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CROP PROTECTION

Growth inhibitory action of acetogenin-rich formulated extracts against *Duponchelia fovealis*

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ABSTRACT: *Duponchelia fovealis (Zeller, 1847) is a new pest of strawberry crops worldwide. To develop alternative strategies for its management, we assessed the lethal toxicity and growth inhibitory action of formulations prepared from ethanolic seed extracts of pre-selected species of Annona (Annona mucosa Jacq., Annona muricata L., and Annona sylvatica A. St.-Hil.), which were previously characterized by their high content of annonaceous acetogenins. In addition, the extracts were compared to a limonoid-based bioinsecticide [Azamax® 1.2 EC (azadirachtin + 3-tigloil-azadiractol), positive control] on D. fovealis larvae. Aqueous emulsions prepared from ethanolic seed extracts of A. mucosa and A. sylvatica and a limonoid-based bioinsecticide had low lethal toxicity to D. fovealis larvae; nevertheless, they caused a pronounced inhibition of their larval development. Thus, the combined effects of lethal and sublethal toxicity of acetogenin-rich formulations and the limonoid-based commercial bioinsecticide may offer a route to new control strategies of D. fovealis in strawberry crops, especially in organic-based production systems.*
Key words: *Annona mucosa, Annona sylvatica, bioactivity, botanical insecticides.*

Ação inibidora do desenvolvimento de extratos formulados ricos em acetogeninas sobre *Duponchelia fovealis*

RESUMO: *Duponchelia fovealis (ZELLER, 1847) é uma nova praga do morangueiro em todo o mundo. Visando desenvolver alternativas para seu manejo, objetivou-se avaliar a toxicidade letal e a ação inibidora do desenvolvimento de formulações preparadas a partir de extratos etanólicos de sementes de espécies pré-selecionadas de Annona (Annona mucosa Jacq., Annona muricata L. e Annona sylvatica A. St.-Hil.), ricos em acetogeninas, em comparação com um bioinseticida à base de limonoides [Azamax® 1,2 EC (azadiractina + 3-tigloil-azadiractol), controle positivo] sobre lagartas de D. fovealis. Emulsões aquosas preparadas a partir de extratos etanólicos de sementes de A. mucosa e de A. sylvatica e um bioinseticida à base de limonóide apresentaram baixa toxicidade letal para larvas de D. fovealis; no entanto, eles causaram uma inibição pronunciada de seu desenvolvimento larval. Assim, os efeitos combinados de toxicidade letal e subletal de formulações ricas em acetogeninas e do bioinseticida comercial à base de limonoides podem oferecer um caminho para novas estratégias de controle de D. fovealis em cultivos de morango, especialmente em sistemas de produção de base orgânica.*
Palavras- chave: *Annona mucosa, Annona sylvatica, bioatividade, inseticidas botânicos.*

INTRODUCTION

Duponchelia fovealis Zeller (Lepidoptera: Crambidae) is originally from the Mediterranean region and the Canary Islands, and currently its occurrence was reported in Europe, Asia, Africa, North America (CABI, 2021), and South America (ZAWADNEAK et al., 2016). It is considered one of the most important pests of strawberry crops [*Fragaria* × *ananassa* Duch. (Rosaceae)] in several producing countries, mainly in Portugal (FRANCO & BATISTA, 2010), Italy (BONSIGNORE & VACANTE, 2010), Turkey (EFIL et al., 2014), and Brazil (ZAWADNEAK et al., 2016). *Duponchelia fovealis* is characterized by high polyphagia and its capacity to damage vegetative and reproductive parts of plants, as well as strawberry fruits at different ripening stages (EFIL et al., 2014; ZAWADNEAK et al., 2016).

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In Brazil, there are no synthetic insecticides registered for *D. fovealis* management (AGROFIT, 2020), which has driven the search for effective, sustainable, and compatible alternatives with organic and/or ecological production systems. The main alternatives include the use of entomopathogenic fungi (BAJA et al., 2020) and beneficial macrorganisms (PIROVANI et al., 2017; ARAUJO et al., 2020). Moreover, botanical insecticides are promising alternatives (KRINSKI et al., 2014) for the management of *D. fovealis*; however, their use is still poorly studied and explored.

