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Active response of soybean to defoliator *Anticarsia gemmatalis* Hübner: strategies to overcome protease inhibitor production

Respuesta activa de las plantas de soya contra el defoliador Anticarsia gemmatalis *Hübner: estrategias para sustituir la producción de inhibidores de las proteasas*

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ABSTRACT

Endogenous mechanisms of plant resistance to herbivorous insects could be adapted to enable the development of alternative strategies for pest control. Activation of the lipoxygenase (LOX) pathway by jasmonic acid, the hormone responsible for activating genes encoding protease inhibitors (PI), is one of the main direct defenses of plants against insects. In this study, soybean cultivars at the V3 stage that were either susceptible or resistant to herbivorous insects were used to investigate changes in the activity of LOX and concentration of PIs in response to 24- or 48-h feeding by fourth and fifth instar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae. LOX activity (0.0-0.007 nm s^{-1}/mg) and the PI concentration (60 to 125 mg of trypsin inhibitor / mg of protein and 90 to 120 mg of trypsin inhibitor / mg protein after 24 and 48 h after attack, respectively) both increased in response to damage caused by *A. gemmatalis*. Proteolytic activity decreased by approximately 50% in cultivars with different degrees of resistance. Raised enzyme profiles in the midgut of *A. gemmatalis* may be related to tryptic enzymes that increased, compensating for the inhibition of proteases by entomotoxic substances of the soybean cultivars.

Key words: plant defense, lipoxygenases, velvet caterpillar.

RESUMEN

Los mecanismos de resistencia endógenos de las plantas contra insectos herbívoros pueden permitir el desarrollo de estrategias alternativas para el control de plagas. La activación de la vía de las lipoxigenasas con la producción de ácido jasmónico, hormona responsable por la activación de los genes inhibidores de proteasa, es una de las principales formas de defensa directa de las plantas contra los insectos. En el presente estudio, cultivares de soya en estadio V3, susceptibles y resistentes a insectos herbívoros fueron utilizadas para investigar las alteraciones en la actividad de LOX y la concentración de PIs, en respuesta al ataque de orugas en cuarto y quinto instar de Anticarsia gemmatalis *(Lepidóptera: Noctuidae) por 24 y 48 horas. La actividad de LOX (0,00 a 0,007 nm s^{-1}/mg) y la concentración de inhibidores de proteasa (60 a 125 mg de inhibidor de tripsina/ mg de proteína y 90 a 120 mg de inhibidor de tripsina/mg de proteína después de 24 y 48 horas del ataque respectivamente) aumenta en respuesta a los daños ocasionados por* A. gemmatalis*. La actividad proteolítica disminuyó aproximadamente en 50% en los cultivares con diferentes grados de resistencia. El aumento del perfil enzimático del intestino medio de* A. gemmatalis *puede estar relacionado al aumento de las enzimas trípticas para compensar la inhibición de proteasas por sustancias entomotóxicas de los cultivares de soya.*

Palabras clave: *defensa de plantas, lipoxigenasas, oruga-de-soja.*

Introduction

Pests can significantly limit the productive potential of soybean throughout its life cycle. *Anticarsia gemmatalis* Hübner, 1818 larva (Lepidoptera: Noctuidae), one of the main pests of soybean, can consume 90 cm^2 of leaves, equivalent to 2.1 times their weight, every 24 h during the larval period (Miklos *et al.*, 2007).

Host plant resistance is compatible with other methods of pest management, because it can impact the biology and/or behavior of herbivorous insects

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negatively (Dunse *et al*., 2010a). However, these insects can evolve defense strategies to use or metabolize the toxic substances produced by the plants. This coevolution biochemistry shows how a toxic plant can defend itself and how phytophagous insects can overcome these defenses by detoxifying or sequestering the plant toxins (Michereff *et al.*, 2014). Using cultivars with a moderate level of resistance to herbivores and that have a positive effect on natural enemy attraction, it would be possible to keep pests at low infestation levels without causing economic damage to the producer, thus contributing to the ecological sustainability of agricultural systems (Michereff *et al.*, 2014).

