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Lethal and Sublethal Effects of Pyrrolizidine Alkaloids of *Senecio rudbeckiaefolious* against *Diatraea saccharalis* F. Larvae

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KEY WORDS

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ABSTRACT *Diatraea saccharalis* Fabricius (Lepidoptera: Crambidae) is the most damaging sugarcane pest in the Northwestern Argentina and an important pest in corn in the Pampas region. Larvae perforate the stem of the cane forming galleries or tunnels in its interior, which decreases the flow of water and nutrients and facilitates the entry of pathogens. Infestations by this insect provoke significant economic losses. Given the environmental and human health impact of control measures based on the application of agrochemicals, the current study was based on the use of bioactive natural products, such as plant secondary metabolites. The objective of this work was to study the alkaloidal composition of a methanolic extract from *Senecio rudbeckiaefolious* Meyen and Walp (Asteraceae) and to evaluate its lethal and sublethal effects against the larvae of sugarcane borer, *D. saccharalis*. The major components detected in the pyrrolizidine alkaloids (PAs) fraction were senecionine and integerrimine. Repellency and toxicity bioassays were carried out with larvae fed on a diet supplemented with an aqueous solution of PAs (50–200 mg/L). No repellent effect was observed. Concentration-dependent mortality and a marked inhibitory action of larval growth were recorded. The results suggested that PAs could be promising biopesticidal compounds for *D. saccharalis* control.

INTRODUCTION

The sugarcane borer, *Diatraea saccharalis* known as "stem borer," has a widespread distribution throughout the Western Hemisphere and is a pest of many crop plants (Fogliata *et al*., 2016). In Northwestern Argentina, it is the most damaging sugarcane (*Saccharum officinarum* Linnaeus; Poaceae) pest and causes significant losses in sugar and ethanol production (Salvatore *et al*., 2010). In addition, in central Argentina (Pampas region), it is the

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most important pest in corn (*Zea mays*) (Serra and Trumper, 2006; Fogliata *et al*., 2017).

Sugarcane is the most important saccharine crop in the world, responsible for 70% of total sugar production. In Argentina, sugarcane cultivation is concentrated in the Northwestern area and is affected by different pests throughout its phenological cycle.

D. saccharalis causes the highest losses in sugarcane cultivation annually (Fogliata *et al*., 2016). The duration of the life cycle of *D. saccharalis* depends on the time of the year, temperature, and humidity (Osores *et al*., 1981). The adult moths deposit clusters of eggs on the leaves of the plants. The eggs hatch and the small larvae feed on the leaf sheaths and then perforate the stem of the cane forming galleries or tunnels in its interior, which decreases the flow of water and nutrients (Serra and Trumper, 2006). In addition, these tunnels facilitate the entry of pathogens (fungi and bacteria). The galleries and the associated rotting cause a decrease in the weight of the canes and in the sucrose content (de Romero *et al*., 2008; Salvatore *et al*., 2009; Pérez *et al*., 2010; Pérez Rico and Martínez Torres, 2011). Pupation occurs within chambers constructed by mature larvae (Salvatore *et al*., 2010). After this period, adults emerge as butterflies with pale yellow front wings. The females attract the males for copulation through the release of pheromones. After copulation, the females lay the eggs on the leaves, restarting the cycle (Pérez *et al*., 2010).

Despite many investigations on transgenic crops that are currently being developed, there are no commercial cane varieties resistant to *D. saccharalis.* Biological control, a practice used in Brazil, is not applied in the Northwestern Argentina since the parasitoids introduced from there could not be established, probably due to inappropriate environmental conditions (Salvatore *et al*., 2009). Worldwide, the management of *D. saccharalis* is mainly by chemical pesticides (Wilson *et al*., 2017). In the United States, this practice is used when 5% of plants infected with small larvae are found (<0.5 cm). Sugar mills using the aforementioned methodology did not give positive results in our country (Salvatore *et al*., 2009). As a measure of cultural control, it is recommended to keep the field clean from Russian grass (*Sorghum halepense*) and other grasses, since they are alternative hosts where the populations of *D. saccharalis* reproduce in the absence of sugar cane crop. The extensive and long-term application of synthetic insecticides has resulted in accumulating their residues in food, milk, water, soil, and other environmental components, thus causing adverse health effects to humans and ecosystems (Mossa *et al*., 2018). Therefore, a general concern is to find alternatives that offer crop yields similar to those obtained with synthetic insecticides, without compromising public health and the environment (Abudulai *et al*., 2001; ÁvalosGarcía and Pérez-Urria Carril, 2009).

