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Biochemical Responses of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) in Soybean Cultivars Sprayed with the Protease Inhibitor Berenil

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ABSTRACT: The damage caused by *Anticarsia gemmatalis* motivates this study on the adaptive mechanisms of the insect to soybean. The lipoxygenase pathway produces and releases jasmonic acid, involved in the regulation of the plant defense genes, which encodes protease inhibitor (PI) production. Three soybean cultivars IAC-18, IAC-24, and Foscarin-31 were sprayed with water and berenil, a synthetic inhibitor, at 0.60 and 1.0% (w/v) and then infested with *A. gemmatalis* larvae. The lipoxygenase (LOX) activity increased in the leaves of Foscarin-31, IAC-18, and IAC-24 by 87, 81, and 78%, respectively, after 24 h of *A. gemmatalis* damage. IAC-18 revealed the lowest increase in PI when compared to the other cultivars. Protease, amidase, and esterase activities in soybean larvae dropped drastically after berenil application. PIs may be included in the control strategies of *A. gemmatalis* in soybean by lowering the digestive enzyme activity in the larval midgut, thus affecting insect growth and development.

KEYWORDS: *Anticarsia gemmatalis, berenil, plant−insect interaction, protease inhibitors, protein entomotoxic*

INTRODUCTION

The constantly increasing global use of pesticides can cause serious environmental and human health problems. Entomotoxic proteins,[1] in particular plant defense products, such as digestive enzyme inhibitors and lectins, can be used in pest control,[2,3] because they can modify the food intake, triggering post-digestive changes. Chemical substances with post-digestion effects, including antinutritional effects by protease inhibitors (PIs), can lower the herbivore growth and/or survival rate.[4]

Jasmonic acid, an intermediate compound of the lipoxygenase (LOX) pathway, when produced and released, activates the defense genes that code for the PI production.[5] This indicates the importance of developing pest control programs by employing digestive enzymes and studying the sensibility of the pests to these inhibitors.

PI ingestion reduces protein degradation in the insect midgut and the amino acid availability for growth, development, and reproduction.[6,7] PIs may also indirectly affect insect development by increasing the production of digestive proteases to compensate for the lower amino acid levels.[8,9] Also, PIs are resistant to proteolysis and are active under various intestinal pH conditions. However, the insects can reduce or avoid the impact of PIs by producing proteases desensitized to them or increasing their food intake.[8,10]

Knowledge about digestive proteases is vital for the use of PIs in pest control. Serine proteases are active in the physiological processes, including the activation of protein digestion in melanization, antibacterial activity, and immune response in insects.[11,12] These compounds are the main digestive enzymes of Lepidoptera, including *Anticarsia gemmatalis*, a major pest of soybean.[13]

Berenil is a synthetic trypsin inhibitor with insecticide potential. The objective was to evaluate the biochemical responses of *A. gemmatalis*-infested plants of three soybean cultivars treated with berenil.

MATERIALS AND METHODS

Rearing and Damage by Insects. The *A. gemmatalis* eggs were obtained from the National Research Center of Soybean (CNPSo) in Londrina, Paraná (PR), Brazil, and kept in the Laboratory of Insects (LI) of the Department of Biochemistry and Molecular Biology (DBB) of the Federal University of Viçosa (UFV) at 25 ± 2 °C temperature and 70 ± 10% relative humidity. The *A. gemmatalis* larvae were incubated at 25 °C with a relative humidity of 60 ± 10% and photoperiod of 14 h and fed an artificial diet.[14]

At the V3 stage, soybean plants in the first trifoliate received the fourth instar larvae of *A. gemmatalis*. After 24 h, the first leaf of the three trifoliolar leaflets of each plant were collected and stored at −80 °C. The caterpillars were collected, and their midguts were removed for analysis.