Several tropical plants have been identified as potential sources of secondary compounds (allelochemicals) that could be used in the preparation of botanical insecticides, including species of the Annonaceae family (KRINSKI et al., 2014; RIBEIRO et al., 2016). Some genera of this family, such as *Annona*, show biosynthesis and accumulation of high acetogenin concentrations in their seeds (KRINSKI et al., 2014; RIBEIRO et al., 2016) and, thus, they are potential biomass sources for botanical insecticides elaboration. Anonnaceous acetogenins promote significant levels of lethal toxicity, as they act on cellular respiration (mitochondria), inhibiting the enzyme NADH – ubiquinone oxidoreductase and, consequently, reducing the ATP production. This action on respiratory process lead to the insect death by energy deprivation (TORMO et al., 1999). Furthermore, acetogenins-rich derivatives also showed significant sublethal effects to exposed individuals, such as growth inhibition and changes in both feeding habits (fagodeterrence) and host selection behavior (KRINSKI et al., 2014). These effects could support Integrated Pest Management (IPM) programs, as they compromise population growth and dynamics of target pests over generations (RIBEIRO et al., 2015; BERNARDI et al., 2017; SOUZA et al., 2019).

In this study, we evaluated lethal toxicity and growth inhibitory action of aqueous emulsions formulations prepared from ethanolic extracts, rich in acetogenins, and obtained from seeds of pre-selected species of *Annona* (*Annona mucosa* Jacq., *Annona muricata* L., and *Annona sylvatica* A. St.-Hil), which were previously characterized by their high content of annonaceous acetogenins (ANSANTE et al., 2015; SOUZA et al., 2017; RIBEIRO et al., 2020). We compared the efficacy of obtained formulations to a commercial limonoid-based bioinsecticide [Azamax® 1.2 EC (azadirachtin + 3-tigloil-azadiractol), positive control] on *D. fovealis larvae*, using ingestion bioassays (dietary exposure assessment).

MATERIALS AND METHODS

Insects

The insects used in the bioassays were obtained from a population of *D. fovealis* established from larvae collected in commercial strawberry crops in the municipality of São José dos Pinhais, Paraná State, Brazil (25°37'S; 49°04'W). In the laboratory, the insects were kept on an artificial diet following the method proposed by Zawadneak et al. (2017), under controlled conditions (temperature: 25 ± 2ºC, RH: 70 ± 10%, and photophase of 14 h).

Botanical extracts: biomass sources and procedure of preparation and formulation

Detailed information on the origin of *Annona* species used in the study (Table 1) is reported in the voucher specimens, previously identified by Prof. Dr. Renato Mello-Silva [Department of Botany, Institute of Biosciences/University of São Paulo (IB/USP)]. The specimens were deposited at the herbarium of the Biological Sciences Department of the "Luiz de Queiroz" College of Agriculture/University of São Paulo (ESALQ/USP), in Piracicaba, São Paulo State, Brazil, under the numbers 120985 (*A. mucosa*), 121205 (*A. sylvatica*), and 121892 (*A. muricata*).

For the preparation of ethanolic crude extracts, seeds were collected from ripe fruits, dried in an oven with forced air circulation at 38 °C for 48-72 h, and ground in a knife mill until obtaining a fine powder, which was stored in airtight glass containers in a freezer (-10 ºC) until use. Ethanolic extracts were obtained by cold maceration technique, using ethanol analytical grade (99.5%) as a solvent at ratio 5:1 (v v^{-1}) (CARVALHO et al., 2020; MIOTTO et al., 2020; RIBEIRO et al., 2020). For that, the powder was added to the solvent at this ratio, stirred for 10 min, and kept at rest for 3 d. Afterward, the solution was paper-filtered, and the remaining cake was again subjected to the same extraction process. This procedure was repeated three more times. The remaining solvent in the filtered samples was removed in a rotary evaporator at 50 °C and at a pressure of -600 mmHg. After complete evaporation of the solvent in an airflow chamber, the extraction yield for each species of *Annona* was determined.

For the preparation of aqueous emulsion formulations, ethanolic extracts were solubilized in acetone: methanol (1:1, v v^{-1}) at 100 g L^{-1}, with the addition of emulsifier Tween® 80 at concentration of 10 g L^{-1}.

Bioassays

All bioassays were performed under controlled conditions (temperature: 25 ± 2 ºC, RH:

Table 1 - Treatments evaluated against larvae of *Duponchelia fovealis* in ingestion bioassay (incorporation in artificial diet). AEEE= Aqueous emulsion of ethanolic seed extract (pre-commercial formulations).

