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Full Length Research Paper

Toxicity of copaiba extracts to armyworm (*Spodoptera frugiperda*)

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The objective of this study was to evaluate the effects of methanolic extracts from leaves, peels, seeds and pulps from fruits of *Copaifera langsdorffii* on *Spodoptera frugiperda.* Extracts derived from leaves and fruit peels were more toxic to *S. frugiperda* than the others. Hence, they were added to the artificial diet and used in further experiments with the second instar larvae of the insect, which presented larval growth reduction, prolonged period of development, increased mortality, and lower fertility and fecundity of adults. Lower egg viability was also observed when the insect was treated with extracts of leaves and fruit peels in the larvae stage. Moreover, when subjected to ultrastructural analysis under a scanning electron microscope, such eggs showed abnormalities in the aeropylar and micropylar regions. Both extracts also increased the excretion of protein in the insect feces and inhibited trypsin activity in the *in vitro* test. Consequently, *C. langsdorffii* presents potential to be used in the development of new products to control the fall armyworm.

Key words: *Copaifera langsdorffii*, natural products, trypsin inhibitor, botanical insecticide.

INTRODUCTION

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a polyphagous insect widely distributed in the tropical and subtropical regions of America (Andrews, 1988), which causes damage to crops such as corn and cotton. The chemical method usually employed to control this insect has favored the selection of populations resistant to pesticides (Yu, 2006; Yu and McCord, 2007; Virla et al., 2008) which justifies the search for new molecules to control *S. frugiperda*.

In the search for new molecules active against insects, several plant species, especially those from the Fabaceae family, stands out as insecticidal producers. One of the first botanical insecticides used was rotenone, isolated from a plant species belonging to this family (Morallo-Rejesus, 1986). Among the effects caused by Fabaceae on insects are the reduction in oviposition and digestive enzymes (Ramos et al., 2008). Regarding inhibition of enzymatic processes, molecules capable of inactivating the trypsin enzyme of insects have been survival (Okwute et al., 2009), growth interruption, mal-formations (Innocent et al., 2008) and inhibition of isolated from several plants of Fabaceae (Gomes et al., 2005; Oliveira et al., 2007; Macedo et al., 2009). Specifically regarding *S. frugiperda*, inhibition of enzymatic processes has been reported as the mode of action by some botanical insecticides, among which the crude extract from Sapindus saponaria seeds is an example. The substances present in this extract reduced trypsin activity, increased the mortality of caterpillars and the larval weights were reduced (Santos et al., 2008). Also worth mentioning is the inhibition of trypsin from *S. frugiperda* by a substance isolated from the seeds of *Poecilanthe parviflora* (Garcia et al., 2004).

In this study, copaiba (*Copaifera langsdorffii* Desf.) (Fabaceae) is reported as a promising alternative for the control of *S. frugiperda*, since this species has presented

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toxic activity against other insects (Mendonça et al., 2005). Although, the mechanism of action of this plant is not clear, apparently the inhibition of trypsin may be involved (Krauchenco et al., 2001). Thus, lethal and sublethal effects on *S. frugiperda* by extracts from leaves, peels, seeds and pulps of fruits from *C. langsdorffii* were studied in this work.

MATERIALS AND METHODS

Insect cultures

All insects used in the experiments were reared in the laboratory. The larvae were fed with an artificial diet (Greene et al., 1976) and adults received food in the form of an aqueous 10% (v/v) honey solution. Only second posture larvae were used to begin the experiments.

Collection of the plant and preparation of extracts

The plant materials were collected from the campus of Universidade Federal de Lavras, MG, Brazil, in the month of August at 9 h, and samples were sent for identification at the ESAL Herbarium, where they are deposited under number 05437. For the preparation of extracts, the leaves, peels, seeds and pulps from fruits of *C. langsdorffii* were chopped, macerated in methanol for 48 h and then filtered through cotton wool plugs. More of methanol was added to the insoluble residue and this procedure was repeated until the liquid phase presented a clear coloration,showing completion of extraction. The liquid phases were combined, concentrated in a rotary evaporator until complete elimination of solvent and were then freeze-dried, giving rise to the dried plant extracts.

Assay with first instar caterpillars of *S. frugiperda*

The dried plant extracts were first dissolved in an aqueous 0.01 g/ml Tween 80 solution and incorporated into the artificial diet described by Greene et al. (1976). The final concentration of each extract in the diet was 1,000 µg/ml. In order to avoid possible compound degradation in the extracts, their incorporations were performed at the end of the preparation of the diet when the temperature reached near 55°C. Portions of the extract-containing diet were previously weighed and transferred to 50 ml plastic containers, where they were offered to newly hatched caterpillars. Aliquots of the diet remained under the same conditions at which the experiment was conducted in order to estimate water loss by difference in weights. Treatments consisted of diet plus the extracts of leaves, fruit pulp, fruit peel and seeds of *C. langsdorffii.* Diet plus water or aqueous solution of Tween 80 at 0.01 g/ml was used as the control. The experiment was conducted in a randomized design, with six treatments and six replications; each plot consisted of six larvae kept individually, with a total of 36 insects per treatment.

The biological parameters evaluated were: survival during the larval stage at every three days, weight and food intake of larvae after seven days of the experiment assembly, duration of larval period, and pupal weight and survival. A correction factor for water loss was used to calculate food consumption: $[1 – a/2] \times [W – (L + bL)]$, where “a” is the initial weight of the diet portion; “b” is the average weight loss rate; W is the diet weight introduced and “L” is the weight of diet not consumed (Cohen, 2004).

Data concerning the consumption and weight of larvae, duration of larval stage, pupal weight and survival of insects in the pupal stage were subjected to a normality test using the Shapiro-Wilk test, through the package Mvnorm test of the R® software (R Development Core Team, 2009). Afterward, data were submitted to ANOVA and the Scott-Knott test, using the Laercio software R® package (R Development Core Team, 2009). Data associated with the survival of insects during the larval stage were subjected to survival analysis, applying the Weibull model with the survival package of the R® software (R Development Core Team, 2009). After selecting the most appropriate mathematical model by the residue analysis, a contrast analysis was carried out to verify the similarity between the treatments employed and clustering peers. Lethal time to 50% of the population (LT_{50}) for each group was also calculated.

Assay with the second instar caterpillars of *S. frugiperda*

Extracts from leaves and fruit peels of *C. langsdorffii* were dissolved in an aqueous 0.01 g/ml Tween 80 solution and incorporated into an artificial diet. The final concentrations of each extract in the diet were 300, 600, 1,000 and 1,300 µg/ml. Portions of the extract-containing diet were transferred to 50 ml plastic containers, where they were offered to three-day old second instar caterpillars that had been previously maintained on an artificial diet. The experimental design was completely randomized with six treatments, since the experiment with the extract of leaves was conducted separately from that performed with the peel extract. Diet plus water or aqueous solution of Tween 80 at 0.01 g/ml was used as the control. Six replicates were employed for each treatment and the experimental plot consisted of six caterpillars kept individually.

To construct the caterpillar growth curve, the insects were weighed seven days after beginning the experiment, and the subsequent weighings were made every two days until the 17th day after the initiation of the experiment. Cumulative survival, duration of the larval stage, duration and survival in the pupal stage, pupal weight and sex ratio of adults [sr = Number of females / (Number of females + males)] were also assessed. At adult emergence, five couples from each treatment were individualized in five PVC cages (10 cm high × 10 cm diameter) and fed with an aqueous 10% (v/v) honey solution. Adult longevity was determined by daily observations and their eggs were collected daily for quantification. Eggs from the second day of oviposition were used to determine the viability, while those from the third day were observed in a scanning electron microscope in order to find out morphological abnormalities on their surfaces. The periods of pre-oviposition and oviposition were also recorded.

Values associated with the growth curve of larvae were analyzed by the dose-response curve (drc) software R® package (R Development Core Team, 2009) using the logistic model and Lack-of-fit test. With regard to the cumulative survival, duration of the larval stage, survival and duration in the pupal stage, pupal weight, sex ratio of adults, pre-oviposition and oviposition periods, longevity of males and females, number of eggs per female and egg viability data were subjected to ANOVA as described above and means followed by standard deviations (SD) were presented by descriptive statistics using the software package gplots R software (R Development Core Team, 2009).