Application of the Berenil Inhibitor on Plants. Seeds of the soybean varieties were obtained from the Agronomic Institute of Campinas (IAC), São Paulo, Brazil. Three soybean cultivars [*Glycine max* (L.) Merrill], Foscarin-31 (susceptible control), IAC-18 (moderate resistance), and IAC-24 (antibiosis resistance), were grown to the V3 stage without application of any products. Three soybean plants were placed per pot containing 4.0 kg of soil in a

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greenhouse. The experiment had a completely randomized design with three treatments in 1 × 1 × 1 m organza-covered cages containing six pots, each having three plants. These plants were sprayed with water and the adhesive spreader 0.01% Triton X-100 (control) or with an aqueous solution containing the inhibitor berenil (Sigma-Aldrich, St. Louis, MO) at 0.06 and 1.0% with a manual pressure sprayer nozzle. Larvae were put on the soybean plants at the time of spraying berenil to simulate field conditions. Plants removed from the cages for biochemical analyses were not returned to them.

Enzyme Extract from the Larval Midgut. Velvetbean caterpillars were dissected, and their midguts were removed and placed in 10^{-3} M HCl at 4 °C in 2 mL plastic tubes. The enzyme extract was obtained by cell disruption during 9 cycles of freezing in liquid nitrogen and thawed in a water bath at 37 °C.[9] Next, 1 mL fractions of the extracts were centrifuged in 2 mL plastic microtubes at 100000*g* for 45 min at 4 °C. The supernatant, containing soluble material, was removed and maintained at −20 °C for analysis. The precipitate containing the insoluble fraction of the membrane-bound enzyme was solubilized in 10 mL of 10^{-3} M HCl with 0.5% Brij 35 and centrifuged.[15] The supernatant collected was stored at −20 °C for analysis.

Determination of the Protein Concentration. Using 0.2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) as the standard, we obtained the protein concentration of the enzyme extract of *A. gemmatalis*.[16]

Determination of Protease Activity in *A. gemmatalis* Midgut. Protease activities were determined[17] using the substrate 2% (w/v) azocasein (Sigma-Aldrich, St. Louis, MO) in 0.1 M Tris-HCl buffer at pH 8.0. The reaction mixture, with 50 μL of substrate and 60 μL of enzyme extract, was incubated for 30 min at 37 °C. Then, with the addition of 240 μL of trichloroacetic acid (TCA) at 10% (w/v), the reaction was stopped. Next, the samples were homogenized by vortexing, maintained on ice for 15 min, and centrifuged at 8000*g* for 5 min at 25 °C to remove the precipitated protein. An aliquot of 240 μL of supernatant was poured into tubes containing 280 μL of 1 M NaOH. The protease activity was monitored in a spectrophotometer at 440 nm. The experiment was conducted with three replicates, in triplicates.

Determination of Amidolytic Activity in *A. gemmatalis* Midgut. The amidolytic activity was evaluated by employing the chromogenic substrate *N*-benzoyl-L-arginyl-*ρ*-nitroanilide (L-BApNA) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 1.2 mM at 25 °C in 0.1 M Tris-HCl buffer at pH 8.2 containing 20 mM $CaCl_2$.[18] The reaction mixture consisted of 0.5 mL of substrate, 0.5 mL of buffer, and 10 μL of enzyme extract. The initial rates of trypsin-like serine proteases were determined by the formation of the product *ρ*-nitroanilide, by measuring the increase in absorbance at 410 nm versus time (2.5 min) and using the molar extinction coefficient of 8800 M^{-1} cm^{-1} for the calculations. The experiments were performed with three replicates, in triplicates.

Determination of Esterolytic Activity in *A. gemmatalis* Midgut. The esterolytic activity was determined with the substrate *N*-*α*-*ρ*-tosyl-L-arginine methyl ester (L-TAME) (Sigma-Aldrich, St. Louis, MO) at a final concentration of 0.1 mM at 25 °C in 0.1 M Tris-HCl buffer at pH 8.2 containing 20 mM $CaCl_2$.[19] The initial rates of trypsin-like serine proteases were determined by the absorbance at 247 nm versus time (2.5 min) and using the molar extinction coefficient of 540 M^{-1} cm^{-1} for the calculations. The experiment was conducted with three replicates, in triplicates.