Treatments	Description	Tested Concentration[a]	Origen
AEEE *Annona mucosa*	Aqueous emulsion of *Annona mucosa* ethanolic seed extract	2,000 mg kg^{-1}	Laboratory preparation (pre-commercial)
AEEE *Annona muricata*	Aqueous emulsion of *Annona muricata* ethanolic seed extract	2,000 mg kg^{-1}	Laboratory preparation (pre-commercial)
AEEE *Annona sylvatica*	Aqueous emulsion of *Annona sylvatica* ethanolic seed extract	2,000 mg kg^{-1}	Laboratory preparation (pre-commercial)
Azamax® 1.2 EC	Limonoid-based bioinsecticide [azadirachtin (6,220.15 mg L^{-1}) + 3-tigloil-azadiractol (2,596 mg L^{-1})] extracted and purified from seeds of *Azadirachta indica* L[a]	4,000 mg kg^{-1}	UPL Brazil (Campinas, São Paulo State, Brazil)

[a]Concentration: g or mL of commercial product.

70 ± 10%, and a photophase of 14 h) in a completely randomized design. The treatments tested and the discriminatory concentrations are detailed in Table 1. The limonoid-based formulation (Azamax® 1.2 EC, azadirachtin + 3-tigloil-azadiractol, 12 g of a.i. L^{-1}) was used as a positive control (Table 1). As a negative control, we used the respective solvents used for solubilization of the formulated extracts.

To assess lethal toxicity of the formulated derivatives, an initial screening was performed using the discriminatory concentration of 2,000 mg kg^{-1} on *D. fovealis* larvae (Table 1), while the limonoid-based formulation was tested at a concentration of 4,000 mg kg^{-1} (Table 1) (ANSANTE et al., 2015; BERNARDI et al., 2017). The treatments were incorporated at the end of the preparation stage of the artificial diet when the temperature was close to 50 ºC to avoid possible degradation of thermolabile compounds. After incorporation, the diet was poured with the aid of a 10 mL syringe into Elisa® plates containing 24 cells each, at 1.5 mL of artificial diet per cell. After diet solidification and cooling in a flow chamber, a 3rd instar caterpillar of *D. fovealis* from the laboratory rearing was inoculated per cell with the aid of a fine-tipped brush. Each treatment comprised five repetitions (Elisa® plates with 24 cells), totaling 120 larvae per treatment.

Larvae mortality was evaluated daily for 7 d. Larvae that did not respond to touch of a fine brush for one min of observation were considered dead. In addition, surviving larvae were weighed (mg) on an analytical balance (0.0001 g) on the 7th day.

Based on the initial screening, the most promising treatments were selected and submitted to a new bioassay to estimate the concentration needed to kill 50 and 90% (LC_{50} and LC_{90}, respectively) of *D. fovealis* larvae. For that purpose, six concentrations were tested (range: 0 - 8000 mg kg^{-1}). The bioassay method, mortality criteria, exposure time, and weighing procedure of surviving larvae were the same used in the initial screening. Again, for each treatment (concentration) five repetitions were used, and each repetition consisted of a 24-cell Elisa® plate (n = 120).

Data analysis

Generalized linear models (GLM) belonging to the exponential distribution family (NELDER & WEDDERBUM, 1972) were used for the analysis of the variables studied. When differences between treatments were significant, multiple comparisons (Tukey *post hoc* test, $P < 0.05$) were performed with the glht function using the Multicomp package, with adjustment of P values. All analyses were performed using the statistical software "R" version 2.15.1 (R DEVELOPMENT CORE TEAM, 2012). The binomial model with a

complementary log-log link function (gompit model) was used to estimate lethal concentrations (LC_{50} and LC_{90}), using the Probit package with the statistical software SAS version 9.2 (SAS Institute, 2011).

RESULTS AND DISCUSSION

In the initial screening, aqueous emulsions formulations prepared from ethanolic seed extracts of three *Annona* species, at diagnostic concentration of 2,000 mg kg^{-1}, as well as the limonoid-based bioinsecticide, at concentration of 4,000 mg kg^{-1}, provided mortality levels of *D. fovealis larvae* below 30% after 7 d of exposure on a treated artificial diet (Figure1).