Botanical insecticides are considered to be an effective alternative for pest control. Their characteristics include low-residue and high-performance, fewer poisonous side effects, and good compatibility with the environment. Resistance to biopesticides in target organisms is not easily generated, unlike many cases of their chemical counterparts (Singh *et al*., 1996; Leng *et al*., 2011), which affect insect populations, decrease the survival, the development, and the reproduction rate (Carlini and Grossi-de-Sá, 2002; Bosly, 2013). Insecticidal activities have been attributed to several families of secondary plant metabolites such as alkaloids, phenolic compounds, terpenoids, and essential oils (Koul *et al*., 2008; 2016; Rattan, 2010; Ge *et al*., 2015; Guerriero *et al*., 2018; Kortbeek *et al*., 2019).

Our research group has recently obtained high mortality rates in *Plodia interpunctella* larvae fed on diet supplemented with pyrrolizidine alkaloids (PAs) isolated from *Senecio rudbeckiaefolius* Meyen & Walp (Asteraceae). The genus *Senecio* (Asteraceae) includes more than 1500 species widely distributed throughout the world (Yang *et al*., 2011). *S. rudbekiaefolius* is a perennial shrub that grows in the mountains of Southern Peru, Bolivia, and Northwestern Argentina and it is used in traditional medicine in Peru. In Argentina, no medicinal use has been reported. No data about the chemical composition of this species have been reported. PAs are well known for their deleterious effects on herbivore insects and vertebrates. These compounds are reported as feeding deterrents and toxic to herbivore insects (Hartmann, 1999; Hartmann and Ober, 2000; Macel *et al*., 2005; Domínguez *et al*., 2008; Ober and Kaltenegger, 2009). In vertebrates, they can have hepatotoxic, pneumotaxic, and carcinogenic effects (Nuringtyas *et al*., 2014). Thus, the objective of the present work was to obtain PAs from *S. rudbeckiaefolius* and to evaluate their lethal and sublethal effects against larvae of *D. saccharalis*.

MATERIALS AND METHODS

Pyrrolizidine Alkaloids

Plant material was collected in the city of Tafí del Valle (S 26°51′9.9″ and W 65°42′35.39″), Tucumán Province. The dry aerial parts were extracted with methanol in Soxhlet equipment and the solvent evaporated in rotavapor. The acidified residue (H_2SO_4, 1N) was extracted with ether; the aqueous phase was alkalized to pH 10–11 and extracted with chloroform. The resulting product was dried with Na_2SO_4, filtered, and evaporated. The obtained extract was analyzed by gas chromatography coupled to mass spectrometry (GC-MS), whose experimental conditions were: Perkin Elmer

Elite-5MS column (5% Phenyl Methyl Siloxane, 30 m × 0.25 mm × 0.25 µm); carrier gas: He, flow: 1.0 mL/min; and programmed temperature: 130°C (0°C), 130–275°C (3°C/min), and 275°C (2 min).

Insects

Neonate larvae (<24 h old) of *D. saccharalis* were used. The larvae were obtained from the breeding stock of the Laboratory of the Agricultural Zoology Section of the Estación Experimental Agroindustrial Obispo Colombres and maintained in a chamber under controlled conditions of 25 ± 2°C, 70–75% RH, and a photoperiod of 14L:10D. Initial collections were made from sugar cane in the locality of Overo Pozo (S 26°50′4.4″ and W 64°52′8″) in Tucuman Province.