Obtaining Leaf Extracts. A crude extract of soybean leaves was prepared at 4 °C.[20] The leaves were weighed, frozen in liquid nitrogen, and crushed using a mortar. The powder obtained was homogenized in 50 mM sodium phosphate buffer at pH 6.5 in a ratio of 1:3 (w/v) and centrifuged at 17200*g* for 60 min at 4 °C according to the modified methodology.[21] From the supernatant or crude extract, the total protein concentration, LOX activity, and concentration of PIs were determined.

Determination of LOX Activity. The action of LOX on linoleic acid (Sigma-Aldrich, St. Louis, MO) was determined by the formation of the conjugated double-bond system of hydroperoxide formed by the increase in the absorbance at 234 nm.[22] The substrate was prepared from a stock solution of 10 mM sodium linoleate. The LOX activity was determined in the reaction mixture containing 1.0 μL of crude sap extract and 4.0 μL of the sodium linoleate stock solution in 1.0 mL of 50 mM sodium phosphate buffer at pH 6.5, and the initial rates were determined by measuring the absorbance at 234 nm versus time (2.5 min) using a molar extinction coefficient of 25 000 M^{-1} cm^{-1} for the calculations. The experiment was conducted with three replicates, in triplicates.

Determination of the PIs. The PI in the crude extract of soybean leaves was determined using bovine trypsin (Sigma-Aldrich, St. Louis, MO).[23] In the presence of inhibitors, the trypsin activity was determined with 50 μL of leaf extract, 500 μL of 0.1 M Tris-HCl at pH 8.2 containing 20 mM $CaCl_2$, and 50 μL of 4.7×10^{-5} M trypsin solution in a test tube. The control of the enzyme was 550 μL of 0.1 M Tris-HCl at pH 8.2 containing 20 mM $CaCl_2$ and 50 μL of 4.7×10^{-5} M trypsin solution added in another test tube. Mixtures of tubes (test and control enzyme) were incubated for 5 min at 25 °C. After incubation, 500 μL of the incubation mixtures, test and control, was removed and placed in another tube containing 500 μL of 0.1 M Tris-HCl at pH 8.2 with 20 mM $CaCl_2$ and 500 μL of solution of 1.2 mM L-BApNA. The absorbance of each solution was determined at 410 nm for 2.5 min. The analyses were performed with three replicates. The results were converted to milligrams per gram of trypsin-inhibited protein, employing the equation: milligrams of trypsin inhibited per gram of protein $A \times B/C \times 1000 \times P$, where A is the absorbance at 410 nm of the control minus the absorbance at 410 nm of the sample, B is the sample dilution, P is the concentration in grams per milliliter of the protein extracts, and C is 0019 (trypsin factor, i.e., the product of the action of 1 mg of active trypsin L-BApNA substrate resulting in a reading absorbance at 410 nm of 0.019).

Statistical Analyses. The model chosen was based on increasing the determination correlation coefficient (adjusted R^2) in relation to the complexity of the model, relative simplicity, and high F values (and mean squares) using two-dimensional (2D) table curve. The statistical analyzes were performed using the Statistical Analysis System (SAS).[24]

RESULTS

The LOX in the leaves of the three soybean cultivars (Figure 1) showed no activity at time zero (beginning of the attack), with an increase in values after 24 h of damage by *A. gemmatalis*. However, Foscarin-31 (susceptible standard) showed a higher percentage of increase over time 0 h, at 87%, followed by IAC-18 and IAC-24, at 81 and 78%, respectively. The larval damage caused by this pest did not affect the LOX activity in cultivars.

