PLANT PROTECTION - Article

Annona crassiflora Mart. (Annonaceae): effect of crude extract of seeds on larvae of soybean looper *Chrysodeixis includens* (Lepidoptera: Noctuidae)

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ABSTRACT: The effect of a crude extract of *Annona crassiflora* was evaluated on larvae of the soybean looper, *Chrysodeixis includens* (Lepidoptera: Noctuidae) in different stages of larval development. The extract was prepared with seeds of *A. crassiflora* fruits collected in the state of Mato Grosso, Brazil. The extract was diluted in water and solubilizer agent at the concentrations of 0.5, 1.0, 2.0, 4.0, and 8.0%; it was tested by ingestion of treated leaves and contact. The mortality rate of topically treated caterpillars of first, third, and fifth instars was significantly higher than that of the control group. For first instar caterpillars, mortality was observed within the first 24 h after application, while, for the remaining instars, it was significant after 72 h.

For caterpillars fed treated leaves, no differences in mortality rates were observed within the first 120 h. These caterpillars were monitored until the end of the larval stage, during which mortality rates increased for first and third instar caterpillars. The mortality rate of fifth instar larvae was not significantly different between treatments. The treatment by ingestion was not efficient over a short period of time, but reduced the number of caterpillars that completed their development, decreasing the number of insects in the following generation. We concluded that the crude extract of *A. crassiflora* affected the development of *C. includens* and is a promising compound for the control of this pest.

Key words: araticum, bioinsecticide, marôlo, phytoinsecticide.

INTRODUCTION

The soybean looper, *Chrysodeixis* (=*Pseudoplusia*) *includens* (Walker) (Lepidoptera: Noctuidae), has been reported infesting approximately 70 species of plants, among them soybean, cotton, beans, sunflower, tobacco, passionflower, tomato, and several vegetables (Moscardi et al. 2012). In the main soybean producing states of Brazil, soybean looper was considered a secondary pest, naturally controlled by parasitoids and entomopathogenic fungi (Sosa-Gómez et al. 2003). However, since 2003, *C. includens* has become the main pest in several states (Bueno et al. 2009; Moscardi et al. 2012). This increase in the population levels of *C. includens* may have been caused by the increased use of fungicides required for the control of the Asian soybean rust. This consequently decreased the populations of entomopathogenic fungi, which naturally controlled soybean looper, such as *Nomuraea rileyi* (Farlow), and resulted in an increase in populations of this pest in many soybean producing states (Sosa-Gómez et al. 2003, Sosa-Gómez 2006).

In most cases, the control of this caterpillar consists in the application of chemical insecticides. This method, however, is often inefficient, as *C. includens* caterpillars stay on the abaxial surface of leaves and do not get in direct contact with the insecticide (Oliveira et al. 2010). Consequently, pesticide applications is often drastically increased, reducing the populations of the natural enemies and promoting the selection of resistant populations (Bueno et al. 2007). Therefore, pest control plans should use techniques and strategies based on the integrated pest management (IPM) that are less harmful to natural enemies and man.

Among the different IPM approaches, the use of plants with toxic properties to phytophagous insects has several advantages compared to synthetic compounds, such as rapid degradation in the environment, low toxicity to mammals, lower cost, and some are selective to natural enemies (Moreira et al. 2006). Promising results with plant extracts for the control of pest insects have drawn attention to several plant families, such as Meliaceae, Rutaceae, Asteraceae, Annonaceae, Piperaceae, Canellaceae, among others (Jacobson 1989; Isman 2006).

Among plant families with potential insecticidal properties, species of Annonaceae are of special interest due to the presence of compounds known as "annonaceous acetogenins", which have proven insecticidal activities and can be an alternative for the control of defoliating lepidopterans (Alali et al. 1999; Isman and Seffrin 2014).