Repellency Tests

Repellency tests were carried out to evaluate the response of larvae of *D. saccharalis* to PAs. Trials were performed using rectangular experimental arenas (10.5 × 7.5 cm) divided into three equal size zones (a middle one and two lateral) along the long axis. At each lateral zone, either an olfactory key (15 µL of PAs diluted in distilled water at concentrations of 100, 150, 200, and 500 mg/L) or the corresponding control (15 µL of distilled water) were placed on filter paper strips of 2.0 × 1.0 cm. In the middle zone of the arena, a single first-instar was released. After 1 h, the location of the larva in the arena (i.e. the selection of a given lateral zone) was recorded.

Experiments were conducted at 25 ± 2°C. In all cases, the olfactory key and control sources were replaced after each trial. Forty-eight larvae were used per treatment. Each larva was used only once.

Toxicity Bioassays

Bioassays were carried out by forced intake tests using a multispecies commercial diet (Southland Products Inc.) with the addition of the 1000 mg/L PAs stock solution to obtain the final concentrations of 50 mg/L, 100 mg/L, 150 mg/L, and 200 mg/L. For the preparation of a liter of diet, 930 mL of boiling water was added to 162 g of dry diet. Then, it was blended for 3–4 min with a hand mixer for controls, diet without extract (C0) was used. Dead larvae due to mishandling problems were discarded. For the inoculation, trays were used (CD International Inc. Bio-Assay tray Pitman), each containing 128 individual cells divided into eight groups of 16 cells. In each cell, 1 mL of hot diet (<50°C) was placed with or without the addition of the natural product to be tested, the diet was allowed to cool, solidify, and kept under laminar flow for 1 h to reduce the risks of contamination. Each cell was inoculated with one neonate or L1 larva obtained from different egg batches to ensure a high genetic variability, using a soft brush to avoid any larval damage. Then, an adhesive cover (Bio-Assay Tray Lid-16 cells Frontier) was placed on each group of 16 cells to prevent the larvae from escaping. These covers have micropores that allow normal respiration. Per diet, six groups of 16 cells (n = 96) were used. The trays, once inoculated, were kept in a chamber under controlled temperature, humidity, and photoperiod conditions. The evaluation was made 7 and 14 days after inoculation under a binocular magnifying glass and the number of live larvae, dead larvae, and their larval stage was recorded. The surviving larvae on day 7 were transferred to test tubes with a normal diet. From the information obtained on day 14, the mortality and the number of surviving larvae in each instar were calculated. The tests were continued until the formation of pupae for evaluation of sublethal effects (malformations, growth inhibition, etc.).

Data Analysis

In repellency tests, the preference of insects for either lateral side of the arena (control vs. experimental) was tested against a random distribution (i.e., 50% of choices for either side of the arena) by means of Chi-square test of goodness of fit. $P > 0.05$ was considered non-significant. Individuals that remained in the middle zone without making a decision were excluded from the analysis. The number of surviving larvae in each treatment concentration and control was compared using a Chi-square test with Xlstat statistical software. Furthermore, mortality results were evaluated using the probit (dose-response) analysis by the Polo-Plus software (LeOra, 2002). This allowed for establishing the 50% lethal concentration (LC_{50}), its respective 95% confidence limits, and the slope (± SE) of the regression line.

Sublethal effects of PAs on growth inhibition were analyzed with a Chi-square test to compare the number of survived larval stages in treated and control experiments using Xlstat program.

RESULTS

Pyrrolizidine Alkaloids

The analysis of the mass spectra allowed for the identification of the PAs based on the fragmentation patterns, the retention times and by comparison of the corresponding mass spectrums with those provided in the spectrometer software and with those reported in the literature (Stelljes *et al*., 1991; Trigo *et al*., 2003). The main alkaloids identified in *S. rudbeckiaefolius* were senecionine (71%), integerrimine (25.6%), seneciphylline (1.1%), senecivernine (0.8%), platyphylline (0.8%), and neoplatyphylline (0.5%) (Fig. 1).