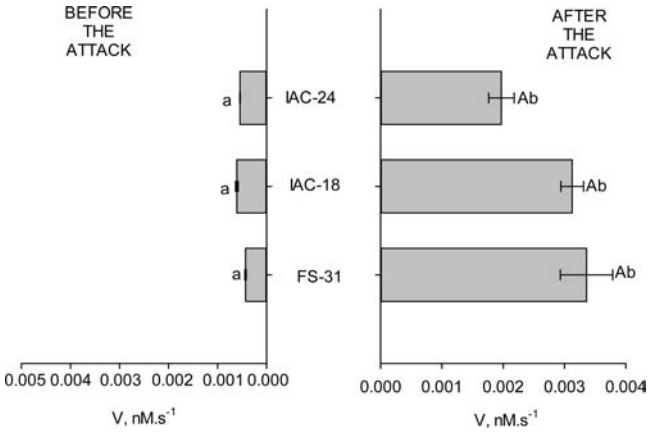

Figure 1. LOX activity in the leaves of the soybean cultivars IAC-24, IAC-18, and Foscarin-31 before and after damage by *A. gemmatalis* (Lepidoptera: Noctuidae) larvae. Means (n = 3) followed by the same lowercase letter horizontally and the same capital letter vertically show no difference at 5% probability by Tukey's test.

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The leaves of three cultivars showed significantly increased production of the PIs after insect damage ($p < 0.05$) (Figure 2).

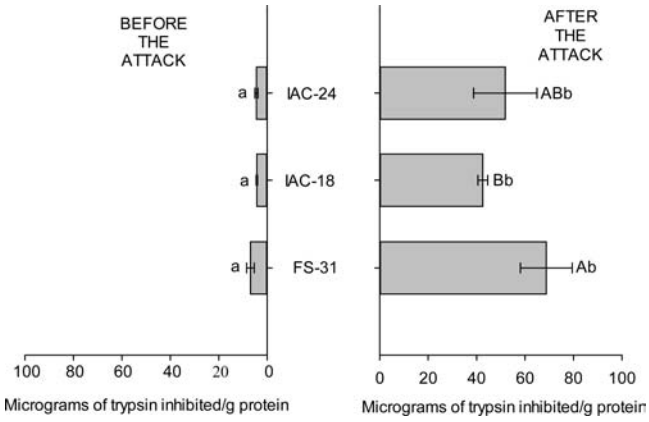

Figure 2. Levels of PIs in the leaves of soybean cultivars IAC-24, IAC-18, and Foscarin-31 before and after damage by *A. gemmatalis* (Lepidoptera: Noctuidae) larvae. Means ($n = 3$) followed by the same lowercase letter horizontally and the same capital letter vertically show no difference at 5% probability by Tukey's test.

Significantly lowered PI production was seen in IAC-18 ($p < 0.05$), while the susceptible Foscarin-31 revealed a value not significantly different ($p > 0.05$) to IAC-24, the cultivar with antibiosis resistance.

The total protease activity in the midgut of *A. gemmatalis* larvae fed Foscarin-31 and IAC-24 cultivars without the application of the inhibitor berenil was found to be similar, i.e., not significantly different ($p > 0.05$). However, the proteolytic activity in larvae fed with IAC-18 was approximately 65% lower ($p < 0.05$) when compared to the other two cultivars (Figure 3). Berenil, at concentrations of 0.6 and 1%, reduced protease activity in the larval midgut (>90%) in all three cultivars. The application of synthetic inhibitor superimposed the intrinsic effect of the cultivars used.