Endogenous mechanisms of plant resistance to insect herbivores are alternative strategies for controlling pests (Dunse *et al.*, 2010b; Scott *et al.*, 2010). The production of jasmonic acid in the lipoxygenase (LOX) pathway can activate genes encoding protease inhibitors (PI), which is a direct defense mechanism of plants against herbivorous insects (Farmer & Ryan 1992; Shivaji *et al.*, 2012). PI are promising for plant defense, because when ingested they block digestive proteases in the insect intestine. This limits the release of amino acids from the diet and affects the synthesis of proteins required for insect growth, development and reproduction (Zhu-Salzman & Zeng 2008).

The aim of this study was to determine the biochemical aspects and development of *A. gemmatalis* and the physiological responses to five soybean cultivars with different degrees of resistance to insects.

Materials and Methods

The experiments were performed in the Laboratory of Enzymology, Biochemistry of Proteins and Peptides of the Institute of Biotechnology Applied to Agriculture (BIOAGRO) and in the Entomology Laboratory (LCI) of the Department of Biochemistry and Molecular Biology (DBB) of the Federal University of Viçosa (UFV) in Viçosa, Minas Gerais, Brazil.

Rearing the insects

Eggs of *A. gemmatalis* were obtained from the National Research Center of Soybean (CNPSo) in Londrina, Paraná State, Brazil and kept in the LCI of the DBB/UFV at 25 ± 2 ºC and 70 ± 10% relative humidity (RH). Larvae *A. gemmatalis* were kept in an incubator at 25 ºC, 60 ± 10% RH and a 14-h photoperiod, and fed an artificial diet (Hoffman *et al.*, 1985).

Soybean seedlings

Seeds of soybean cultivars [*Glycine max* (L.) Merrill] were obtained from the Agronomic Institute of Campinas (IAC) of São Paulo State, Brazil. The soybean varieties used were as follows: IAC-PL1, which is susceptible to insects, and IAC-17, IAC-18, IAC-19 and IAC-24, which show different levels of resistance to insects (Lourenção *et al.*, 1997). Seedlings were grown in 4.0-kg pots with soil to the V3 stage, with no foliar application of any product, and with three soybean plants per greenhouse. The first three trifoliate leaves of each plant were collected, frozen in liquid nitrogen and stored at -80 ºC until they were used to evaluate LOX activity and PI production.

Damage by *A. gemmatalis* larvae

The V3-stage soybean plants were subjected to damage by fourth- and/or fifth-instar *A. gemmatalis* larvae. One larva was placed on the first trifoliate of each plant. After 24 h and 48 h, the three leaflets of the first trifoliated leaf were collected, frozen in liquid nitrogen and stored at -80 ºC for use in obtaining the plant extracts. Larvae were then taken off the plants and their midguts removed for enzymatic analysis.

Enzyme extracts of the larva midgut

The midguts of *A. gemmatalis* larvae were extracted after being dissected and placed in HCl 10^{-3} M at 4 ºC in 2 mL plastic tubes. The enzyme extract was obtained by cell disruption during nine cycles of freezing in liquid nitrogen and thawing in a water bath at 37 ºC. After the cycles, fractions of 1 mL of the extract were centrifuged in 2 mL plastic tubes with caps at 100,000 *g* for 30 min at 4 ºC. The supernatant containing soluble material was removed and kept at –18 ºC until the protein concentration and enzymatic activity was determined.

Determination of protein concentration

The protein concentration of the enzyme extract of *A. gemmatalis* was obtained (Bradford, 1976),

using bovine serum albumin (BSA) 0.2 mg/mL as standard.