The highest mortality rates were reported for formulations based on the ethanolic seed extract of *A. mucosa* (27.5%) and *A. sylvatica* (23.3%), which did not differ from each other nor from the limonoid-based bioinsecticide (20.0%) used as a positive control (Figure 1). It was not possible to estimate lethal concentrations (LC_{50} and LC_{90}) due to the low mortality of larvae in the same evaluation period (~ 48%) even at maximum concentrations tested for the most promising treatments selected in the initial screening.

Despite reduced lethal toxicity, inhibition of larval development of *D. fovealis* was significant (Figure 2). After 7 d of feeding on an artificial diet treated with formulations based on ethanolic seed extract of *A. mucosa* and *A. sylvatica*, larval weight reduced by approximately 80 and 66%, respectively, when compared to the negative control (Figure 3). In turn, the limonoid-based bioinsecticide (Azamax® 1.2 EC) reduced larvae weight by 50% compared to larvae exposed to the negative control. In addition, larvae of surviving insects showed deformation when submitted to the artificial diet containing formulated extracts of *A. mucosa* (28%) and *A. sylvatica* (23%) (Data not analyzed) (Figure 4).

Results from laboratory bioassays (dietary exposure assessment) indicated growth inhibitory action of the aqueous emulsion prepared

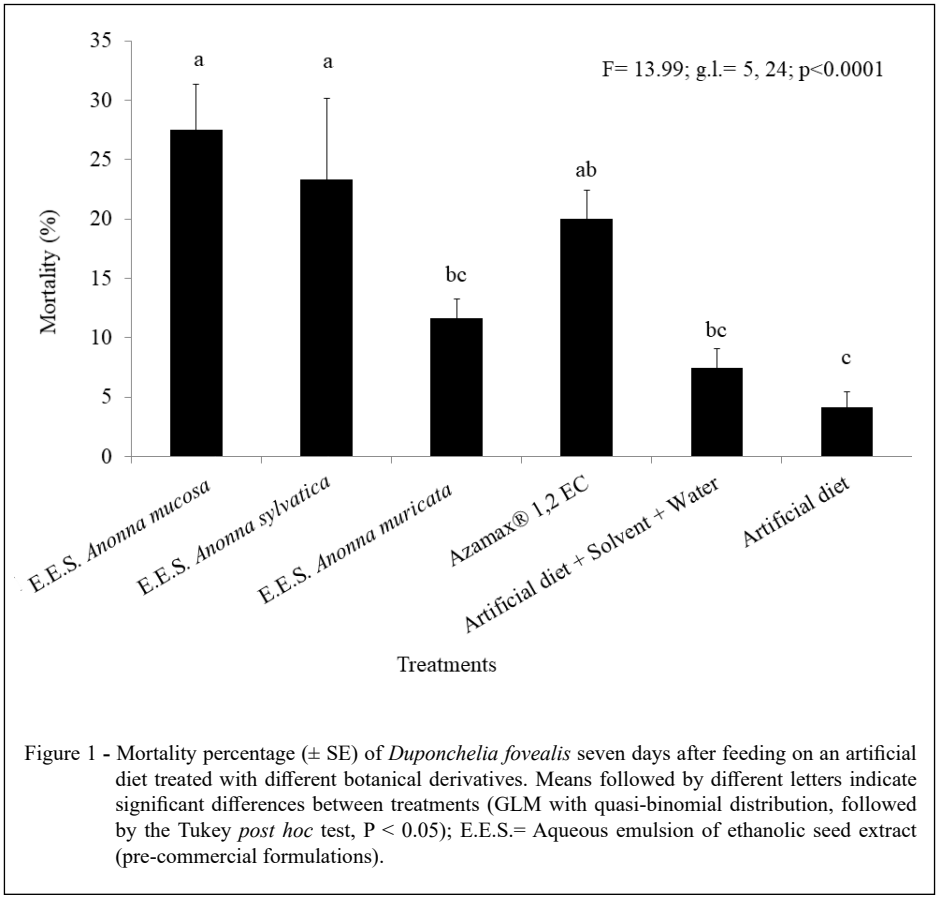

Figure 1 - Mortality percentage (± SE) of *Duponchelia fovealis* seven days after feeding on an artificial diet treated with different botanical derivatives. Means followed by different letters indicate significant differences between treatments (GLM with quasi-binomial distribution, followed by the Tukey *post hoc* test, P < 0.05); E.E.S.= Aqueous emulsion of ethanolic seed extract (pre-commercial formulations).

