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Protease inhibitory, insecticidal and deterrent effects of the trypsin-inhibitor benzamidine on the velvetbean caterpillar in soybean

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ABSTRACT

The recognition of protease inhibitors with insecticidal activity is important as a basis for the development of mimetic peptides with potential use as biorational insecticides. We sprayed benzamidine on soybean plants and assessed whether this potent synthetic trypsin-inhibitor has protease inhibitory, insecticidal and deterrent effects on the velvetbean caterpillar *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae). Activity of trypsin inhibition in soybean leaves was increased and total proteolytic activity in the midgut extract from larvae fed on these leaves was reduced by benzamidine. Different concentrations of benzamidine sprayed on the plant caused approximately 50 % of larval mortality, and larval choice and moth preference and oviposition were all negatively affected. Low concentrations of benzamidine increased mortality and hindered insect choice and oviposition as well as higher doses. Since many synthetic protease inhibitors are usually expensive, small doses of benzamidine may be effective to protect soybean against *A. gemmatalis* attack. Our results highlight the potential of synthetic protease inhibitors for insecticidal and deterrent purposes in insect pest management.

Key words: protease inhibitor, synthetic trypsin-inhibitor, insect performance, insect preference, *Anticarsia gemmatalis*.

INTRODUCTION

To counter herbivory, plants have evolved morphological traits, secondary metabolites and proteins that have toxic, repellent, and/or antinutitional effects on herbivorous insects (Fürstenberg-Hägg et al. 2013). Proteinase inhibitors (PIs) cover one of the most abundant classes of defensive proteins in plants. Proteinase inhibitors bind to the digestive enzymes in insect

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gut and inhibit their activity, thereby reduce protein digestion, resulting in the shortage of amino acids for the herbivore (War et al. 2012). Traits and mechanisms underpinning plant resistance and tolerance to herbivores have shown considerable utility in pest control and potential to improve the sustainability of crop protection (Mitchell et al. 2016). For example, transgenic plants with increased PIs expression may have detrimental effects on pest growth and development (Ryan 1990, Chen 2008). Ingestion of potent synthetic PIs can be lethal for insects (Moreira et al. 2011), even though the usage of synthetic inhibitors for insecticidal purpose remains poorly explored.

Crop domestication artificially selects plants to increase their suitability to human requirements, such as taste, yield, storage, and cultivation practices. Plant defensive traits can be lacking or be expressed weakly in domesticated plants as consequence of this selection for other desirable traits (Chaudhary 2013, Chen et al. 2015). For example, the genetic elimination of lipoxygenase isozymes (LOX) from soybean (*Glycine max* (L.) Merrill) seeds was a way to overcome the problems associated with the undesirable beany flavor of soybean products (Fortunato et al. 2007). Compounds derived from LOX activity are precursors of jasmonic acid, which would in turn activate transcription of genes encoding for PIs (Farmer and Ryan 1992). The PIs correspond to about 6% of the total protein present in soybean seeds and they are represented by the Boward-Birk trypsin and chymotrypsin inhibitor (BBI) and mainly by the Kunitz trypsin inhibitor (KTI) (Brandon et al. 1993). An impairment of PIs synthesis poses a particular challenge for crop protection as it suggests that modern varieties of soybean would perform poorly in systems with restricted pesticide use. Because LOX and PIs are lacking in seeds of mutant lines, we do not know exactly to what extent breeding programs have decreased the presence of these proteins in leaves, compromising soybean resistance and tolerance to folivorous insects.

Soybean fields usually cover vast areas and, like any other monoculture, they suffer severely from the attack of insect pests, especially the velvetbean caterpillar, *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) (Bortolotto et al. 2015). In the present study, we sprayed the synthetic PI benzamidine on a soybean line that lacks LOX and KTI in seeds and assessed whether this method have protease inhibitory, insecticidal and deterrent effects on the velvetbean caterpillar. The recognition of PIs with insecticidal activity is important as a basis for the development of mimetic peptides with potential use as biorational insecticides (Nicholson 2007, Ishaaya and Horowitz 2009). Benzamidine [$C_6H_5C(=NH)NH_2$] is a strong reversible competitive inhibitor of trypsin, trypsin-like enzymes and serine proteases. Benzamidine was tested against the velvetbean caterpillar with the expectation of an insecticidal activity of this potent trypsin inhibitor.