Protease activity in the *A. gemmatalis* midgut

Protease activity was determined using the substrate azocasein 2% (w/v) in Tris-HCl 0.1 M pH 8.0 (Tomarelli *et al.*, 1949) in a reaction mixture of 50 µL substrate and 60 µL enzyme extract, which was then incubated for 30 min at 37 ºC. The reaction was stopped by adding 240 µL trichloroacetic acid 10% (w/v). The samples were then homogenized by vortexing and maintained at rest on ice for 15 min, followed by centrifugation at 8000 *g* for 5 min at 25 ºC to remove the precipitated protein. An aliquot of 240 µL of supernatant was transferred to tubes containing 280 µL NaOH 1 M. Protease activity was monitored in a spectrophotometer at 440 nm. The experiment was conducted in triplicate.

Amidase and Esterase activity in the *A. gemmatalis* midgut

Amidase activity was evaluated using the chromogenic substrate N-α-benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BApNA) at a final concentration of 1.2 mM at 25 ºC in Tris-HCl 0.1 M pH 8.2 containing 20 mM $CaCl_2$ (Erlanger *et al.*, 1961). The reaction mixture comprised 0.5 mL substrate, 0.5 mL buffer and 10 µL enzyme extract. Initial rates of trypsin-*like* serine proteases were determined by the formation of *p*-nitroanilide by measuring the absorbance increase at 410 nm over 2.5 min, using a molar extinction coefficient of 8800 $M^{-1}.cm^{-1}$ for the calculations. The experiments were performed in triplicate.

Esterase activity was assessed using the substrate N-α-*p*-tosyl-L-arginine methyl ester (L-TAME) at a final concentration of 0.1 mM at 25 ºC in Tris-HCl 0.1 M pH 8 containing 20 mM $CaCl_2$ (Hummel, 1959). Initial rates of trypsin-*like* serine proteases were determined by measuring the absorbance at 247 nm over 2.5 min using a molar extinction coefficient of 540 $M^{-1}.cm^{-1}$ for the calculations. The experiment was conducted in triplicate.

Cysteine protease activity in the *A. gemmatalis* midgut

The activity of cysteine proteases was determined by adapting the method of Erlanger *et al.* (1961) (Mendonça *et al.*, 2012). The reaction mixture included 0.5 mL Tris-HCl 0.1 M pH 8.0, 1 mM dithiothreitol, 10 µL enzyme extract and 0.1 mL of a 10 mM benzamidine inhibitor, which was then incubated for 15 min at room temperature. To the mixture was added 0.5 mL L-BApNA to a final concentration of 1.2 mM. Initial rates of cysteine proteases were determined by measuring the absorbance at 410 nm versus time (2.5 min) using a molar extinction coefficient of 8800 $M^{-1}.cm^{-1}$ for the calculations. The experiment was conducted in triplicate.

Leaf extract

Crude leaf extract was prepared at 4 ºC by weighing soybean leaves, which were then immediately frozen in liquid nitrogen and crushed in a mortar. The powder obtained was homogenized in a 50 mM sodium phosphate buffer, pH 6.5 at a ratio of 1:3 (w/v) and centrifuged at 17,200 *g* for 60 min at 4 ºC, following modifications of the methodology of Batista *et al.* (2002). The total protein concentration, LOX activity and the concentration of PI were determined in the supernatant, named the 'crude extract'.

LOX activity

The activity of LOX on linoleic acid was determined in a stock solution of 10 mM sodium linoleate containing 1.0 µL crude leaf extract and 4.0 µL of a stock solution of sodium linoleate in 1.0 mL sodium phosphate buffer 50 mM, pH 6.5. This activity was measured by forming a system of conjugated double bonds in the hydrogen peroxide, increasing absorbance at 234 nm (Axelrod *et al.*, 1981). 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 in triplicate.