Ultrastructural analysis of eggs from *S. frugiperda*

Eggs of the third posture from couples of *S. frugiperda* obtained in the test with second instar larvae were subjected to ultrastructural analysis in a scanning electron microscope (SEM). The standard protocol described by Alves (2004) was used to prepare all samples. Initially, eggs were fixed with the Karnovsky reagent for 24 h and then we performed a post-fixation with a solution of osmium tetroxide at 0.01 g/ml in a 0.05 M cacodylate buffer. Samples were

then washed with distilled water and dehydrated with aqueous solutions of acetone in increasing concentrations (25, 50, 75, 90 and 100%). Subsequently, samples were dried using the Critical Point Balzers CPD 030 apparatus. After drying, they were mounted on aluminum and gold stubs and brought to the SCD 050 Balzers evaporator to complete the preparation step. Samples were observed under a Leo Evo 40 SEM, using the Leo User Interface software to generate images.

Quantification of proteins in the feces of *S. frugiperda*

Feces collected at the time of pupation from the assay with the second instar larvae were grouped to form composite samples, each of these consisting of feces from an experimental plot. Samples were freeze-dried and submitted to protein extraction according to the method adapted from Ferreira et al. (2008). Then, the protein content of each extract was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) at concentrations varying from 0 to 20 µg/mg as standard. Each composite sample was extracted three times and the absorbance measure at 594 nm was performed in triplicate. Data were submitted to F test and regression analysis using the R software (R Development Core Team, 2009).

Determination of the trypsin inhibitory activity by extracts from leaves and fruit peels of *C. langsdorffii*

Digestive enzymes were obtained according to the method adapted from Oppert et al. (2005). For this, 4th instar larvae of *S. frugiperda* kept under laboratory rearing were immobilized on ice. Then, the anterior end and posterior intestine were removed, leaving the mid gut that was homogenized in a Potter homogenizer at a ratio of two intestines per 8 ml of distilled water at 4°C. The homogenate was filtered through a 100 µM nylon sieve and centrifuged at 30,000 × g for 10 min at 4°C. The supernatant consisted of the enzyme extract used in the trypsin inhibition tests.

Trypsin inhibition was determined by measuring the residual enzyme activity toward the substrate N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) (Erlanger et al., 1961; Kakade, 1974). The experiment was conducted in a randomized design and treatments consisted of methanol solutions of *C. langsdorffii* leaf extract at 25, 50, 100, 200 and 400 µg/ml, and *C. langsdorffii* fruit peel extract at 75, 125, 250, 500 and 1,000 µg/ml, which were determined in previous tests. The reactions proceeded in the absence or presence of plant extracts, enzyme extract of *S. frugiperda* and substrate. The enzyme extract (200 µl) and *C. langsdorffii* extracts dissolved in methanol (200 µl) were poured into glass tubes. Then, 0.87 mM BAPNA (800 µl) in DMSO containing 0.1 M Gly-NaOH buffer at pH 9.7 was added to the content of each tube, which was incubated at 30°C for 30, 60, 90 or 120 min. The reaction was stopped by the addition of 200 µl of an aqueous 30% (v/v) acetic acid solution, and absorbances were measured at 410 nm. Four replications were used, each represented by a glass tube. Finally, the results were expressed as percentage inhibition of trypsin by comparing the reaction rate in controls and in treatments with extracts. Data analysis was performed by F test and regression analysis using the R software (R Development Core Team, 2009).

RESULTS

Assay with first instar caterpillars of *S. frugiperda*

The extract from *C. langsdorffii* leaves was the most toxic to *S. frugiperda* caterpillars since it presented LT50 of 13.17 days and caused insect mortality of 73.4% by the end of the evaluation period. Under the same conditions, the extracts from seeds, pulps and peels from fruits of *C. langsdorffii* showed mortality of about 34% by the end of the assessment, with LT50 of 43.49 days (Figure 1). Seven days after the start of the experiment, there was no effect of extracts on food consumption by *S. frugiperda* larvae, which ranged from 491.3 ± 23.65 to 596.4 ± 32.56 mg. However, insects that developed on a diet containing extracts from *C. langsdorffii* seeds and peels of fruits were respectively 1.62 and 4.29 times lighter than those fed on the control diet containing Tween 80 (Table 1). The extracts from the *C. langsdorffii* seeds, peels, and pulps of fruits, increased the duration of the *S. frugiperda* larval stage. However, these treatments did not affect the pupal stage duration, pupal weight or the noctuid survival in the pupal stage (Table 2).

Assay with second instar caterpillars of *S. frugiperda*

The *C. langsdorffii* fruit peel extract reduced the percentage of *S. frugiperda* larva cumulative survival ($F = 5.5425$, $p \leq 0.0010$). While the control treatments with water and Tween 80 afforded average values of 97.2 ± 5.44 and 86.1 ± 6.21%, respectively; values for the fruit peel extract ranged from 63.9 ± 16.38 to 80.6 ± 19.48%. This extract also caused an increase of 2.6 days in the duration of the larval stage ($F=12.2750$, $p \leq 0.0013$) and reduced the pupal weight ($F=3.6754$, $p \leq 0.0103$). No difference between the treatments and controls was observed in the survival of insects during the pupal stage ($F = 1.1274$, $p \leq 0.3673$) and the sex ratio of adults ($F = 1.1274$, $p \leq 0.3673$) (Figure 2).

Water and Tween 80 (controls) showed values of 97.2 ± 6.80 and 94.4 ± 8.60%, respectively for the cumulative survival of insects in the larval stage, while *C. langsdorffii* leaf extract afforded values that ranged from 36.7 ± 18.26 to 80.5 ± 12.54% ($F = 11.1090$, $P \leq 0.0000$). Plant extracts also increased the duration of the larval stage to values ranging from 23.0 ± 1.09 to 30.9 ± 4.15 days ($F = 34.9780$, $p \leq 0.0000$), and reduced pupal weight to values between 197.2 ± 16.54 and 240.2 ± 14.9 mg ($F = 12.1270$, $p \leq 0.0000$). Nevertheless, no differences were observed for the survival in pupal stage ($F = 0.5778$, $p \leq 0.6814$) and sex ratio of adults ($F = 1.4430$, $p \leq 0.2493$) (Figure 3). Regarding the growth curve of larvae fed on diet containing the *C. langsdorffii* leaf extract, the contrast analysis allowed the formation of four groups. The first of them, formed by water and Tween 80 (controls), presented an average maximum weight of 0.4894 g, and the second group, comprising the extract at 300 and 600 µg/ml showed an average maximum weight of 0.2475 g. At 1,000 and 1,300 µg/ml, the extract reduced the maximum weight to 0.1414 and 0.0649 g, respectively. The extract of fruit peels was not so efficient since larvae