The insecticidal effect of annonaceous compounds has been reported for several lepidopteran pests such as *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae: Plusiinae) (Leatemia and Isman 2004; Seffrin et al. 2010; Ribeiro et al. 2014), *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) (Saito et al. 2004; Álvarez-Colom et al. 2007; Blessing et al. 2010; Ribeiro et al. 2016), *Tuta absoluta* Meyrick (Lepidoptera: Gelichiidae) (Silva et al. 2007), *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) (Fontana et al. 1998; Saito et al. 2004), *Pseudaletia sequax* Franclemont (Lepidoptera: Noctuidae) (Fontana et al. 1998); *Plutella xyllostela* Linnaeus (Lepidoptera: Plutellidae) (Leatemia and Isman 2004; Dadang and Prijono 2009; Trindade et al. 2011) and *C. includens* (Massarolli et al. 2016).

However, few studies have focused on potential for insect control of the *Annona crassiflora* Mart. commonly known as marôlo or araticum (Lorenzi 2002). The insecticidal properties of this species have been examined for only 1 species of mosquito, *Aedes aegypti* (Linnaeus 1762) (Diptera: Culicidae) (Rodrigues et al. 2006; Omena et al. 2007; Costa et al. 2013), 3 species of stink bugs, *Dipetalogaster maxima* (Uhler) (Hemiptera, Reduviidae) (Coelho et al. 2009), *Euschistus heros* (Fabr. 1798) (Heteroptera: Pentatomidae) (Oliveira and Pereira 2009; Silva et al. 2013), *Tibraca limbativentris* Stal (Hemiptera: Pentatomidae) (Krinski and Massaroli 2014), and 1 lepidopteran species, *S. frugiperda* (Prates et al. 2000).

Given the few studies on *A. crassiflora* and its potential presented over other species of insects, this study evaluated the effects of the crude extract of *A. crassiflora* seeds on larvae of *C. includens* on 3 instars under laboratory conditions.

MATERIAL AND METHODS

Extract preparation

The plant material was collected in areas of Cerrado in the municipality of Tangará da Serra, Mato Grosso state, and identified. Samples were deposited in the TANG Herbarium of the Mato Grosso State University, Tangará da Serra campus.

Seeds were dried in an air circulating oven at 40 °C for 72 h and then ground in a knife mill. The powder obtained was taken to laboratory and lipids were removed during 3 days with organic solvent chloroform-methane (2:1) with a Soxhlet extraction apparatus heated at 60 °C until exhaustion. A rotatory evaporator was used to remove solvents and obtain the crude extract at 40 °C. From this extract, dilutions were prepared for the concentrations used in experiments.

Chrysodeixis includens colony

Eggs and caterpillars of *C. includens* used in the experiments were obtained from the colony maintained at the laboratory, in a climatized room at 25 ± 3 °C and 70 ± 10% relative humidity. Adults were maintained in PVC cages of 100 mm × 200 mm internally lined with sulphite paper. The top end of the cages was covered with voil fabric and a rubber band and the bottom end was closed with a Petri dish lined with filter paper and a container with food (10% honey solution) and water.

Eggs were used in bioassays and to maintain the colony. For the maintenance of the colony, a substrate with eggs (sulphite paper or voil fabric covering the cage) was cut in stripes and placed on lids of 145-mL plastic containers containing an artificial diet, as described by Greene et al. (1976). Newly-emerged caterpillars remained in this container until the third instar, when they were transferred to 50-mL cups covered with an acetate lid and containing the same diet. Three larvae were placed per cup where they remained until they reached the pupal stage. Pupae were transferred to PVC cages where they remained until the end of the development cycle.

Bioassays

Randomized experiments were carried in climatized chambers (BOD) at 25 ± 1 °C and 70 ± 10% RH and 12-h photoperiod. The extract of *A. crassiflora* was tested at the concentrations of 0.5; 1.0; 2.0; 4.0 and 8.0%. These concentrations were prepared by diluting the extract in 10% polysorbate 80 (Tween 80°) and water, as well as mixing for 5 min with a magnetic shaker. Thus, 2 control groups were used: one with water and another with water and solubilizer agent. Each treatment consisted of 15 replicates with 1 caterpillar each.