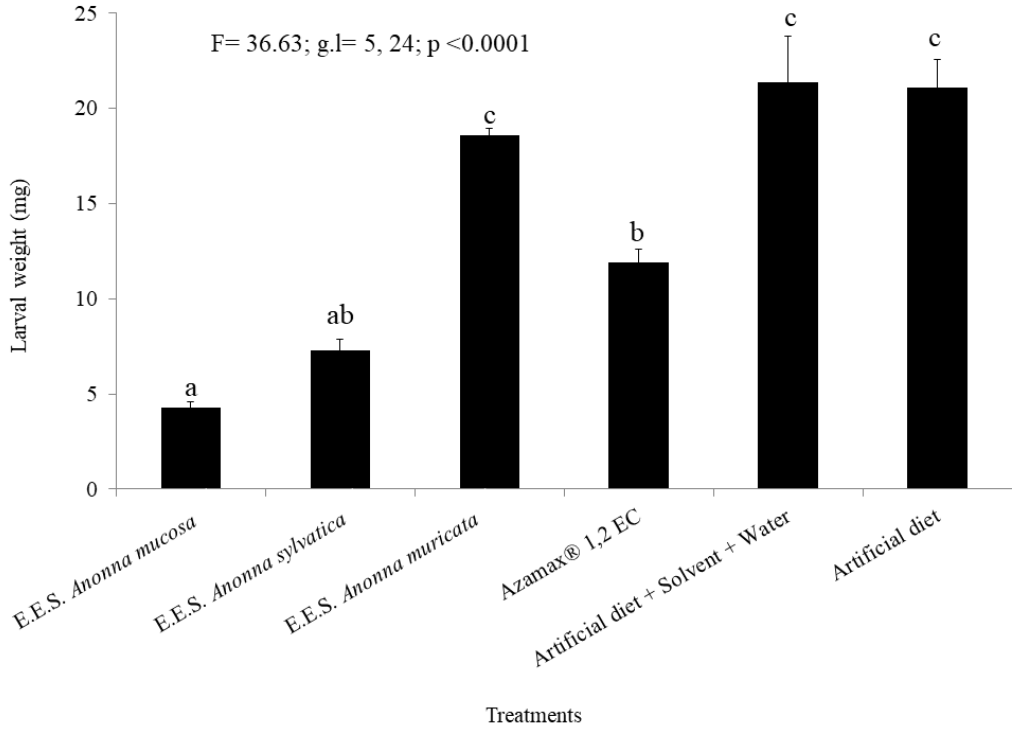

Figure 2 - Larval weight (± SE) of *Duponchelia fovealis* seven days after feeding on an artificial diet treated with different botanical derivatives. Means followed by different letters indicate significant differences between treatments (GLM with Gaussian distribution, followed by the Tukey *post hoc* test, P<0.05); E.E.S. = Aqueous emulsion of ethanolic seed extract (pre-commercial formulations).

from ethanolic seed extract of *A. mucosa* and *A. sylvatica* on *D. fovealis larvae*. Several studies have demonstrated the lethal toxicity of *A. mucosa* derivatives to other pest species, including *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) (RIBEIRO & VENDRAMIM, 2017; RIBEIRO et al., 2020), *Panonychus citri* (McGregor) (Prostigmata: Tetranychidae) (RIBEIRO et al., 2014a), *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) and *Myzus persicae* (Sulzer) (Aphidomorpha: Aphididae) (RIBEIRO et al., 2014b), *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (ANSANTE et al., 2015), *Helicoverpa armigera* (Lepidoptera: Noctuidae) (SOUZA et al., 2019), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) (BERNARDI et al., 2017), *Zaprionus indianus* Gupta (Diptera: Drosophilidae) (GEISER et al., 2019), and *Tetranychus urticae* (KOCH, 1836) (Prostigmata: Tetranychidae) (MIOTO et al., 2020).