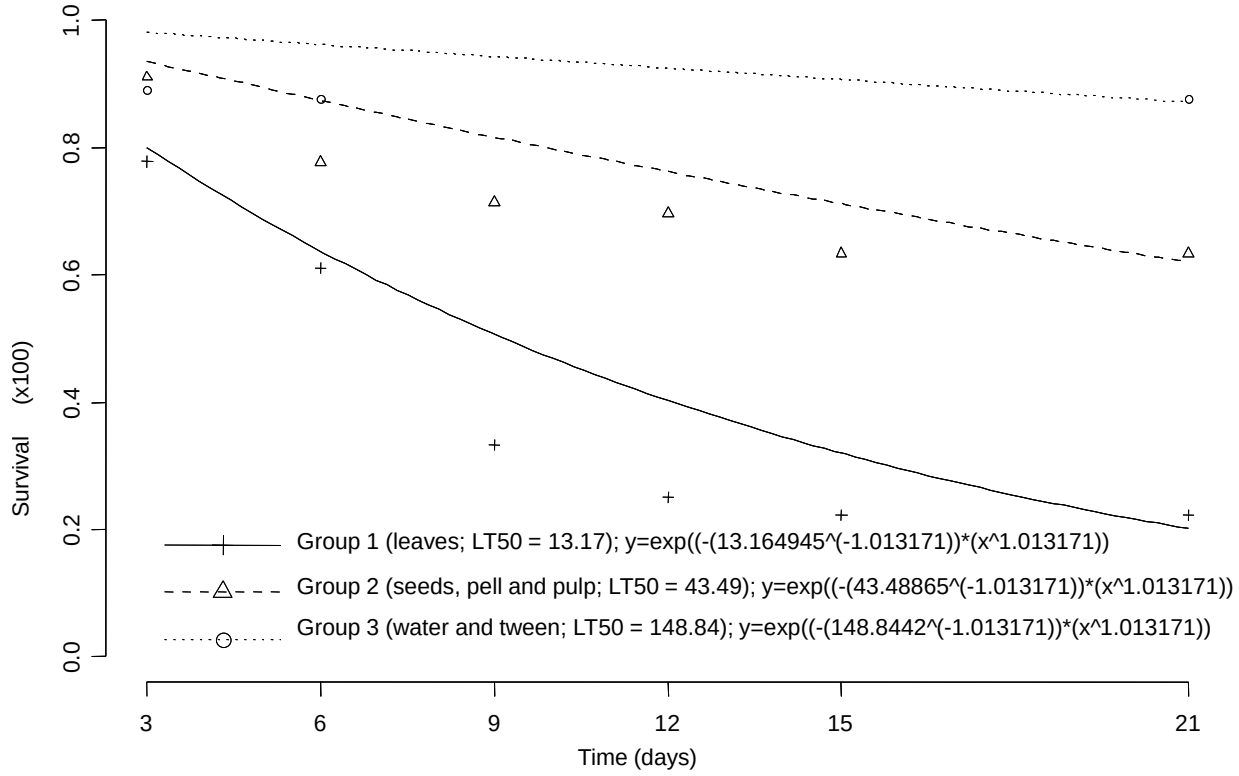

Figure 1. Survival over time of *Spodoptera frugiperda* first instar larvae fed with an artificial diet containing extracts from leaves and peels, seeds, and pulp of *Copaifera langsdorffii* fruits. y = exp((μ)^(-α)*(x^α), where y = survival; μ = LT50; α = 1.013171; and x = time (days).

Table 1. Means (±SE) for food consumption and weight of *Spodoptera frugiperda* caterpillars after seven days of exposure to diets containing extracts of *Copaifera langsdorffii.*

Treatment	Food consumption (mg)[1]	Weight of caterpillars (mg)[2]
Water	491.3 ± 23.65	56.3 ± 3.36[a]
C. langsdorffii – fruit peel	560.6 ± 17.20	12.4 ± 5.60[c]
C. langsdorffii – leaves[3]	-	-
C. langsdorffii – fruit pulp	596.4 ± 32.56	49.5 ± 8.46[a]
C. langsdorffii – fruit seed	525.9 ± 29.59	32.8 ± 6.75[b]
Tween 80-1%	514.0 ± 18.98	53.2 ± 4.98[a]
p <	0.0710	0.0001
F	2.7413	10.703

[1]These results are not significant. [2]Means followed by the same letter do not differ by the Scott-Knott test. [3]This treatment caused high mortality of larvae.

fed on diet containing such extract at 300 and 600 µg/ml showed an average maximum weight of 0.3934 g, while at 1,000 and 1,300 µg/ml the average maximum weight was 0.3303 and 0.2965 g, respectively (Figure 4).

Furthermore, larvae fed on a diet containing extracts of *C. langsdorffii* fruit peels at 1,000 and 1,300 µg/ml resulted in adults with pre-oviposition periods of 4.3 ± 0.96 and 3.5 ± 0.58 days, respectively while water and Tween 80 (controls) afforded values of 2.5 ± 0.57 and 2.8 ± 0.5 days, respectively ($F = 5.6571$, $p \leq 0.0026$). The longevity of males ($F = 1.3500$, $p \leq 0.2888$) and females ($F = 1.6848$, $p \leq 0.1892$) was not affected by the extract, but the oviposition period and the number of eggs per female were reduced in all concentrations evaluated. Egg viabilities were also reduced by the extracts, which afforded values from 56.8 ± 9.33 to 69.0 ± 6.28% of hatching. Even the lowest concentration of *C. langsdorffii* fruit peels extract tested was able to reduce *S. frugiperda* egg viability: treatment with the 300 µg/ml extract caused 56.8 ± 9.33% hatching, compared to 84.2 ± 9.86 and 83.1

Table 2. Means (± SE) for the duration of larval and pupal stages, pupal weight, and survival in the pupal stage of *Spodoptera frugiperda* first instar larvae fed with artificial diet containing extracts of *Copaifera langsdorffii*.

Treatment	Duration of larval stage (days)[1]	Duration of pupal stage (days)[2]	Pupal weight (mg)[2]	Survival in the pupal stage (%)[2]
Water	23.2 ± 0.24[b]	11.6 ± 0.24	308.5 ± 3.03	89.7 ± 4.72
C. langsdorffii – fruit peel	27.9 ± 1.53[a]	11.4 ± 0.35	310.0 ± 10.0	100.0 ± 0.00
C. langsdorffii – leaves[3]	-	-	-	-
C. langsdorffii – fruit pulp	26.0 ± 1.08[a]	14.9 ± 0.34	316.9 ± 9.81	100.0 ± 0.00
C. langsdorffii – fruit seed	26.1 ± 1.40[a]	12.2 ± 0.59	314.8 ± 5.08	76.3 ± 10.19
Tween 80 -1%	23.9 ± 0.29[b]	11.5 ± 0.58	304.8 ± 5.42	87.8 ± 5.81
p ≤	0.0001	0.2445	0.4195	0.0634
F	18.458	1.4135	0.6714	3.0278

[1]Means followed by the same letter, in each column, do not differ by the Scott-Knott test. [2] These results are not significant. [3]This treatment caused high mortality of larvae.

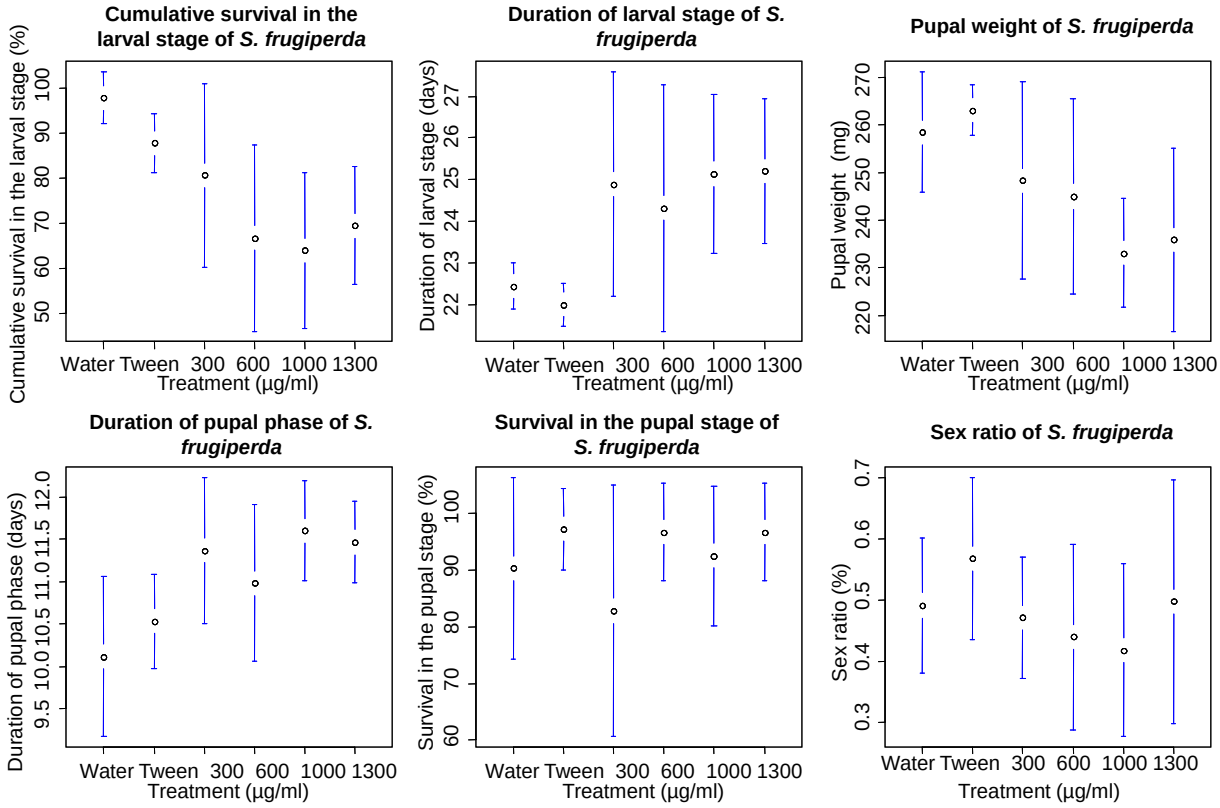

Figure 2. Mean (± SD) for cumulative survival and duration of the larval stage, pupal weight, duration and survival in the pupal stage, and sex ratio of *Spodoptera frugiperda* second instar larvae fed on an artificial diet containing extract from *Copaifera langsdorffii* fruit peels at 300, 600, 1,000 and 1,300 µg/ml. Water and Tween 80 were used as controls.