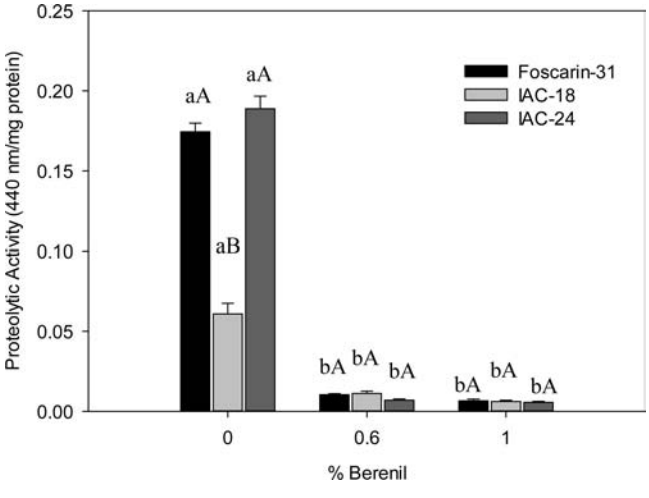

Figure 3. Protease activity in the midgut of *A. gemmatalis* (Lepidoptera: Noctuidae) larvae fed soybean leaves sprayed with PI berenil. The same capital letters show no difference at 5% probability by Tukey's test between cultivars with the same dose of berenil ($n = 18$). The same lowercase letters show no difference at 5% probability by Tukey's test with different doses of berenil in the same cultivar ($n = 18$).

The amidolytic (Figure 4) and esterolytic (Figure 5) activities in the midgut of *A. gemmatalis* larvae fed with the

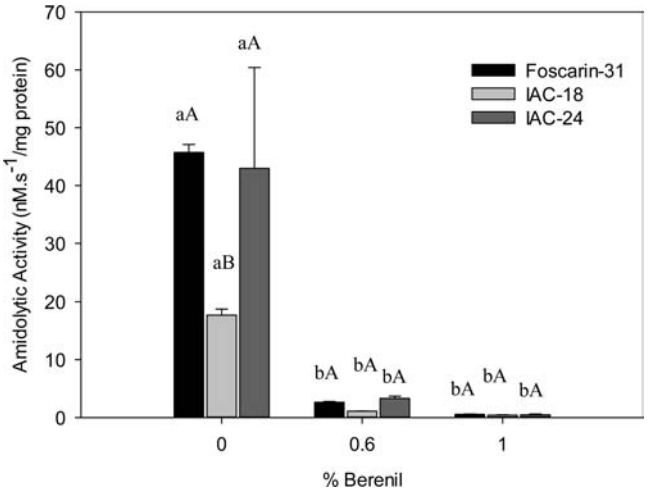

Figure 4. Amidolytic activity in the midgut of *A. gemmatalis* (Lepidoptera: Noctuidae) larvae fed soybean leaves sprayed with the PI berenil. The same capital letters show no difference at 5% probability by Tukey's test between cultivars with the same dose of berenil ($n = 18$). The same lowercase letters show no difference at 5% probability by Tukey's test with different doses of berenil in the same cultivar ($n = 18$).

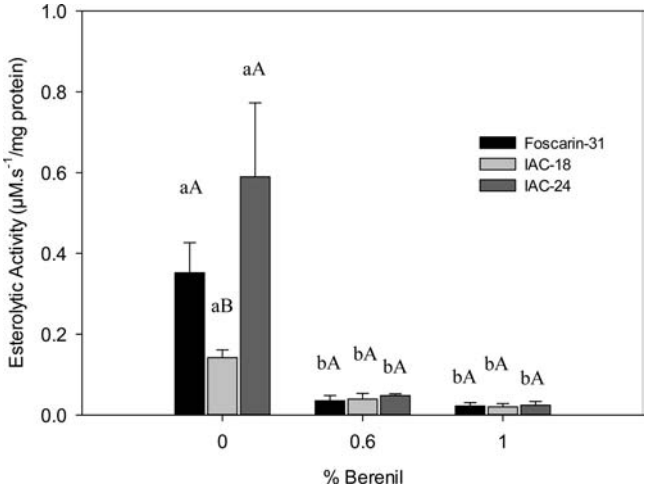

Figure 5. Esterolytic activity in the midgut of *A. gemmatalis* (Lepidoptera: Noctuidae) larvae fed soybean leaves sprayed with the PI berenil. The same capital letters show no difference at 5% probability by Tukey's test between cultivars with the same dose of berenil ($n = 18$). The same lowercase letters show no difference at 5% probability by Tukey's test with different doses of berenil in the same cultivar ($n = 18$).