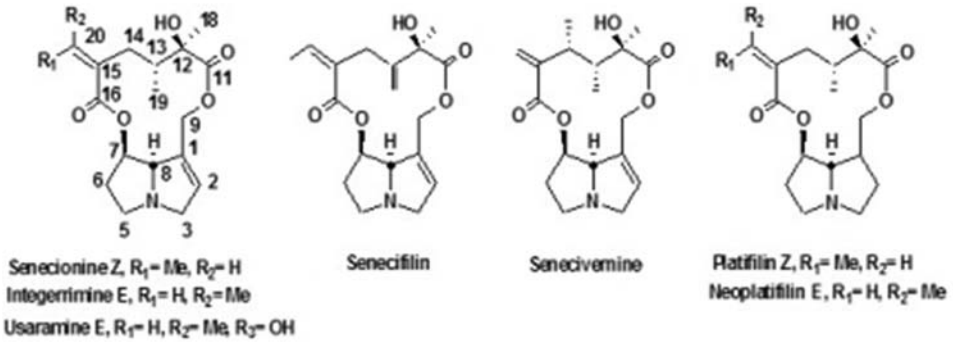

Fig. 1. Chemical structures of main pyrrolizidine alkaloids identified in *Senecio rudbeckiaefolius*.

Repellent Activity

Repellency tests were carried out to evaluate the response of larvae of *D. saccharalis* to PAs at concentrations from 100 to 500 mg/L. After 1 h, larvae were distributed at random in the experimental arena in all cases, which implies that PAs do not exert an attractive or repellent effect on *D. saccharalis* ($P > 0.05$).

Lethal Effects

To evaluate the toxic effects of PAs on the larval development of *D. saccharalis*, forced intake tests were performed. On day 14, concentration-dependent toxic effects with high percentages of mortality were observed. Significant differences were found ($X^2 = 117.4$, $P < 0.0001$) in the percentages of mortality between the different groups (Fig. 2). The LC_{50} value estimated by probit analysis was 113.30 (95.40–131.54) mg/L and the slope was 2.65 ± 0.39.

Sublethal Effects

On day 7 of the trial, a slight decrease in growth was recorded in the larvae fed with diet supplemented with PAs. Results on day 14 show a potent growth-inhibitory effect, in particular from the PAs concentration of 100 mg/L (Fig. 3). Significant differences were found ($X^2 = 139.58$, $P < 0.0001$) in the number of surviving larvae in each instar between the groups (Fig. 4). While control larvae were mostly in the L3 and L4 stages, almost 90% of the larvae treated with PAs reached the L2 stage only. The tests were conducted until pupae formation. While control larvae reached pupal stage, the significant growth-inhibitory effect was observed in surviving larvae treated with PAs, depending on the concentrations used. Most of the larvae fed on a diet with 50 mg/L PAs reached the L3 stage and almost all the larvae under treatment of 100–200 mg/L died; the few surviving larvae were in L2 stage (Fig. 5). During the toxicity tests, there were few signs of intake, tunneling or presence of feces in all treatments, these effects being more pronounced at the highest concentration of treatment (150 and 200 mg/L) (Fig. 6).

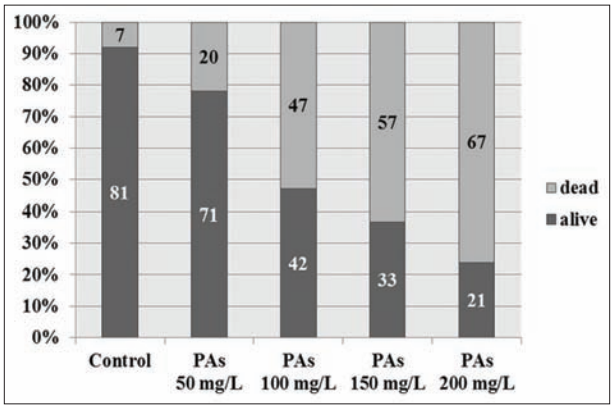

Fig. 2. Dead and survived *Diatraea saccharalis* larvae fed on artificial diet supplemented with pyrrolizidine alkaloids (50–200 mg/L) after 14 days.