For *D. fovealis*, the low lethal toxicity after dietary exposure of an artificial diet containing aqueous emulsions from the tested *Annona* seed extracts and with the limonoid-based bioinsecticide (Azamax® 1.2 EC) was also observed when synthetic insecticides, based on lambda-cyhalothrin, milbemectin, cyromazine, thiamethoxam, methoxyfenozide, deltamethrin, alpha-cypermethrin, acetamiprid, thiamethoxam + lambda-cyhalothrin and phenpropatrin in laboratory bioassays were used (DOS SANTOS et al., 2019). This low efficacy of the products may be, probably, associated to the high capacity of metabolization and detoxification of active ingredients after ingestion of this insect pest, as verified for larvae of *S. frugiperda* (BAI-ZHONG et al., 2020), which is a hypothesis to be further evaluated. This fact is reinforced by the low increase in mortality (approximately 50% mortality) of larvae when using the maximum concentration of 8,000 mg kg^{-1} of the aqueous emulsion of both formulated extracts. At this concentration, these derivatives showed pronounced lethality for the other species tested (RIBEIRO et al.,

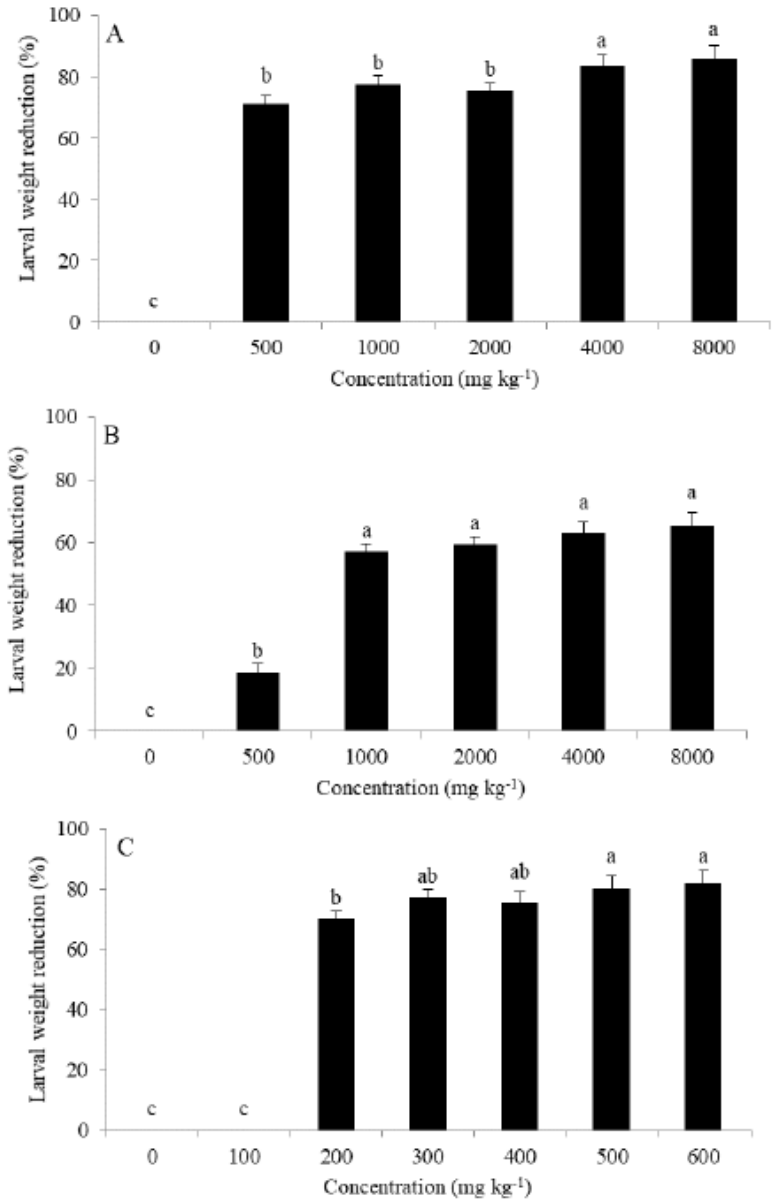

Figure 3 - Larval weight reduction (%) (± SE) of *Duponchelia fovealis* seven days after feeding on an artificial diet treated with different botanical derivatives. (A) AEEE *Annona mucosa*; (B) AEEE *Annona sylvatica* and (C) Azamax® 1.2 EC.

Means followed by different letters indicate significant differences between treatments (GLM with Gaussian distribution, followed by the Tukey *post hoc* test, $P < 0.05$).

2014a, 2014b; ANSANTE et al., 2015; BERNARDI et al., 2017; RIBEIRO & VENDRAMIM, 2017; GEISLER et al., 2019; SOUZA et al. 2019; MIOTO et al., 2020), leading to the total mortality of exposed arthropods, in most cases.