± 9.71%, observed in the controls, water and Tween 80, respectively (F = 4.6188, p ≤ 0.0043) (Figure 5).

The extract of *C. langsdorffii* leaves caused no changes in the pre-oviposition (F = 0.7143, p ≤ 0.5919) and oviposition periods (F = 0.7788, p ≤ 0.5520), as well as in the longevity of females (F = 2.4344, p ≤ 0.0809) and males (F = 1.6595, p ≤ 0.1988). However, females had reduced fertility (F =7.6818, p ≤ 0.0006), since values for water and Tween 80 were 1895.4 ± 285.99 and 1969.0 ± 518.20 eggs per female, respectively whereas for the extract they ranged from 1008.4 ± 489.86 to 1073.6 ± 179.40 eggs per female. Similarly, as observed for the extract from fruit peels, the smallest concentration of the extract of leaves that reduced egg viability was 300 µg/ml with 53.5 ± 2.75% hatching (F=13.6150, p ≤ 0.0000) (Figure 6).

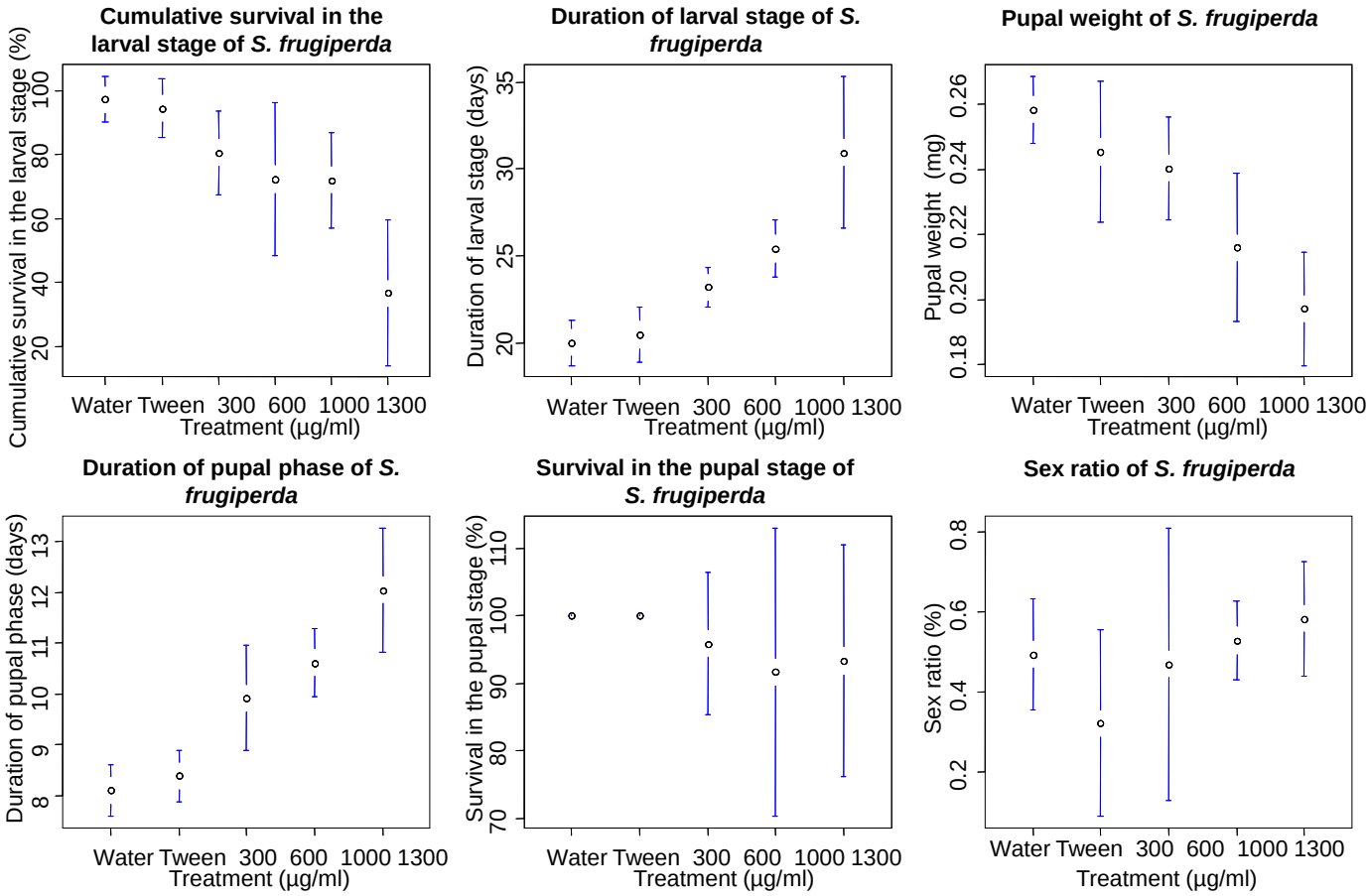

Figure 3. Means (± SD) for cumulative survival and duration of the larval stage, pupal weight, duration and survival in the pupal stage, and sex ratio of *Spodoptera frugiperda* second instar larvae fed on an artificial diet containing extract from *Copaifera langsdorffii* leaves at 300, 600 and 1,000 µg/ml. Water and Tween 80 were used as controls.

Ultrastructural analysis of eggs from *S. frugiperda*

Eggs from females originated from larvae fed on a diet containing extracts from *C. langsdorffii* leaves showed malformations of the eggshell in the micropylar and aeropylar region of the chorion (Figure 7). When the extract of fruit peels was used, cracks and abnormal deposition of material on the chorion of eggs were observed (Figure 8). These changes could be detected for both peel and leaf extracts at lowest concentration (300 µg/ml). Meanwhile, a search for correlation between egg abnormality and hatching was not carried out since the arrangement of *S. frugiperda* eggs in layers does not allow the quantitative determination of malformed eggs (Adamski et al., 2005).

Quantification of proteins in the feces of *S. frugiperda*

The amount of protein in the feces of *S. frugiperda* larvae increased with the concentration of the extract from copaiba fruit peels in the diet, ranging from 2.99 ± 0.09 to 4.32 ± 0.20 µg/mg ($F = 57.6000$, $p \leq 0.0000$), which are higher values than those observed for water and Tween 80 (controls). Similarly, there was a correlation between the concentration of copaiba leaf extract and protein concentration in the feces of insects ($F = 130.9$, $p \leq 0.0000$), which ranged from 2.83 ± 0.07 to 3.90 ± 0.14 µg/mg for insects treated with the plant extract, while for water and Tween 80 (controls), average values were 2.35 and 2.41 µg/mg ($F=0.1628$, $p \leq 0.6951$), respectively (Figure 9).