Determination of protease inhibition

PI in leaf crude extract were determined using bovine trypsin (Kakade *et al.*, 1974). The trypsin activity in the presence of inhibitors was determined using 50 µL leaf extract, 500 µL Tris-HCl 0.1 M pH 8.2, with 20 mM $CaCl_2$ and 50 µL trypsin solution 4.7×10^{-5} M in a test tube. The control treatment was

performed using 550 µL 0.1 M Tris-HCl, pH 8.2, with 20 mM $CaCl_2$ and 50 µL trypsin solution 4.7 × 10^{-5} M. The test tubes were incubated for 5 min at 25 ºC. From each tube, 500 µL of the incubation mixture was removed and added to another tube containing 500 µL 0.1 M Tris-HCl, pH 8.2, with 20 mM $CaCl_2$ and 500 µL 1.2 mM L-BApNA. The absorbance of each solution was determined at 410 nm for 2.5 min. The experiment was performed in triplicate. The results were converted to mg trypsin inhibited per gram of protein according to

Equation 1: mg trypsin inhibited/gram of protein= A × B/(C × 1.000 × P),
where A is the absorbance at 410 nm of the control–410 nm absorbance of the sample, B is sample dilution, P is the concentration in g/mL of protein extracts, and C is 0.019 (trypsin factor; i.e. the product of the action of 1 µg of active trypsin on L-BAPNA results in an absorbance reading of 0.019 at 410 nm).

Statistical analyses

Data relating to proteolytic and trypsin-*like* amidase activities were combined for analysis. Data were subjected to analysis of normality and variance with the R Program at a reliability level of 95%.

Results

The activity of LOX (Figure 1) after 48 h of feeding by *A. gemmatalis*, increased significantly with cultivar type (P= 0.0189), time (P< 0.0001) and with the cultivar × time interaction (P= 0.0174). LOX showed no activity at time zero (beginning of the attack) in resistant (IAC-17, IAC-18, IAC-19 and IAC-24) and susceptible (IAC-PL-1) cultivars to *A. gemmatalis*. IAC-18, which is moderately resistant to *A. gemmatalis*, presented a lower rise in lipoxygenase activity when compared the other.

The production of PI during the 48 h period increased in all cultivars after larval damage (Figure 2), but without interactions of cultivar parameters or cultivar x time interaction (P= 0.0561 and P= 0.5481, respectively), differing from that observed for the time parameter (P= 0.0001).

Proteolytic activity in the midgut of larvae *A. gemmatalis* decreased with time after feeding (Table 1). Activity showed a greater reduction in IAC-19 (P<0.05), which has standard resistance to insects, compared with the more resistant (IAC-17,

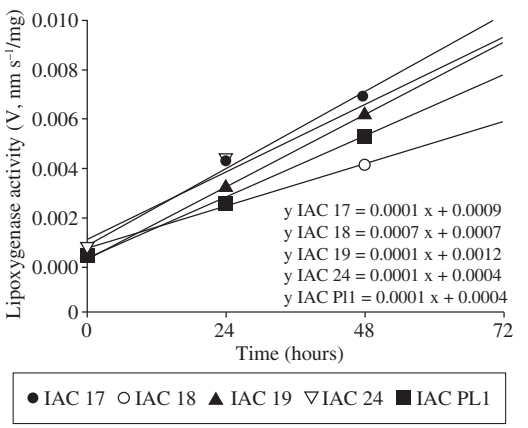

Figure 1. Lipoxygenase activity in leaf extracts of soybean cultivars with different degrees of resistance when damaged by *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Plant*time*cultivars interactions were not significant (gl_{erro}= 44, F= 6.01, P>0.05).

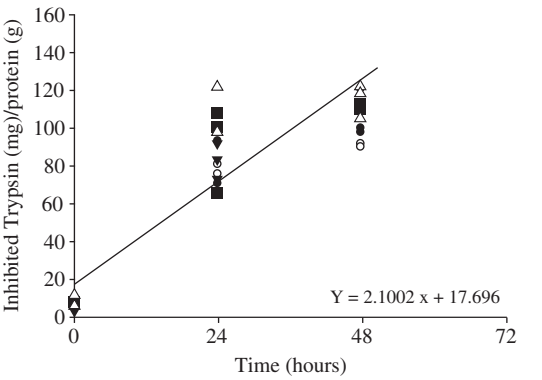

Figure 2. Inhibited trypsin activity with inhibitor from soybean cultivars with different degrees of resistance to insects. Caterpillar*time interaction was not significant (glerro= 44, F= 213.42, P> 0.05).