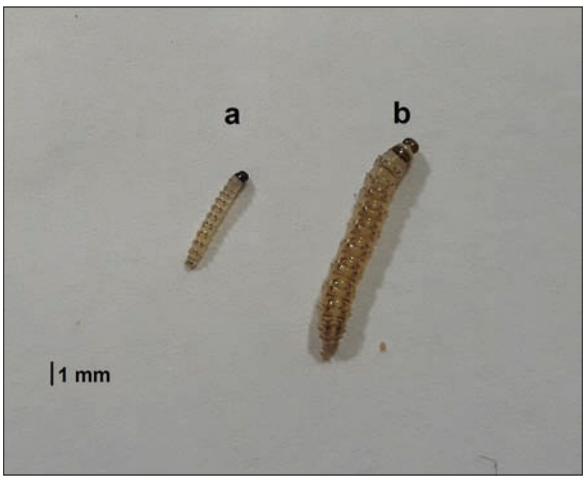

Fig. 3. *Diatraea saccharalis* larvae on day 14 post-treatment. (a) L2 larva after treatment with pyrrolizidine alkaloids 200 mg/L, (b) L4 control larva.

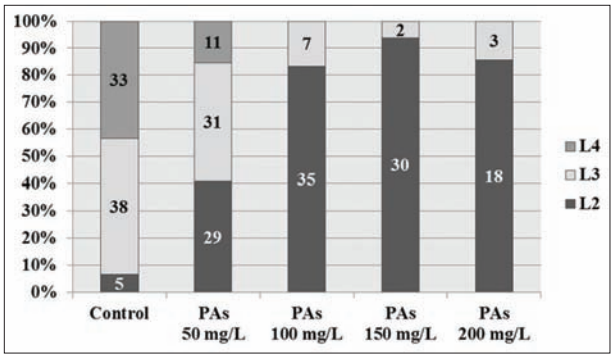

Fig. 4. Survived *Diatraea saccharalis* after feeding artificial diet supplemented with pyrrolizidine alkaloids (50–200 mg/L) after 14 days.

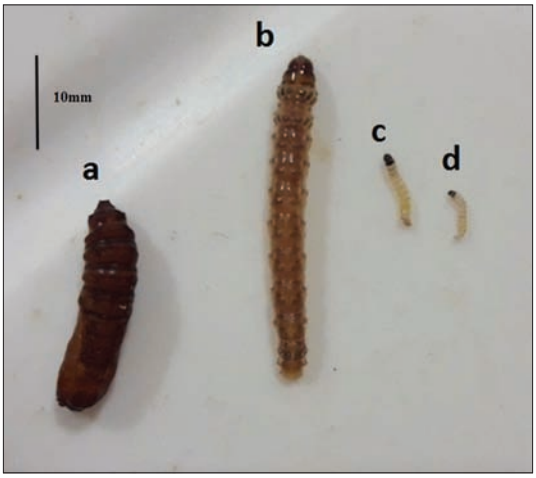

Fig. 5. Growth inhibition of *Diatraea saccharalis* larvae under different treatment conditions. (a) Pupa in controls, (b) L5 larva in controls, (c) L2 larva after treatment with pyrrolizidine alkaloids (PAs) 50 mg/L. (d) L2 larva after treatment with PAs 200 mg/L.

DISCUSSION

Taking into account the growing interest in plants and their chemo-biodiversity as a source of bioactive substances for the development of new phytosanitary products, our work group has conducted research on biological properties of a series of secondary metabolites obtained from regional native flora. The present study is a first report of isolation of PAs from the aerial parts of *S. rudbeckiaefolius* from a methanolic extract. The major components detected in this fraction were senecionine and its stereoisomer integerrimine; together they represent approximately 97% of the alkaloidal fraction.