Determination of the trypsin inhibitory activity by extracts from leaves and fruit peels of *C. langsdorffii*

The extract from *C. langsdorffii* fruit peels at 75 to 1,000 µg/ml caused inhibition in the range of 4.7 to 37.7% in *S. frugiperda* trypsin activity ($F = 211.7000$, $p \leq 0.0000$). The leaf extract was a bit more active since concentrations in the range of 25 to 400 µg/ml caused 11.8 to 46.3% inhibition of the enzyme activity ($F = 158.3000$, $p \leq$

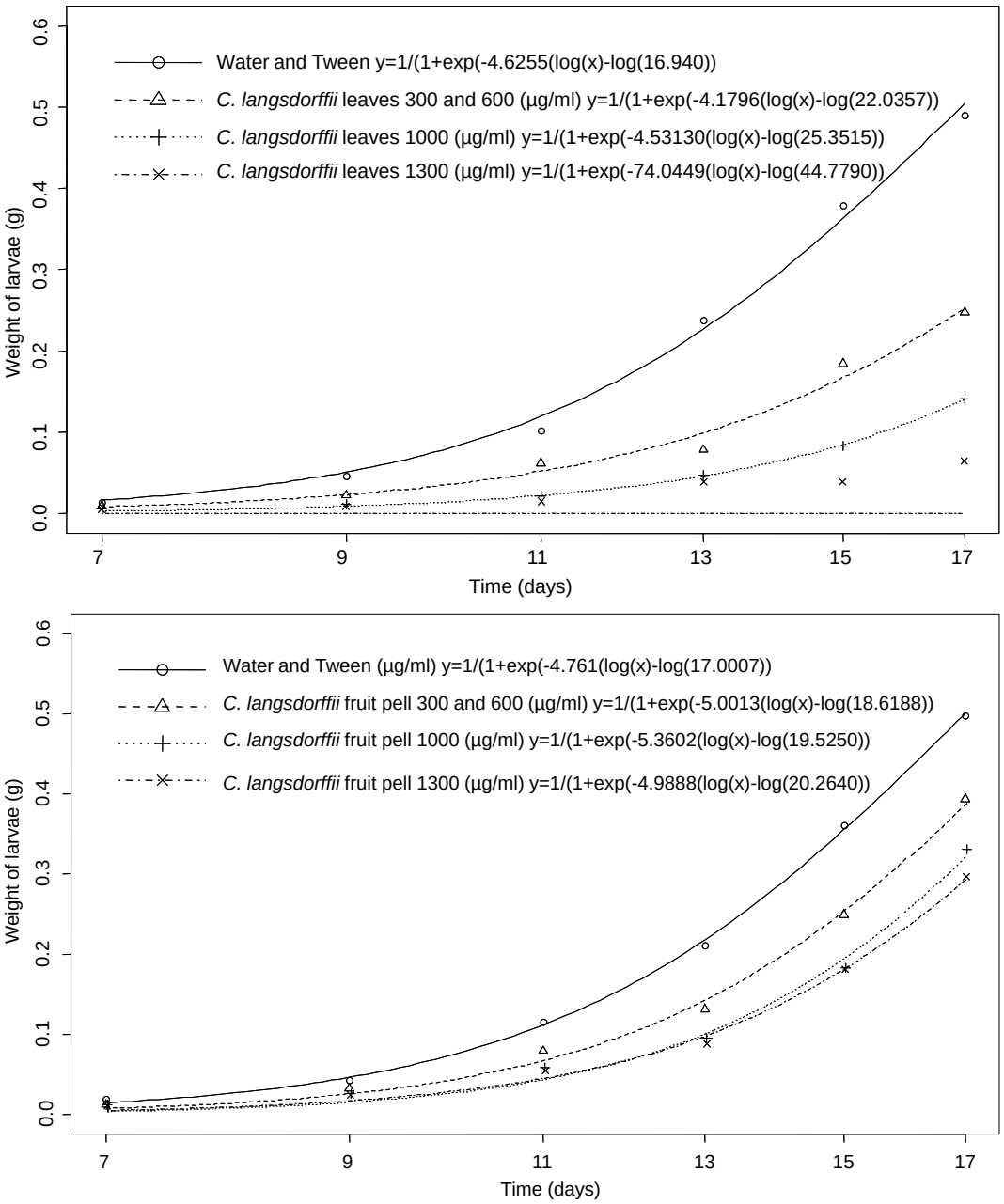

Figure 4. Growth curve of *Spodoptera frugiperda* second instar larvae fed on an artificial diet containing extracts from leaves and fruit peels of *Copaifera langsdorffii*. y = 1/(1+exp(b(log(x)–log(e)), where "b" and "e" are the intercepts and x is the time (days).

inhibition of the enzyme activity (F = 158.3000, p ≤ 0.0000) (Figure 10).

DISCUSSION

The increased mortality of *S. frugiperda* larvae fed on extract-containing diet is in accordance with the reported insecticidal activity by *C. langsdorffii* oil (Mendonça et al., 2005). Furthermore, other plant species belonging to the same genus also present insecticidal activity. *Copaifera reticulata* for example, produces the dipertenes-b-acetoxylabdan-8(17)-13-dien-15-oic acid and alepterolic acid, which reduced the survival of *Aedes aegypti* larvae (Geris et al., 2008). However, the effect of the extracts varies according to the plant part used in their preparation, and that obtained from leaves caused the highest mortality of *S. frugiperda* larvae. This result is in accordance with the described metabolic variation between plant parts (Silva et al., 2002; Harris et al., 2007;

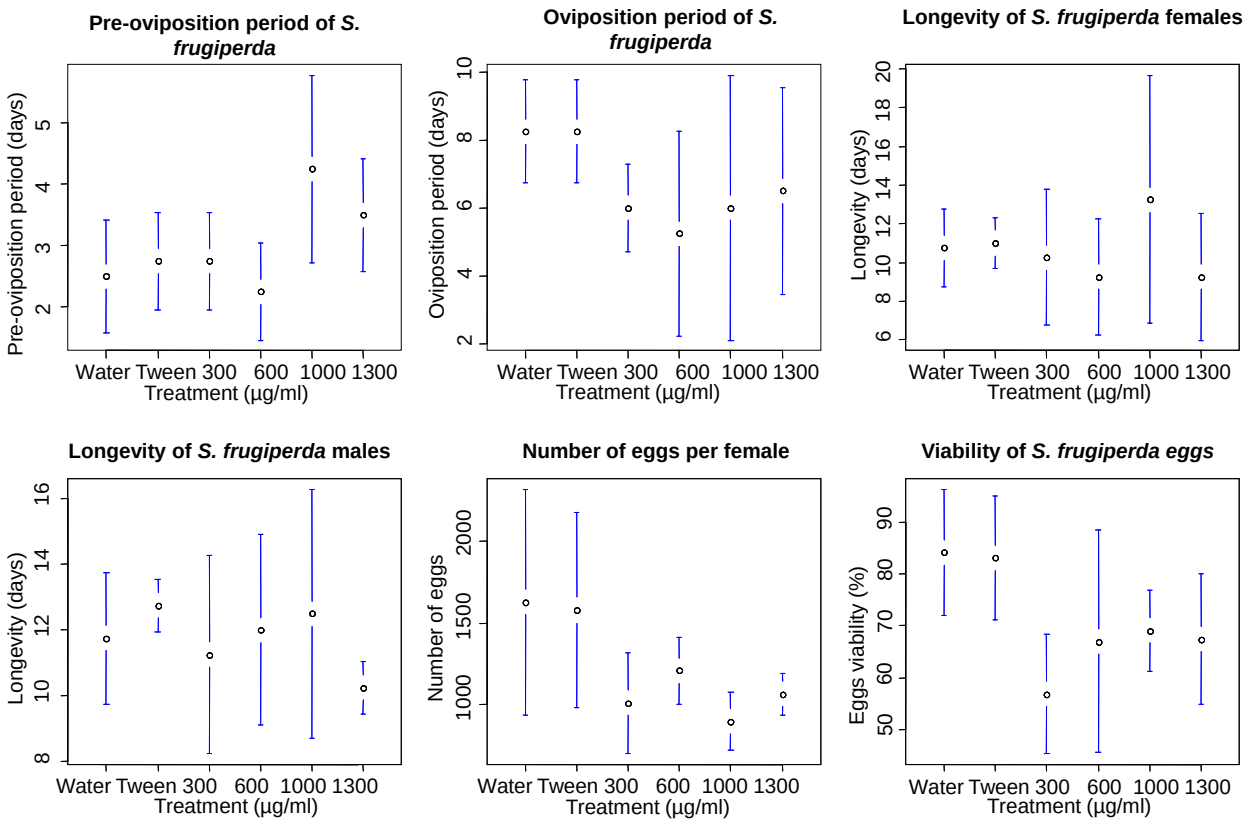

Figure 5. Means (± SD) for the periods of pre-oviposition and oviposition, longevity of females and males, number of eggs per female, and egg viability of adult *Spodoptera frugiperda* that was fed on a diet containing the extracts of *Copaifera langsdorffii* fruit peels at 300, 600, 1,000 and 1,300 µg/ml during the larval stage.