Table 1. Proteolytic enzyme extract from the midgut of velvet bean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae fed soybean cultivars with different degrees of resistance to insects.

Cultivars	Activity Proteolytic (Abs 440 nm/ protein mg)	
	24 hours	48 hours
IAC PL–1	0.047 ± 0.004Aa	0.045 ± 0.017Aa
IAC 17	0.072 ± 0.011Aa	0.062 ± 0.005Aa
IAC 18	0.067 ± 0.006Aa	0.041 ± 0.012Aa
IAC 19	0.136 ± 0.021Ba	0.069 ± 0.015Ab
IAC 24	0.056 ± 0.022Aa	0.054 ± 0.017Aa

IAC-18 and IAC-24) and the susceptible IAC-PL-1 cultivars.

The same capital letters show no difference at 5% probability by Tukey's test between differents cultivars (column). The same lowercase letters show no difference at 5% probability by Tukey's test in differents times in the same cultivar (line).

Amidase activity of trypsin-like did not differ with the type of cultivar (P>0.05), time (P>0.05) and cultivar × time interaction (P>0.05), according to data presented in the table 2. Esterase activity of trypsin-like varied over time (P<0.05), but without an interaction with time (P>0.05), significantly increased within 48 hours, except for the larvae subjected to treatment with cultivar IAC 17 (Table 2).

The same capital letters show no difference at 5% probability by Tukey's test between different cultivars (column). The same lowercase letters show no difference at 5% probability by Tukey's test in differents times in the same cultivar (line).

The activity of cysteine proteases in the gut of larval *A. gemmatalis* was similar across the five cultivars over the different feeding times (P>0.05) (Table 3).

The same capital letters show no difference at 5% probability by Tukey's test between different cultivars (column). The same lowercase letters show no difference at 5% probability by Tukey's test in different times in the same cultivar (line).

Table 2. Amidase and esterase activity of trypsin-like enzyme extract from the midgut of velvet bean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) when fed soybean cultivars with different degrees of resistance to insects.

	Trypsin-like Amidase Activity (V, μM.s^{-1}/ protein mg)	
Cultivars	24 hours	48 hours
IAC PL–1	0.003 ± 0.001Aa	0.005 ± 0.003Aa
IAC 17	0.013 ± 0.003Aa	0.016 ± 0.010Aa
IAC 18	0.019 ± 0.001Aa	0.002 ± 0,000Aa
IAC 19	0.011 ± 0.010Aa	0.009 ± 0.007Aa
IAC 24	0.006 ± 0.001Aa	0.005 ± 0.002Aa
	Trypsin-like Esterase Activity (V, μM.s^{-1}/ protein mg)	
Cultivars	24 hours	48 hours
IAC PL–1	0.105 ± 0.003Aa	1.715 ± 1.610Ab
IAC 17	0.189 ± 0,016Aa	0,059 ± 0,039Ab
IAC 18	0.308 ± 0,277Aa	1.717 ± 1.393Ab
IAC 19	0.333 ± 0.206Aa	1.551 ± 0.523Ab
IAC 24	0.060 ± 0.011Aa	0.411 ± 0,015Ab

Table 3. Cysteine protease activity of the enzyme extract from midgut of velvet bean caterpillar *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) fed soybean cultivars with different degrees of resistance to insects.

	Cysteine protease Activity (V, μM.s^{-1}/ protein mg)	
Cultivars	24 hours	48 hours
IAC PL–1	0.002 ± 0.0003Aa	0.002 ± 0.0001Aa
IAC 17	0.002 ± 0.0002Aa	0.002 ± 0.0001Aa
IAC 18	0.006 ± 0.0003Aa	0.005 ± 0.0003Aa
IAC 19	0.006 ± 0.0002Aa	0.002 ± 0.0001Aa
IAC 24	0.004 ± 0.0002Aa	0.001 ± 0.0005Aa

Discussion

The biological and biochemical responses of *A. gemmatalis* larvae fed susceptible or resistant soybean showed that antimetabolic agents (i.e. those that increase LOX and the PI) produced by this plant can cause physiological changes in the ability of *A. gemmatalis* larvae to digest proteins. However, this insect can adapt to these compounds through the hyperproduction either of enzymes sensitive to these plant inhibitors or of insensitive enzymes, as well as other classes of protease (Marinho *et al.*, 2010; Moreira *et al.*, 2011).