We also evaluated the lethal and sublethal effects of aqueous solutions of the PAs from a methanolic extract

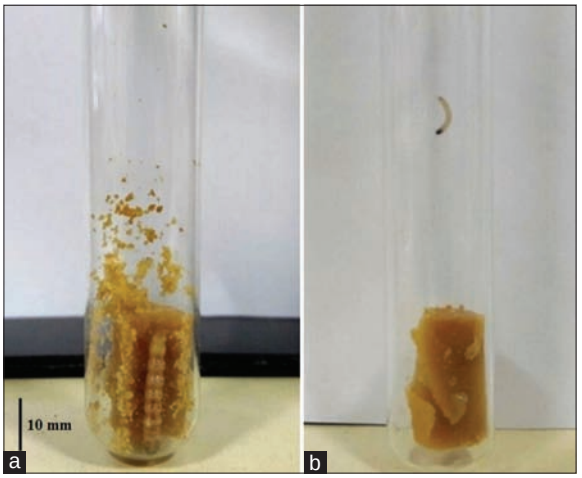

Fig. 6. Larvae of *Diatraea saccharalis* feeding on control and treated diets on day 14. (a) L4 larva (on control diet). (b) L2 larva in treated diet with pyrrolizidine alkaloids (200 mg/L).

of *S. rudbeckiaefolius* on *D. saccharalis* larvae. High percentages of mortality were obtained and this effect was dependent on the concentrations tested, i.e., about 80% at 200 mg/L. Similar results have been recorded for *Plodia interpunctella*, but at slightly lower concentration of 150 mg/L (Unpublished data).

Based on the biosynthetic route, PAs are divided into four main groups: Senecionine-, jacobin-, erucifoline-, and otosenine-like PAs. Most of these compounds occur in two interchangeable forms: Free base (tertiary amine) and N-oxide (Hartmann and Dierich, 1998), except for otosenine-like PAs (Pelser *et al*., 2005). The N-oxide is accepted as the major storage form in plants (Hartmann and Toppel, 1987; Van Dam *et al*., 1995), and the free base form is considered to be more toxic than the N-oxide (van Dam *et al*., 1995; Hartmann and Ober, 2000; Hartmann, 2007; Nuringtyas *et al*., 2014). The toxic effects of the PA mixture on *D. saccharalis* demonstrated in our experiments prove the powerful bioactivity of PAs as free base, since the employed processing technique separates these compounds at such stage. Moreover, the analysis of this fraction by GC-MS confirmed that all alkaloids present in the mixture are found as free bases.

PAs have been investigated as feeding deterrents and toxic compounds in herbivore insects. Macel *et al.* (2005) did not observe the deterrent feeding activity of senecionine in *Spodoptera exigua* larvae even at 3 times the plant concentration. Domínguez *et al.* (2008) studying the effect of senecionine on *Spodoptera littoralis* did not report any effect either. However, senecionine has been reported to significantly reduce larval survival of *Myzus*

persicae and to deter feeding of *Locusta migratoria* (Macel *et al*., 2005; Nuringtyas *et al*., 2014). Although many investigations conducted with senecionine-like PAs show that the toxic effects largely differed among the insect species, most of them agree that senecionine is the least bioactive. Considering that senecionine is the simplest structure of the macrocyclic PAs, from which all other PAs are derived, it is accepted that derived PAs should be more toxic than senecionine (Ehrlich and Raven, 1964; Hartmann and Dierich, 1998). However, our experiments demonstrated that a senecionine-type PAs mixture whose primary component is senecionine proved toxic for *D. saccharalis* larvae. On the other hand, previous results have shown that the antifeedant action of the individual PAs did not correlate with that of the alkaloidal extract. Indeed, Domínguez *et al.* (2008) showed that an alkaloidal extract had a stronger antifeedant effect against *S. littoralis* while its main component was not active. Out of the PAs found in small proportions in the mixture used in our assays, seneciphylline has been described as highly toxic in the previous studies (Macel *et al*., 2005; Narberhaus *et al*., 2005; Nuringtyas *et al*., 2014). These antecedents allow us to suggest that the small quantities of seneciphylline present in the PAs mixture could enhance the toxicity of the alkaloidal fraction.