Sharma et al., 2008; Srivastava et al., 2008; François et al., 2009). In a study with *Citrus aurantium*, for example, the fruit peel extract was more active against adults of *Bactrocera oleae* than the extracts from the mesocarp and endocarp of the fruit (Siskos et al., 2007). Similarly, the extract from senescent *Melia azedarach* leaves was much more effective in controlling *A. aegypti* than the extract of fruits (Coria et al., 2008).

Although no reduction in food intake was observed, insect weights decreased for larvae fed on diet impregnated with the extracts of fruit peel and fruit seed. Apparently, the presence of substances with antibiosis properties in these extracts accounted for such reduction since countless reports can be found in the literature about antibiosis caused by ingestion of substances from natural origin, which affect the activity of digestive enzymes without causing any reduction in food intake (Farias-Rivera et al., 2003; Pavela et al., 2008). Among the classes of substances with this property, those able to inhibit proteinases, lipases and amylases deserve mention (Nathan et al., 2006; Kansal et al., 2008; Silva et al., 2009). *Plathymenia foliolosa* is an example, since a trypsin inhibitor was isolated from the seeds of this plant. This substance caused no change in the dietary intake of *Anagasta kuehniella*, but drastically reduced growth and delayed the development of this insect (Ramos et al., 2008).

Despite the reduction in caterpillar weight by the extracts of seeds, pulps, and peels of *C. langsdorffii* fruits, the corresponding pupa weight was not affected. However, the reduction in caterpillar weight may have caused an increase in the duration of the larval stage, which is disadvantageous to the insect since it increases exposure to natural mortality factors in field conditions. The lower toxicity of chemical agents to the pupa stage has already been reported by other authors, who attribute this behavior to the lower activity of the insect in such stage (Bell, 1978; Jbilou et al., 2006). Similar to what was observed in the present work, Medeiros et al. (2007) noticed an increase in the duration of the larval stage of *Ascia monuste orseis* after the treatment of this insect with the extract of *S. saponaria*, but no reduction in the corresponding pupa weights could be observed. Nevertheless, further studies should be conducted in order to determine the leaf damage caused by *S. frugiperda* after ingestion of plant extracts, since the increase in duration of larval stage does not nullify the deleterious effect of the extracts. Considering that this is a result that has been described in literature after the intake of secondary metabolites for *Spodoptera* spp., the

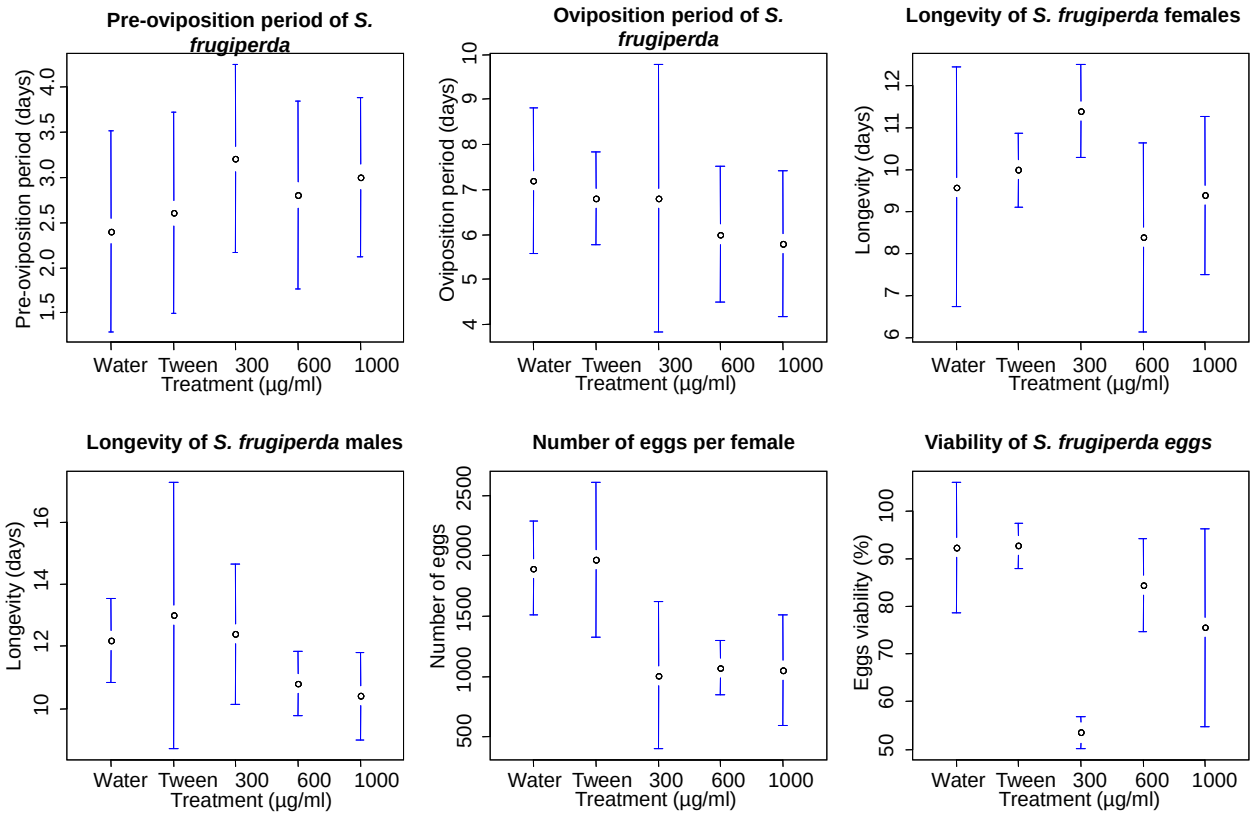

Figure 6. Mean (± SD) for the periods of pre-oviposition and oviposition, longevity of females and males, number of eggs per female, and egg viability of adult *Spodoptera frugiperda* that was fed on a diet containing the extracts of *Copaifera langsdorffii* leaves at 300, 600 and 1,000 µg/ml during the larval stage.

cause may be related to decreased of food intake, presence of enzyme inhibitors, or even with energy expended in metabolic detoxification (Sintim et al., 2009; Roel et al., 2010; Baskar et al., 2011).

Both extracts from leaves and fruit peels reduced the second instar larvae survival, but no significant relationship between their concentrations and the cumulative mortality was observed. These results are similar to those described by Mello et al. (2007), who studied the effect of *Pilocarpus spicatus* essential oil on nymphs of the fifth instar of *Rhodnius prolixus*. The authors could not suggest a relationship between the doses applied to the insect and their effect on mortality. The adverse effects on adults obtained from *S. frugiperda* larvae fed on diets containing the extracts from fruit peels and leaves certainly were caused by substances produced by *C. langsdorffii*. A similar result was described for the extract of *M. azedarach*, which caused an increase in the pre-oviposition period and reduced the oviposition period and the fertility of *Spodoptera littoralis* females (Schmidt et al., 1997). Also, worth mentioning is the reduction in the number of eggs laid by *S. frugiperda* after ingestion during the larval stage of fractions from the extract of *Ipomoea murucoides* (Curzio et al., 2009). Analogously, the fertility of *S. frugiperda* females was reduced after ingestion of proteinase inhibitors present in *Momordica charantia* (Telang et al., 2003).