Foscarin-31 (susceptible) and IAC-24 (antibiosis resistance type) cultivars were similar after 24 h of damage but possessed higher values than that of IAC-18. The two doses of berenil inhibited the trypsin-like activity in all cultivars by more than 90%.

DISCUSSION

Plants have the ability to modify their protein expression after injury by abiotic and biotic factors. For the production of

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defense proteins for protection against pests, the major biomolecules related to plant injuries are stimulated.[25]

The higher LOX activity in the leaves of the cultivars Foscarin-31, IAC-18, and IAC-24 after 24 h of damage by *A. gemmatalis* larvae is due to increased LOX activity, which activates the LOX route as a plant defense mechanism. This increased expression of LOX matches those detected in the plant leaves during or after stress, including mechanical injuries,[26] damage by herbivores,[27,28] and pathogen infection.[29]

The increased PI level in all three soybean cultivars after damage by *A. gemmatalis* larvae was found to be related to jasmonic acid, a product of the LOX pathway related to the induction of the PI genes. The synthesis of the PIs in tobacco leaf cells by jasmonic acid was preceded by increased LOX activity;[30] also, the soybean leaves attacked by the pest or mechanical injury showed higher PI levels.[5] However, the lower PI levels noted in IAC-18 than in the susceptible Foscarin-31 may indicate a defense mechanism and production of a differentiated PI on the first cultivar with a higher efficiency of inhibiting the larval digestive enzymes in the midgut. Guava leaves showed a higher protein concentration and lower PI after 48 h of infestation by *Thyrinteina leucoceraea*, Rindge (Lepidoptera: Geometridae), which is related to the production of other defense proteins in response to herbivore damage.[31]

The total protease activity and trypsin-like similarities in the substrates L-BAρNA and L-TAME of *A. gemmatalis* larvae, without spraying berenil, fed with Foscarin-31 (the susceptible standard) and IAC-24 (the antibiosis-resistant species) produce a similar pattern of response to PI production. Antibiosis is characterized by the deleterious effects in insects caused by plants, including increased mortality during the young stages and a reduction of emergence, size, weight, fecundity, sex ratio, and lifetime of adults.[32] IAC-24 possesses antibiosis resistance to defoliators and sucking insects, as demonstrated by the low viability of the larva, pupa, and egg, deformities in the adults, and fewer eggs per female of *A. gemmatalis*.[33]

The decrease in the digestive enzyme activity in the midgut of *A. gemmatalis* by two concentrations of berenil shows that this insect was affected by the negative effect of this synthetic inhibitor. Berenil, having a diazo bond between two amino benzamidinic rings, is a potent, partly competitive inhibitor, with trypsin-like parabolic behavior. Berenil binds to the active center as a competitive trypsin inhibitor as well as its secondary active site, exhibiting a partially competitive behavior with the substrate.[34,35] *A. gemmatalis* larvae had lower survival and proteolytic activity with increasing doses of berenil on the artificial diet of insects, besides increased consumption and protein digestion.[36] The inhibitory action of PI is attributed to interference in the protein digestion, decreasing the availability of amino acids and proteins, thereby reducing growth, development, and reproduction of insects.[15,9]

Plants have the ability to develop defense mechanisms to reduce damage by herbivores. However, the resistance mechanisms of IAC-18 and IAC-24 differed from each other, warranting a further study of entomotoxic compounds produced by these cultivars and their involvement in plant response to insects. The digestive enzymes of *A. gemmatalis* were found to be sensitive to the berenil inhibitor.

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Notes

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