MATERIALS AND METHODS

INSECTS AND PLANTS

A colony of *A. gemmatalis* was kept in laboratory at 25 ± 2 °C, with relative humidity of 70 ± 10 % and photoperiod of 14 h. The insect eggs were surface-sterilized with UV light (20 W lamps placed 25 cm away) and subsequently transferred to sterile Petri dish until hatching. The newly hatched larvae were transferred to plastic pots in a laminar flow chamber and reared on artificial diet, following Hoffmann-Campo et al. (1985).

The soybean commercial line CAC-1 TN (LOX-/KTI-) were grown to reach the V2 (second node, first trifoliate fully developed) and V3 (third node, second trifoliate fully developed) stages of the vegetative phase, without leaf damages and application of any chemical. The CAC-1 TN (LOX-/KTI-) lacks the genes encoding three LOX

isozymes and did not have KTI in their seeds, which were altered by the Soybean Quality Improvement Program of the Agricultural Biotechnology Research Institute (BIOAGRO) at the Federal University of Viçosa (UFV), Brazil (Fortunato et al. 2004).

PIs IN SOYBEAN LEAVES AND PROTEOLYTIC ACTIVITY IN CATERPILLAR MIDGUT

In a protected greenhouse, soybean seedlings were grown in 5-L plastic pots containing 4.0 kg of soil, each pot with three plants. Twenty four pots, with plants at the V2 stage, were distributed in six mesh cages (1 × 1 × 1 m), four pots per cage. Thus, the experiment was designed with six treatments (cages) and four replicates (pots) (n = 24). When the plants reached the V3 stage, the first trifoliate of each was subjected to a 4^{th} or 5^{th} instar of the velvetbean caterpillar, therefore, three larvae per pot. Immediately after introducing the caterpillars, and to simulate field conditions, the plants were sprayed according to the following conditions: **control treatment** = spray with aqueous solution of the adhesive spreader Triton X-100 at 0.01 %; **inhibitor treatments** = solution of Triton X-100 (0.01 %) with addition of benzamidine at 0.15, 0.30, 0.45, 0.60 and 0.75 %. In sequence, one pot was removed from each treatment (cage) at 6, 12, 24 and 48 hours after spraying. Plants withdrawn from the cages were not returned to them.

Preparation and analyzes of plant extract

Three folioles were collected from each plant, which were frozen in liquid nitrogen and stored at -80 °C for later determination of total protein and PIs in leaf crude extract. The crude extracts were prepared at 4 °C following Ohta et al. (1986). PI in leaf crude extract were determined using bovine trypsin (Kakade et al. 1974). The trypsin activity in the presence of PIs in soybean leaves 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 test tubes were incubated for 5 min at 25 ºC. From each tube, 500 mL 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 chemical analyzes were performed in triplicate for each biological replicate and the results were converted to mg trypsin inhibited per gram of protein (Paixão et al. 2016). The determination of total protein followed Bradford (1976), using bovine serum albumin (BSA) 0.2 mg/mL as standard.

Preparation and analyzes of insect midgut extract

Following Xavier et al. (2005) and Paixão et al. (2013), the three larvae from each plant were water rinsed and ice-chilled for dissection. The midguts were extracted in 10^{-3} M HCl at 4 ºC and placed in 2 mL microtubes. The extracted midguts were obtained by cellular lyses through submission to nine cycles of nitrogen freezing and thawing at 37 ºC in a water bath. Aliquots of 1 ml of midgut extract were centrifuged at 100000 g for 30 min at 4 ºC. The resulting supernatant was collected and stored at -20 ºC for later use.

The bicinchoninic acid method (Smith et al. 1985) was used to determine protein concentration in midgut extract, using 0.2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) as standard. Proteolytic 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. Total protease activity was monitored in a spectrophotometer at 440 nm.