With regard to structural changes in eggs from adults originating from larvae fed with the extracts of leaves and fruit peels of *C. langsdorffii,* similar results were reported by Fila et al. (2002). These authors also observed abnormalities in the eggs of *Spodoptera exigua* that were laid by adults of this insect that originated from larvae exposed to the commercial insecticide fenitrothion. Cracks around the micropylar and aeropylar regions were very similar to those observed in the present study. In order to understand the interaction between the inheritance of chorionic malformations and the insecticide resistance of *S. exigua*, Adamski et al. (2005) studied the eggs of this insect and observed deposition of abnormal material in the chorion that was very similar to that observed in the present study. Since the cement layer of the chorion is composed of lipoproteins and organophosphate pesticides have, in general, the ability to cause disturbances in protein metabolism, the authors suggested that disturbances in the cement glands of females caused by the pesticides, could explain chorion malformation. As the extract of *C. langsdorffii* is also capable of altering the protein metabolism in *S. frugiperda*, it is possible that metabolites produced by this

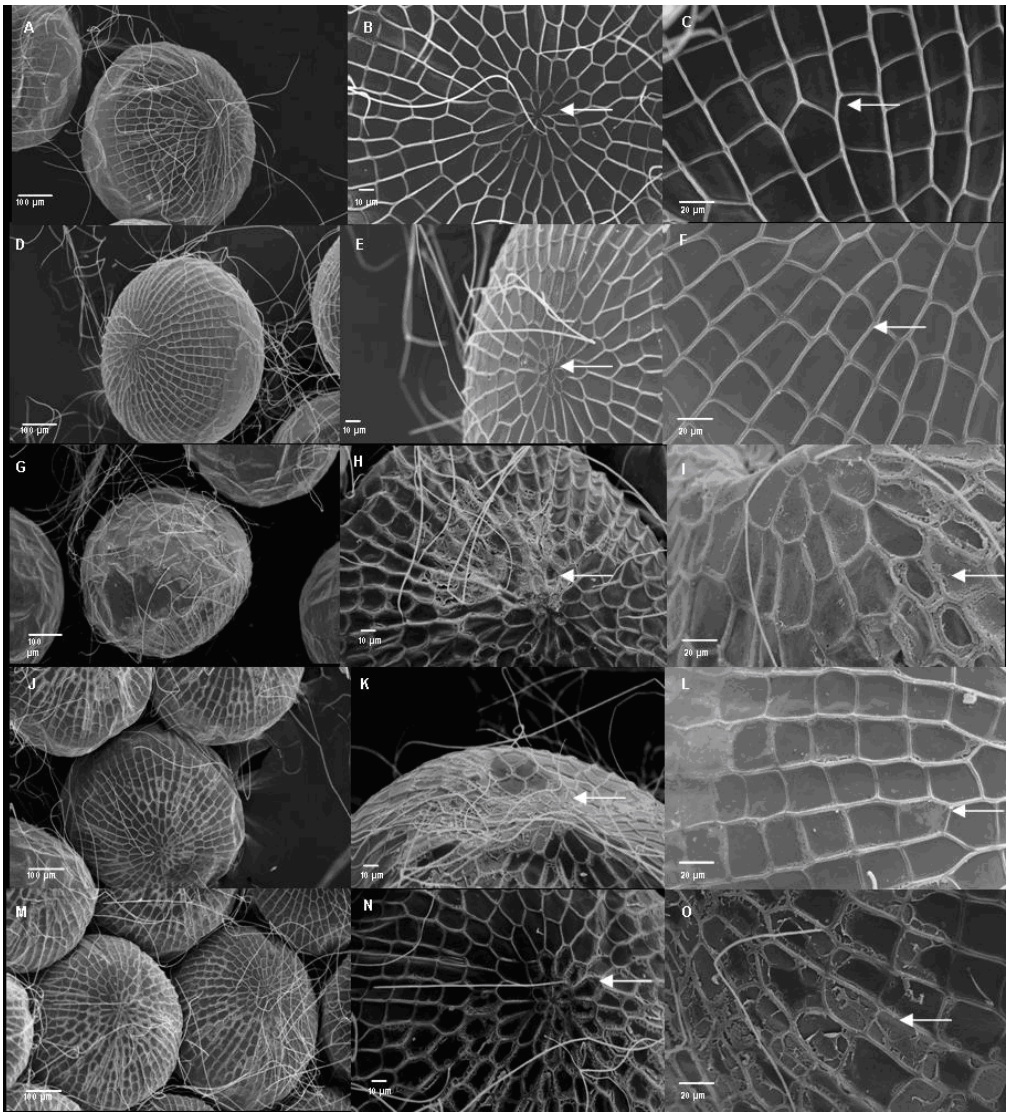

Figure 7. Scanning electron microscopy of eggs from adults of *Spodoptera frugiperda* whose larvae were treated with *Copaifera langsdorffii* leaf extracts. A, B and C: water (arrows indicate aeropylar and micropylar region without morphological changes); D, E and F: Tween 80 (arrows indicate aeropylar and micropylar region without morphological changes); G, H and I: extract of *C. langsdorffii* leaves at 300 µg/ml (arrows indicate aeropylar and micropylar region with morphological changes); J, K and L: extract of *C. langsdorffii* leaves at 600 µg/ml (arrows indicate aeropylar and micropylar region with morphological changes); M, N and O: extract of *C. langsdorffii* leaves at 1,000 µg/ml ppm (arrows indicate aeropylar and micropylar region with morphological changes).

plant are also affecting the cement glands of females. Thus, the active metabolite may alter protein metabolism, being perhaps a trypsin inhibitor member of the Kunitz family, which has been reported from *C. langsdorffii* (Krauchenco et al., 2004).

The increased amount of protein in the feces and the inhibition of trypsin activity also show that one mode of action of the extracts of *C. langsdorffii* against *S. frugiperda* is through the production of protease inhibitors, which can cause effect on insects very similar to those observed in this work (Leo and Gallerani, 2002; Gomes et al., 2005; Bhattacharyya et al., 2007). These results find support in the study by Krauchenco et al. (2001), in which an inhibitor of proteinases was purified from the seeds of *C. langsdorffii*. Similarly, Silva et al. (2001) characterized trypsin inhibitors in the seeds of *C. langsdorffii*. Although, no study has been conducted to purify the active substance(s) against *S. frugiperda* in this work, the results previously described show *C. langsdorffii* as a promising plant for the development of new products to control this insect. It is worth mentioning, that among the compounds of trypsin inhibitor member of