Ruiz-Vásquez *et al.* (2015) have proved that the non-alkaloidal fraction of a methanolic extract of a *Senecio* species (*Senecio kingii*) exerts a powerful antifeedant effect in *S. littoralis* larvae. Considering that the alkaloidal fraction accounted for a maximum of 2% of the methanolic extract, they postulate that the chemistry of the non-alkaloidal fraction could explain most of the antifeedant effects (Ruiz-Vásquez *et al*., 2015) as it was suggested for other *Senecio* species (Joshi and Vrieling, 2005; Domínguez*et al*., 2008). These findings back the need to prove the bioactivity of the methanolic extract of *S. rudbeckiaefolious*, rather than separate the alkaloidal fraction. This would be greatly convenient when suggesting its application as biopesticide, as it implies a more accessible methodology.

Sublethal effects described in *P. interpunctella* and *D. saccharalis* larvae by our group differ widely. While in the tests with PAs on larvae of *P. interpunctella* morphological alterations in the larval development were observed, in particular, pigmentation changes at the mesenteric region. Our results show that PAs induce a significant growth-inhibition effect in larvae of *D. saccharalis*, without evident morphological changes. In fact, while on day 14 of the test the control larvae were mostly in the L3 and L4 stages, almost 90% of the larvae fed with PAs at 200 mg/L had only reached the L2 stage. While in control experiments larvae reached pupa stage, treated larvae stopped their development in L2 instar. This is in accordance with Narberhaus *et al.* (2005), who found that the alkaloid-fed larvae of *Philosamia ricini* grew significantly slower than the control individuals and the entire set of PAs-fed insects died within or before the pupal stage. It should be noted that for the evaluation of lethal and sublethal effects of larvae of *D. saccharalis* at time spans longer than 7 days, the larvae were transferred to test tubes with standard diet. This step responds to the need to mimic in the laboratory the cycle of the insect in the field; when the plague attacks the sugarcane, the females deposit clusters of eggs on the leaves and the small larvae feed from the leaf sheaths. Once they reach L2 stage, they perforate the stem of the cane forming galleries or tunnels inside ("stem-boring"). Therefore, the design of any intake toxicity test must take into account that the insect must make contact with the product to be tested in the first stages of development.

To complement the toxicity tests, PAs repellency tests were carried out at concentrations between 100 and 500 mg/L. The results of the repellency tests, analyzed together with the behavioral observations, allow us to verify that the PAs do not exert a repellent or attractive effect on the insects. In fact, in toxicity tests, it was found that the larvae feed on the diet with PAs during the initial days of the test and subsequently feeding decreased, probably due to their reduced metabolism caused by the toxic effects and by day 14, the larvae moved totally away from the diet (Fig. 5). It is, therefore, possible to attribute the registered toxic effects to the addition of PAs to the diet at the beginning of the larval cycle. Similarly, the previous results have shown that monocrotaline and acetyl usaramine reduced biomass gains of orally injected *S. littoralis* larvae indicating that they acted as strong post-ingestive toxins without antifeedant effects (Domínguez *et al*., 2008).

Toxicity tests of PAs therefore, showed concentration-dependent lethal effects against larvae of *D. saccharalis* and apparently act as physiological toxins. This is also obvious from the marked growth inhibitory effects that could be due to interference with physiological processes like growth hormonal regulations. Repellency tests, together with behavioral observations with regard to food intake, show that this natural product does not exert a repellent or attractive effects on *D. saccharalis* larvae.

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