Environmental Pollution 243 (2018) 809–814

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Azadirachtin impairs egg production in *Atta sexdens* leaf-cutting ant queens☆

Karina Dias Amaral [a, *], Luis Carlos Martínez [b], Maria Augusta Pereira Lima [c], José Eduardo Serrão [b], Terezinha Maria Castro Della Lucia [c]

[a] Department of Entomology, Federal University of Viçosa, Viçosa, MG 36570-000, Brazil
[b] Department of General Biology, Federal University of Viçosa, Viçosa, MG 36570-000, Brazil
[c] Department of Animal Biology, Federal University of Viçosa, Viçosa, MG 36570-000, Brazil

ARTICLE INFO

Article history:
Received 26 June 2018
Received in revised form
12 September 2018
Accepted 12 September 2018
Available online 14 September 2018

Keywords:
Ant control
Formicidae
Neem oil
Vitellogenin
Yolk

ABSTRACT

Leaf-cutting ants are important pests of forests and agricultural crops in the Neotropical region. *Atta sexdens* colonies can be composed of thousands of individuals, which form a highly complex society with a single reproductive queen. Successful control of this species is achieved only if the queen is affected. Few data are available on the lethal or sublethal effects of toxic compounds on leaf-cutting ant queens. Azadirachtin has been claimed as an effective biopesticide for insect control, but its action on leaf-cutting ants has been little explored. This study shows that azadirachtin affects oviposition in *A. sexdens* queens, impairing egg development by decreasing protein reserves. Azadirachtin inhibits the synthesis of vitellogenin, the major yolk protein precursor. The negative effects of azadirachtin on the reproduction of leaf-cutting ant queens suggest a potential use for the control of these insects.

1. Introduction

Leaf-cutting ants (Formicidae: Attini) are defoliating insects that cause damage to agricultural crops and forest areas in the Neotropical region. The ants cut fresh parts of plants to serve as substrate for the symbiotic fungi that they cultivate (Della Lucia and De Souza, 2011; Della Lucia et al., 2014; Britto et al., 2016).

In Brazil, *Atta sexdens* Linnaeus are found in the Southeast and Central regions (Delabie et al., 2011). Their colonies can contain millions of workers divided into various castes but have only one reproductive queen (monogyny) (De Souza et al., 2011).

The most common control method against leaf-cutting ants is the use of chemicals that kill foraging workers; thus, damage to crops is immediately interrupted (Forti et al., 2007). However, it is desirable that the active ingredient also causes the queen's death (Hölldobler and Wilson, 1990; Forti et al., 2000).

The complex structure of ant nests ensures the queen's performance, protecting her from natural enemies and other factors that might threaten her survival (Forti et al., 2011). Queens are usually located in secluded nest chambers, where a large number of workers feed and protect their queen (Hölldobler and Wilson, 2009). Even so, queens can be exposed to sublethal concentrations of toxic compounds applied for chemical control.

Although sublethal concentrations of insecticides do not cause high population mortality, they can significantly affect lifespan, fertility, communication, feeding, and oviposition (Lee, 2000). In eusocial insects, such as leaf-cutting ants, sublethal effects are even more important because, in these societies, individuals are closely related and the success of the colony depends on cooperation and labor division among members (Hölldobler and Wilson, 2009).

In a scenario where the damage of insecticides to the environment and to non-target organisms still needs mitigation (Desneux et al., 2007), the search for new molecules to control insect pests is important. Selective and biodegradable compounds, including "green pesticides," might be an alternative to reduce the use of synthetic insecticides on crops (Isman, 2006; Martínez et al., 2015). The action of plant secondary metabolites on the behavior and survival of leaf-cutting ants has been investigated (Isman, 2006; Britto et al., 2016).

Azadirachtin is a natural triterpenoid insecticide extracted from

☆ This paper has been recommended for acceptance by Charles Wong.

* Corresponding author.
E-mail addresses: karina_damaral@yahoo.com.br (K.D. Amaral), lc.martinez@outlook.com (L.C. Martínez), gutaufv@gmail.com (M.A. Pereira Lima), jeserrao@ufv.br (J.E. Serrão), tdlucia@ufv.br (T.M.C. Della Lucia).

https://doi.org/10.1016/j.envpol.2018.09.066
0269-7491/

neem, *Azadirachta indica* Juss. (Sapindales: Meliaceae), that promotes feeding deterrence, growth regulation, and reproduction inhibition in many insects (Mordue et al., 1998; Mulla and Su, 1999). This compound also affects oviposition in some insects (Sayah et al., 1996; Su and Mulla, 1999a,b; Medina et al., 2004). In addition, azadirachtin has low toxicity to mammals and is rapidly degraded in the environment (Isman, 2006). Azadirachtin was shown to have non-toxic effects on cultured mouse cells but was toxic to *Spodoptera* cells (Reed and Majumdar, 1998).

The female reproductive tract of leaf-cutting ants consists of two ovaries with a pair of lateral oviducts that open into a common oviduct connected to the genital chamber (Antunes et al., 2002; Ortiz and Camargo-Mathias, 2006; Cardoso et al., 2008). Ovaries are composed of functional units, the ovarioles, which are responsible for the production of oocytes. The vitellarium is the largest portion of the ovariole, where oocyte maturation and yolk uptake occur (Bussador do Amaral and Machado-Santelli, 2009; Chapman, 2013). Insect yolk is composed of proteins and lipids. Yolk proteins are derived from vitellogenin, which is produced in the fat body, released into the hemolymph, and then transported to oocytes (Tufail and Takeda, 2008, Azevedo et al., 2011, 2016). Thus, the fat body and reproductive tract are important targets for compounds expected to affect reproduction.