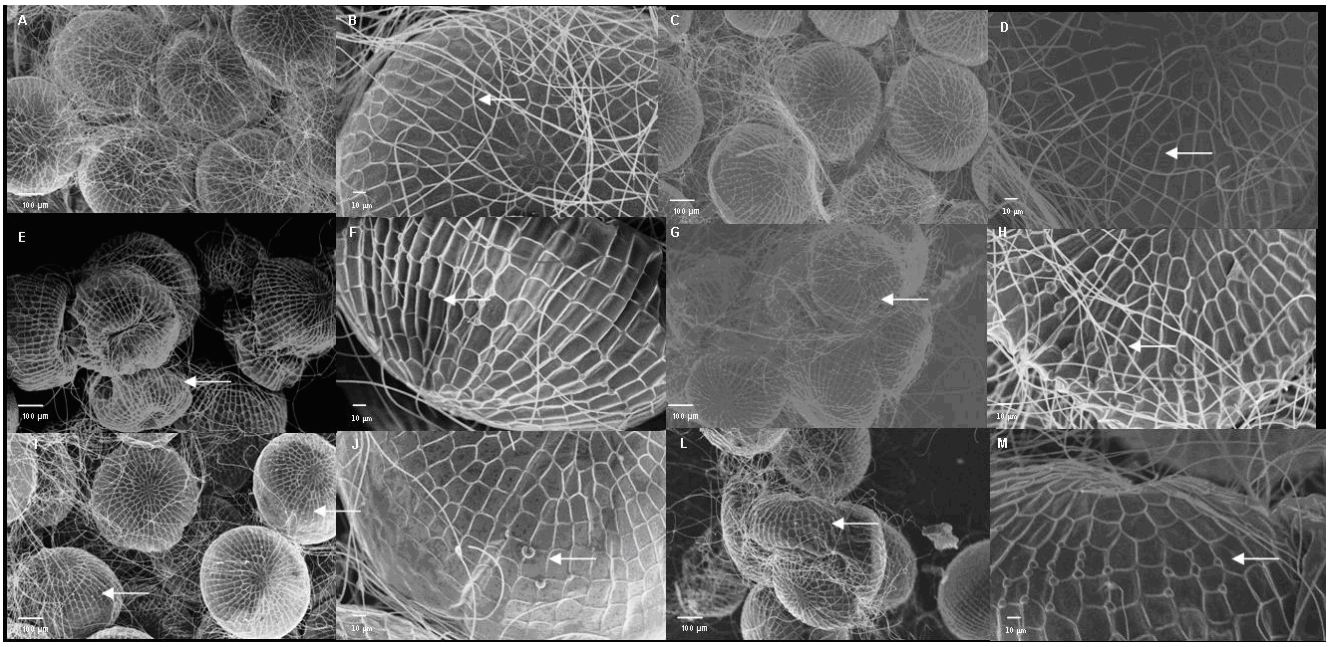

Figure 8. Scanning electron microscopy of eggs from adults of *Spodoptera frugiperda* whose larvae were treated with *Copaifera langsdorffii* fruit peel extracts. A and B: water (arrows indicate areas without morphologic changes); C and D: Tween 80 (arrows indicate areas without morphologic changes); E and F: extract of *C. langsdorffii* at 300 µg/ml (arrows indicate areas with morphological changes); G and H: extract of *C. langsdorffii* at 600 µg/ml (arrows indicate areas with morphological changes); I and J: extract of *C. langsdorffii* at 1,000 µg/ml (arrows indicate areas with morphological changes); L and M: extract of *C. langsdorffii* at 1,300 µg/ml (arrows indicate areas with morphological changes).

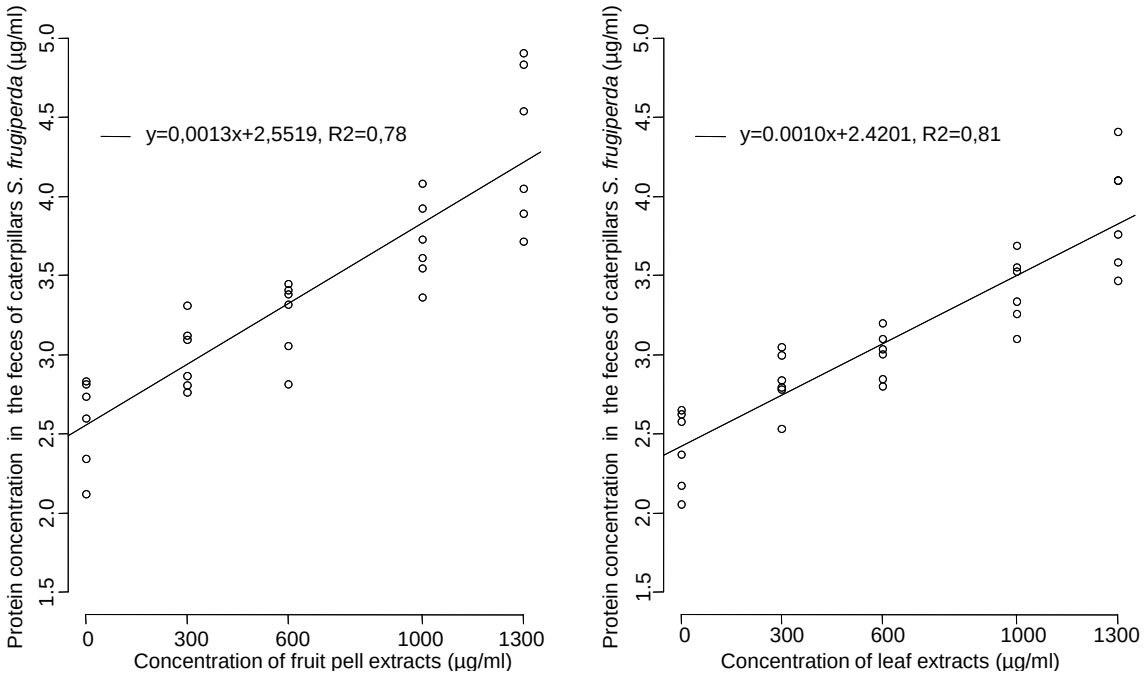

Figure 9. Proteins in the feces of *Spodoptera frugiperda* fed with extracts of *Copaifera langsdorffii* fruit peels and leaves.

Kunitz family isolated from *C. langsdorffii* (Krauchenco et al., 2004) are coumarins (Lima Neto et al., 2008) and caryophyllene (Souza et al., 2011), which possessed activity against *Spodoptera* spp. (Langenheim and Hall,

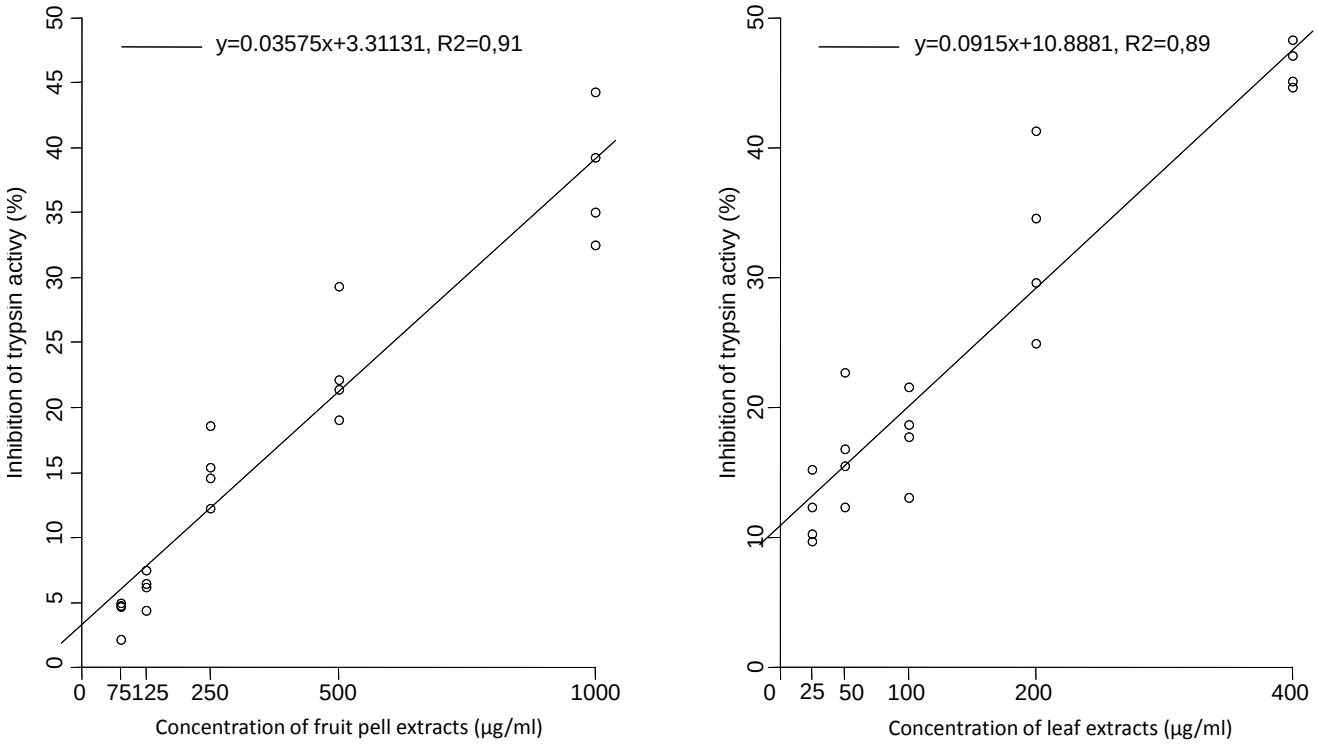

Figure 10. Inhibition of the trypsin from *Spodoptera frugiperda* by extracts from *Copaifera langsdorffii* leaves and fruit peels.

1983; Garcia et al., 2004; Vera et al., 2006).

In conclusion, *C. langsdorffii* is potentially useful for the development of new products to control *S. frugiperda* since the extracts of this plant reduced the survival of the fall armyworm. Furthermore, the extracts caused sublethal effects such as reduced growth, reduced fertility and fecundity, structural changes in the eggs laid by insects treated with the extracts during the larval phase, and changes in protein metabolism. However, since treatment with the extracts caused a prolonged larval stage, further studies should be conducted to evaluate whether this leads to an increase in the damage caused to the plant.

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