The highly specific activity of LOX after injury results from the increased content of PI through activation of the LOX pathway as a plant defense mechanism. Induction of LOX activity was also recorded in passion fruit leaves damaged by the oligophagous *Agraulis vanillae vanillae* (Linnaeus, 1758) (Lepidoptera: Nymphalidae) and the generalist *Spodoptera frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae). The interaction between soybean and *A. gemmatalis* through induction of the LOX pathway enhances the activity of these enzymes and the final products of the route, the PI.

The increase of PI in soybean is the result of a response to the increased activity of LOX, characterizing this defense pathway. Herbivory increased the concentration of PI in leaves of soybean cultivars after 48 h of damage by increasing the activity of LOX. Plants increase the activity of inhibitors in their tissues and the production of active broad-spectrum inhibitors as a defense against insect pests (Tiffin & Gaut, 2011). In addition, they produce highly specific inhibitors, resistant to proteolysis and active under the variable pH conditions of the insect midgut (Christeller, 2005). Inhibitors induced

by plant damage are used as a model system to study plant defense mechanisms (Farmer & Ryan, 1992; Shivaji *et al.*, 2012; Siqueira-Júnior *et al.*, 2008). After 24 h and 48 h of treatment with methyl jasmonate, mechanical wounding or herbivory, passion fruit leaves showed higher accumulation of PI, demonstrated by increased inhibition of trypsin (Siqueira-Júnior *et al.*, 2008).

The highest impact on the activity of proteolytic enzymes in the gut of *A. gemmatalis* fed IAC-19 compared to those fed IAC-17, IAC-18 or IAC-24, suggests that the first cultivar produces more potent and/or multidomain PI against herbivores. The lower proteolytic activity in the standard susceptible cultivar IAC-PL-1 suggests the presence of a PI with a high inhibition constant that reduces the activity of proteolytic enzymes in the gut of *A. gemmatalis* larvae (Lourenção *et al.*, 1997).

The increase in amidase and esterase activity over time in the gut of *A. gemmatalis* larvae fed IAC-19 and in esterase activity in those fed IAC-17, IAC-18 and IAC-24 could be related to increased production of trypsin-like enzymes that offset proteases inhibited by entomotoxic substances in these cultivars. This induces insects to use available amino acids to synthesize more protease rather than for the production of proteins for growth and development (Broadway, 1995). Similarly, the sudden decrease in amidase activity in the gut of larvae fed the cultivars IAC-17 and IAC-18 reinforces a possible inhibitory effect of the PI.

The expression of cysteine proteases in the midgut of *A. gemmatalis* larvae could avoid a host defense that is rich in serine protease inhibitors. The presence of cysteine proteases in the midgut (e.g. digestive enzymes) is an adaption to ingest food rich in serine protease inhibitors (Mendonça *et al.*, 2012). The increased activity of cysteine proteases in the gut of larvae fed on the five soybean cultivars confirms the presence of this adaptive mechanism in *A. gemmatalis*. Although plants have defense mechanisms, insects may metabolize and use such toxic substances to protect themselves against their own natural enemies (Michereff *et al.* 2014).

The changes in soybean biochemistry caused by *A. gemmatalis* larvae serve as a model to produce cultivars that are resistant to this pest. The plant responds to insect damage by activating biochemical defense pathways. In response, the insect adapts its digestive enzymes to maintain proteolysis, which is the major amino acid source in phytophagous insects. This ensures a supply of amino acids for biosynthesis in diets low in nitrogen (Bown *et al.*, 2004). Knowledge of the digestive enzymes of *A. gemmatalis* larvae is essential to develop strategies to control this important insect pest.

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