1967) for 12 h at 5 °C. Then, the samples were dehydrated in a graded ethanol series (70%, 80%, 90% and 95%), embedded in historesin Leica (Leica Biosystems Nussloch GmbH, Heildelberger, Germany) and sectioned at 3 µm thickness in Leica RM2255 microtome. Sections were stained with hematoxylin and eosin and analyzed using a Leica DMLS light microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.5. Transmission electron microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 24, 48 and 72 h. The midgut of *A. gemmatalis* larvae from both treatment and control were dissected and transferred to 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.2 containing 0.2 M sucrose for 4 h at room temperature. Then, the midgut was divided into anterior, middle and posterior region and fragments post-fixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, followed by washing in the buffer and dehydration in a graded ethanol series (70%, 80%, 90% and 99%). The samples were embedded in LR White Resin (Electron Microscopy Sciences, Fort Washington, PA, USA) and ultrathin sections (70–90 nm) were obtained using a glass knife in a Sorvall MT2-BMT2-B ultramicrotome (Sorvall Instruments, Wilmington, DE, USA). Sections were stained with 1% aqueous uranyl acetate and lead citrate (Reynolds, 1963) and examined with a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Jena, Germany).

2.6. Fluorescence microscopy

Larvae of *A. gemmatalis* were exposed to lethal concentration LC_{50} and LC_{90} of squamocin extract for 72 h. The midgut of *A. gemmatalis* larvae from both treatment and control were dissected and transferred to sodium phosphate buffer (PBS). Diluted 1 mM MitoTracker® stock solution (Invitrogen, Waltham, MA, USA) to the 100 nM concentration in PBS buffer and the samples were then transferred to this solution for 2 h at 37 °C. After washing with buffer several times, midguts were transferred to PBS at 37 °C for 20 min. Samples were then transferred to iodide TO-PRO® – 3 (Invitrogen, Waltham, MA, USA) staining solution by diluting 1:1000 (1 µM) in PBS at 37 °C for 40 min. Carefully removed the medium/buffer covering the midguts, and replaced it with freshly prepared, pre-warmed buffer containing 2–4% formaldehyde. The samples were washed in PBS and mounted in slides with 50% sucrose