This study evaluated the effects of azadirachtin on oviposition, ovary histology, and vitellogenin content in the fat body and hemolymph of *A. sexdens* queens.

2. Material and methods

2.1. Insect collection and maintenance

A. sexdens queens were obtained from 6-month-old colonies with a 200 mL fungus garden in Viçosa, Minas Gerais, Brazil. The colony's early months are critical, as the queen has to work continuously with the help of few workers, requiring great energy expenditure. Moreover, in early colonies, queens lay many trophic eggs, which serve as food for herself and the offspring. On average, ants begin to forage and cultivate fungi after 87 days (Hölldobler and Wilson, 2009). These stress factors must be considered when carrying out experiments with *A. sexdens* queens to avoid that they influence the results. It is also very important to standardize the queens' age, a factor that affects oviposition rate. For this reason, we collected queens from 6-month-old colonies, as colonies were already well established and the queens were in full reproductive condition.

Colonies were maintained at 25 ± 5 °C and 75 ± 5% relative humidity under a photoperiod of 12 h in the Insectary of the Department of Entomology at the Federal University of Viçosa, Brazil, according to the protocol established by Della Lucia and Moreira (1993). Colonies received fresh leaves of *Acalypha wilkesiana* Müll. (Euphorbiaceae) daily and water *ad libitum*.

2.2. Insecticide

The insecticide Azamax (12 g L^{-1} azadirachtin), manufactured by E.I.D. Parry Ltd. (India), was purchased from DVA Especialidades (Brazil). The formulation is an emulsifiable concentrate. Azadirachtin belongs to the toxicological class III (moderately toxic) and the environmental hazard potential class IV (not harmful to the environment). Azamax is registered in Brazil by the Ministry of Agriculture, Livestock, and Supply (no. 14807) and is certified internationally and in Brazil for use in organic crops.

2.3. Effects of azadirachtin on oviposition

Fifteen queens were collected and had their oviposition rate assessed. Five queens received 4 μL of a 1.2 mg mL^{-1} azadirachtin on the thorax surface and were fed with a 1:1 (v/v) water and honey solution. Another set of five queens were fed a water and honey diet with 1.2 mg mL^{-1} azadirachtin. The five remaining queens (control) were fed the liquid diet but were not exposed to azadirachtin either by topical application or by ingestion (Araújo and Della Lucia, 1993). Diets were the same throughout the test period. The sublethal azadirachtin concentration of 1.2 mg mL^{-1} (LC_{30}, lethal concentration that kills 30% of the individuals) was determined from previous mortality tests with workers topically exposed to the insecticide. The same concentration was added to the diet because it was accepted by the ants in social immunity tests. Both treatment groups received the nominal exposure concentration of 1.2 mg mL^{-1} azadirachtin; no measurements were performed to determine the actual concentration in each individual after exposure. Queens were individualized in plastic vials and maintained at 25 ± 5 °C and 75 ± 5% relative humidity in the dark. The number of eggs was counted every 12 h during 96 h. At each count, eggs were removed from the vials, according to Della Lucia et al. (1990).

2.4. Histopathological analysis

Fifteen *A. sexdens* queens were selected for analysis; five controls, five queens topically exposed to azadirachtin, and five queens fed a diet containing azadirachtin, as previously described in section 2.3. Queens were dissected in insect saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, and 0.1 M Na_2HPO_4) and their ovaries were fixed with Zamboni's fixative (Stefanini et al., 1967) for 12 h at 5 °C. Then, samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%), embedded in Historesin (Leica Microsystems GmbH, Wetzlar, Germany), and sectioned at 4 μm thickness using a Leica RM2255 microtome (Leica Microsystems GmbH, Wetzlar, Germany). Sections were stained with hematoxylin and eosin and analyzed using an Olympus BX-60 light microscope (Olympus Corporation, Tokyo, Japan).

2.5. Total protein and vitellogenin quantification

Sixteen queens, eight control and eight topically treated with azadirachtin, were used for this analysis. Eight microliters of hemolymph was extracted from each queen. Subsequently, queens were dissected, and the fat body was removed. The hemolymph was diluted in 50 μL of distilled water; and the fat body, in 100 μL of distilled water. Samples were centrifuged at 10000 × *g* for 15 min at 4 °C. The supernatant was collected, and total protein quantification was performed at 280 nm using a NanoDrop spectrophotometer.

2.6. ELISA

The vitellogenin content of the hemolymph and fat body was determined using an enzyme-linked immunosorbent assay (ELISA). Briefly, in a 96-well plate, 5 μg of total protein from the hemolymph or 20 μg of total protein from the fat body was diluted in 0.1 mol L^{-1} phosphate buffered saline, pH 8.0, plus 0.05% (v/v) Tween (PBST) in a final volume of 100 μL, blocked with 3% (w/v) non-fat dry milk in distilled water, and incubated at 4 °C for 16 h. Mouse anti-vitellogenin antibody diluted 1:500 in PBST (Azevedo et al., 2011) was added to each well, and the plate was incubated for 2 h. After washing, horseradish peroxidase-conjugated rabbit anti-mouse IgG (Sigma–Aldrich) diluted 1:9000 in PBST was added, and the plate