Topical application bioassay

Eggs with 24 h were taken from the colony and placed in the controlled conditions previously mentioned. First instar caterpillars were treated after hatching, while in assays with other instars, caterpillars were maintained in 145-mL plastic containers lined with filter paper and fed soybean leaves (var. Tucunaré conventional) obtained from plants grown in a greenhouse until they reached third or fifth instars. After being collected, leaves were washed in tap water and placed in containers with caterpillars, with the petiole wrapped in wet cotton to maintain the turgidity of the leaf.

To evaluate the effect of the extract by topical application, a droplet (1 μL for first instar caterpillars and 2 μL for third and fifth instar caterpillars) was applied to the back of each caterpillar with the aid of a micropipette and for first instar caterpillars under a stereomicroscope. After the application, caterpillars were maintained under controlled conditions. Mortality was assessed daily for 5 days, and the results were compared for 24, 72, and 120 h after application.

Ingestion bioassay

Ingestion tests were carried out by immersion of adaxial surface soybean leaves within a Petri dish containing extract solution, and allowed to air dry with the adaxial side up for approximately 30 min. After this period the leaves were offered to caterpillars for 24 h, when the first evaluation was carried out and replaced by a new untreated leaf.

After the application, larvae were maintained under the temperature and humidity conditions previously mentioned and mortality was evaluated daily for 5 days. Mortality rates were presented for 24; 72 and 120 h after application. Since mortality was not significant after 5 days after treatment, the evaluation on the effect of the extract regarding mortality and deformities was extended until the end of the larval stage.

Statistical analysis

Normality was assessed with the Shapiro-Wilk test. Since the results did not conform to normality requirements, the Kruskal-Wallis test was used with significance set at 5%. Analyses were carried out with the software Statistica 7.0.

RESULTS

Effects of topical application

The crude extract of *A. crassiflora* showed insecticidal properties against *C. includens* caterpillars of all instars evaluated. For first instar caterpillars, mortality rate reached 80.0% at the highest concentration in the first 24 h after application and 86.6% in the following assessments (Table 1). For third and fifth instar caterpillars, mortality rate was not significantly different in the first assessment; however, 72 h after application, mortality rates reached 86.6 and 66.6%, respectively, at the highest concentrations. At 120 h after application, mortality of the third instar caterpillars remained 86.6% for the highest concentration and for the fifth instar caterpillars, the highest mortality recorded was 93.3% for the concentration of 8.0%. For the fifth instar caterpillars, mortality rate increased after 72 h, when caterpillars reached the pre-pupal stage, but not the pupal one (Table 1).

Effect of ingestion

The ingestion of leaves treated with *A. crassiflora* extract by *C. includens* caterpillars of the 3 instars evaluated after 120 h did not have toxic effects (Table 2). Based on these results, the development and mortality

Table 1. Mortality (% ±SD) of *Chrysodeixis includens* after topical application of the extract of *Annona crassiflora* at different concentrations and instars.