Conversely, concentration increase of active ingredients in the artificial diet greatly reduced larval development over time, reaching 80% in larvae submitted to the artificial diet treated with formulated extract from *A. mucosa* seeds. This

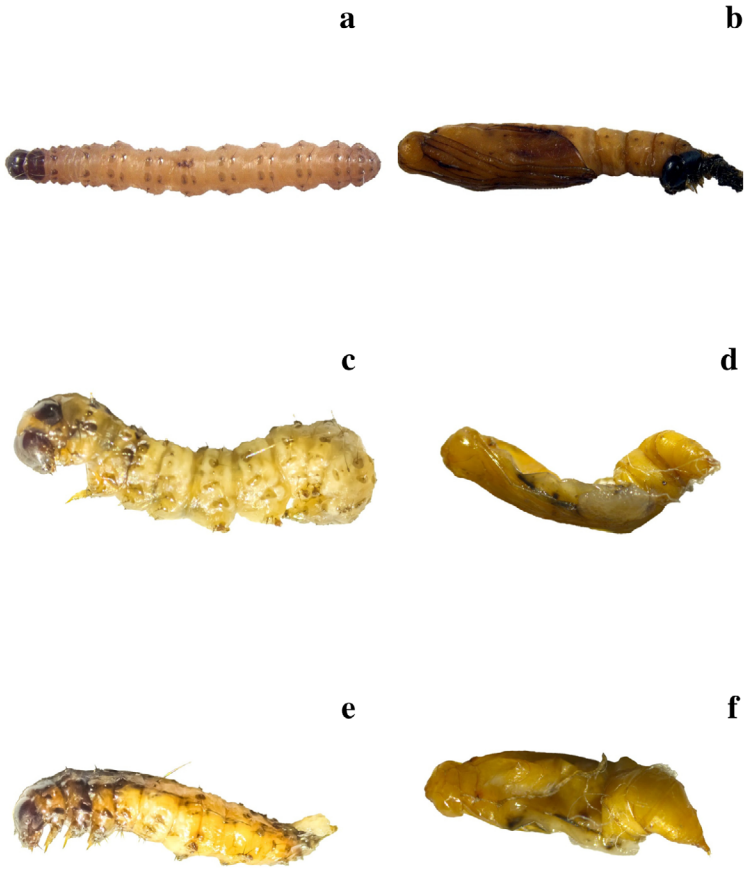

Figure 4 - *Duponchelia fovealis* larvae and pupae control (a, b) and with deformation when submitted to the artificial diet containing formulated extracts (concentration of 8000 mg kg^{-1}) prepared from seeds of *Annona mucosa* (c, d) and *Annona sylvatica* (e, f).

sublethal effect is extremely important for IPM because they can directly affect population density of *D. fovealis* in future generations. This fact is proven by the high larval deformation rate in surviving larvae, preventing them from passing to the pupal stage. Thus, the combined effects of lethal and sublethal toxicity are important tools for IPM programs, since biologically and ecologically alternatives have been studied on *D. fovealis* larvae and with promising results, such as the use of entomopathogenic fungi (BAJA et al., 2020), and natural enemies (PIROVANI et al., 2017; ARAUJO et al., 2020). Thus, future studies could evaluate the integration of biological control agents and botanical insecticides in the framework of *D. fovealis* IPM.

In Brazil, there are no synthetic products registered for the management of *D. fovealis* in strawberry crops. Therefore, due to easy production and rapid degradation of products based on *Annona* derivatives (RIBEIRO et al., 2015) and limonoids (BERNARDI et al., 2012), these products constitute important alternatives for the management of *D. fovealis,* particularly, in organic production systems. Further studies should be conducted under field conditions to evaluate the effectiveness of these products in commercial crops, as well as aspects related to ecotoxicological safety and degradability.

CONCLUSION

Aqueous emulsions prepared from ethanolic seed extracts of *A. mucosa* and *A. sylvatica* and a limonoid-based bioinsecticide have low lethal

toxicity to *D. fovealis* larvae; nevertheless, they have a pronounced inhibition of their larval development.

Derivatives rich in acetogenins (pre-commercial formulations) and a limonoid-based commercial bioinsecticide are promising alternatives for IPM of *D. fovealis*.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

The authors contributed equally to the manuscript.

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