Treatments	1st instar		
	24 h	72 h	120 h
Water	0.0 ± 0.0 b[1]	0.0 ± 0.0 b[1]	6.6 ± 2.5 b[1]
Soluble agent	20.0 ± 4.1 b	20.0 ± 4.1 b	20.0 ± 4.1 b
0.5%	20.0 ± 4.1 b	26.6 ± 4.5 ab	26.6 ± 4.5 ab
1.0%	6.6 ± 2.5 b	13.3 ± 3.5 b	20.0 ± 4.1 b
2.0%	6.6 ± 2.5 b	13.3 ± 3.5 b	20.0 ± 4.1 b
4.0%	6.6 ± 2.6 b	6.6 ± 2.5 b	26.6 ± 4.5 ab
8.0%	80.0 ± 4.1 a	86.6 ± 3.5 a	86.6 ± 3.5 a
H	42.09	41.39	29.10
	3rd instar		
Water	0.0 ± 0.0 a	0.0 ± 0.0 b[1]	13.3 ± 3.5 b[1]
Soluble agent	0.0 ± 0.0 a	6.6 ± 2.5 b	6.6 ± 2.5 b
0.5%	0.0 ± 0.0 a	6.6 ± 2.5 b	33.3 ± 4.8 ab
1.0%	0.0 ± 0.0 a	20.0 ± 4.1 b	46.6 ± 5.1 ab
2.0%	0.0 ± 0.0 a	26.6 ± 4.5 ab	33.3 ± 4.8 ab
4.0%	26.6 ± 4.5 a	60.0 ± 5.0 ab	80.0 ± 4.1 a
8.0%	53.3 ± 5.1 a	86.6 ± 3.5 a	86.6 ± 3.5 a
H	38.76	44.33	34.43
	5th instar		
Water	0.0 ± 0.0 a	13.3 ± 3.5 ab[1]	13.3 ± 3.5 bc[1]
Soluble agent	0.0 ± 0.0 a	0.0 ± 0.0 b	6.6 ± 2.5 c
0.5%	6.6 ± 2.5 a	40.0 ± 5.0 ab	66.6 ± 4.8 ab
1.0%	6.6 ± 2.5 a	40.0 ± 5.0 ab	46.6 ± 5.1 ab
2.0%	6.6 ± 2.5 a	46.6 ± 5.1 ab	73.3 ± 4.5 ab
4.0%	6.6 ± 2.5 a	46.6 ± 5.1 ab	53.3 ± 5.1 ab
8.0%	26.6 ± 4.5 a	66.6 ± 4.8 a	93.3 ± 2.5 a
H	10.50	19.36	35.32

[1]Means followed by the same letter in columns are not significantly different (p > 0.05) according to the Kruskal-Wallis test at 5%.

Table 2. Mortality (% ±SD) of *Chrysodeixis includens* caterpillars 120 h after ingestion of leaves treated with Annona crassiflora and mortality (% ±SD) during the entire larval stage.

Treatments	1st instar		3rd instar		5th instar	
	% Mortality 120 HAA[ns]	% Larval mortality	% Mortality 120 HAA[ns]	% Larval mortality	% Mortality 120 HAA[ns]	% Larval mortality[ns]
Water	6.6 ± 2.6	13.0 ± 3.5 b[1]	6.6 ± 2.6	13.0 ± 3.5 c[1]	0.0 ± 0.0	0.0 ± 0.0
Soluble agent	0.0 ± 0.0	13.0 ± 3.5 b	6.6 ± 2.6	20.0 ± 4.1 bc	6.6 ± 2.6	6.6 ± 2.6
0.5%	6.6 ± 2.6	66.0 ± 4.8 ab	20.0 ± 4.1	66.0 ± 4.8 abc	0.0 ± 0.0	26.0 ± 4.6
1.0%	33.3 ± 4.9	66.0 ± 4.8 ab	13.3 ± 3.5	80.0 ± 4.1 ab	13.3 ± 3.5	26.0 ± 4.6
2.0%	20.0 ± 4.1	73.0 ± 4.5 ab	26.6 ± 4.6	86.0 ± 3.5 a	33.3 ± 4.9	33.0 ± 4.9
4.0%	46.6 ± 5.1	93.0 ± 2.5 a	6.6 ± 2.6	46.0 ± 5.1 abc	20.0 ± 4.1	60.0 ± 5.0
8.0%	60.0 ± 5.0	73.0 ± 4.5 ab	33.3 ± 4.9	86.0 ± 3.5 a	26.6 ± 4.6	33.0 ± 4.9
H	24.6	35.51	7.64	34.97	12.32	17.55

[ns]Non-significant according to the analysis of variance at 5%; [1]Means followed by the same letter in columns are not significantly different (p > 0.05) according to the Kruskal-Wallis test at 5%. HAA = hours after application.

of caterpillars were evaluated until the end of the larval development.

In the treatments with first and third instar caterpillars until the end of the larval stage, mortality increased, reducing the number of caterpillars that completed development. For first instar caterpillars treated with *A. crassiflora* extract at the concentration of 4.0%, mortality rate was 93.3% at the end of the larval phase. For third instar caterpillars, mortality rate reached 86.0% for the highest concentration (Table 2). For fifth instar caterpillars, mortality was not significantly different between treatments even at the end of the development cycle (Table 2).

DISCUSSION

The extract of *A. crassiflora* showed satisfactory results at the concentration of 8.0% for all instars and at 4.0% for third instar caterpillars treated topically. However, when ingested, mortality was negligible throughout the cycle, with no short-term response, but the development of a new generation was halted, corroborating the potential for insect control recorded for other species of Annonaceae (Alali et al. 1999; Ribeiro et al. 2013; Ribeiro et al. 2016; Massarolli et al. 2016).

The insecticidal effect of *A. crassiflora* was also observed against other species. For *A. aegypti* larvae treated with methanol, hexane, dichloromethane extracts and the hexane fraction at the concentrations of 1.0 mg·mL^{-1}, mortality reached 90.0% after 24 h (Costa et al. 2013).

Prates et al. (2000) obtained similar results to those found in our study. These authors offered an artificial diet with ethanol extract of *A. crassiflora* seeds at the concentration of 10 mg·mL^{-1} to *S. frugiperda* and observed a mortality of 56.7%. Caterpillars that did not die continued to be monitored and the development of those that ingested the extract was negatively affected. Although the characteristics evaluated in the present study were not the same as those by Prates et al. (2000), *A. crassiflora* interfered in the larval development of *C. includens*. In *E. heros*, *A. crassiflora* had an antifeedant effect in soybean pods treated with methanol extract at the concentration of 4.0% (Oliveira and Pereira 2009).

In other species of Annonaceae, effects have also been described after the ingestion of the extract after 7 days of application, as reported by Leatemia and Isman (2004). These authors evaluated the effect of the crude extract of *A. squamosa* seeds on *P. xylostella* (Lepidoptera: Plutellidae) and observed a high mortality of caterpillars after 12 days of feeding on leaf disks treated with the extract.

The composition of species of Annonaceae include acetogenins, which are potential stomach poisons, and can be efficient in the control of chewing insects such as lepidopterans (Isman 2006). Based on this toxic properties, patents have been issued for annonaceous acetogenins for the control of chewing and sucking insects (Mikolajczak et al. 1988).

Several authors reported insecticidal properties for other species of Annonaceae against different insect species (Fontana et al. 1998; Seffrin et al. 2010; Trindade

et al. 2011; Ribeiro et al. 2013; Costa et al. 2013). These studies corroborate that isolated annonaceous acetogenins interfere in the development of lepidopterans (Alali et al. 1999; Morales et al. 2004; Castillo-Sánchez et al. 2010).

Annonaceous acetogenins are strong inhibitors of ATP production (Adenosine triphosphate) in the mitochondrial complex I (NADH ubiquinone oxidoreductase) in insects, and this reduction in ATP production may lead cells to apoptosis (programmed cell death) (Lewis et al. 1993; Ahammadsahib et al. 1993; Tormo et al. 1999). In addition, acetogenins have characteristics of compounds that interfere in the hormonal activity of lepidopterans. Blessing et al. (2010) and Prates et al. (2000) reported morphological alterations in caterpillars and pupae of *S. frugiperda*, similar to the observed in the present study in *C. includens.*

Castillo-Sánchez et al. (2010) pointed out that the secondary metabolites of species of Annonaceae are promising to control several pest insects, with variations in the concentrations used for each type of insects. In addition, these authors indicated the need to conduct semi-field and field studies to support the results obtained in the laboratory, as well as assess the effects of these metabolites against natural enemies of these pests.

Based on the properties already described from other species of Annonaceae and the positive results obtained for *A. crassiflora* in our study, this species has the potential to control all instars of *C. includens* evaluated, especially by topical application, which had a faster response compared to ingestion, which was also efficient throughout time, preventing the development of the next generation of the insect.

In view of our findings, further studies are needed to develop cultivation techniques for *A. crassiflora,* allowing for the possible use of this plant by the food industry, while residues can be used in further studies or even to produce bioproducts such as phytoinsecticides.

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