DEVELOPMENTAL PERFORMANCE OF CATERPILLARS

First instar larvae of *A. gemmatalis* were reared in plastic trays that were 50 x 40 x 10 cm and covered by a white cloth bound with elastic bands on the edges. Ten larvae were placed per tray, being six trays per treatment (n = 36). Insects were kept in a climatic chamber at 25 ± 2 °C, relative humidity of 65 ± 10 % a and a 14 h photoperiod. The larvae were fed with soybean leaves, which were sprayed with benzamidine 12 h early, following the six treatments (concentrations from 0.00 to 0.75 %) described in the section 2.2 above. The leaves had petioles wrapped in moist cotton. The food was replenished daily and the trays were cleaned by removal of feces and waste. The caterpillars were fed until the pupal stage. Weight and mortality of caterpillars were evaluated every two days.

HERBIVORE PREFERENCE

Soybean seedlings were individually grown in plastic tubes 8 cm diameter x 20 cm length. Seedlings at the V3 stage were placed in five mesh cages (1 × 1 × 1 m), six plants per cage equidistantly arranged in a circular manner, inserted in styrofoam plates. Thus, the experiment was designed with six treatments (plants) and five replicates (cages) (n = 30). The plants in each cage had previously been sprayed with a solution of Triton X-100 (0.01%) with addition of benzamidine at 0.00; 0.15; 0.30; 0.45; 0.60 or 0.75 % (concentration treatments). We used this design to assess the preference of both larvae and adults of *A. gemmatalis*. Thirty 4th instar larvae were released in the center of the arena (cage). The number of larvae per plant (treatment) was assessed after ten hours (larval choice). Ten adult females were released per cage. The number of individuals resting per plant was observed after two hours (moth preference). Because of the nocturnal habits of the moths, the test was carried out at night (9:00 p.m.), in a dark room. In order not to affect insect behavior, we used an ultraviolet flashlight. Twenty-four hours after moth release, the plants were removed from the cages and number of eggs per plant were counted (oviposition preference).

DATA ANALYSIS

Since the trypsin inhibition by soybean leaves and the proteolytic activity in the midgut extract from *A. gemmatalis* larvae fed on soybean leaves were measured at four periods over time (hours after spraying the different doses of benzamidine), data was submitted to ANOVA considering the repeated measurements structure. As post ANOVA procedure, we planned on regression analysis for studying concentration effect. Larval mortality, larval choice, adult preference for resting and oviposition preference were submitted to one-way ANOVA as a function of benzamidine doses. Then, the averages were compared using Holm–Sidak's HSD test with $p < 0.05$. Data analysis was performed using the statistical package Sigma Plot, version 12.0, Systat Software Inc. 2011.

RESULTS AND DISCUSSION

Trypsin inhibition by PIs present in the leaf extract was affected by the concentration of benzamidine sprayed on the plant ($F_{5\text{-}23} = 8.5$, $p < 0.001$); however, time after spraying was not significant ($F_{3\text{-}23} = 1.5$, $p = 0.26$). Thus, inhibitory activity was continued for 6 to 48 hours after spraying, although we did not measure it for a longer time. The causal relation between benzamidine concentration and trypsin inhibition was non-linear, following the exponential equation $y = 105\ [1\text{-}\exp(-10x)]$. Trypsin inhibition initially increased with the smallest concentrations of benzamidine but remained constant as the

concentration was gradually increasing. Spraying benzamidine at 1.5 and 3.0 % w/v respectively increased 75 and 100 times the inhibitory activity in soybean leaf extract. Spraying higher benzamidine concentrations did not improve trypsin inhibition (Fig. 1).

The soybean line CAC-1 TN, which lacks LOX and KTI in their seeds, showed to have endogenous trypsin inhibition in the leaves, which were taken from the control plants, without benzamidine spraying (Fig. 1). This natural occurrence of PIs in soybean leaves may be constitutive or induced by caterpillar damage (Fortunato et al. 2007, Paixão et al. 2016). However, we found that the presence of the velvetbean caterpillar on the plant did not increase trypsin inhibition over time. Because endogenous trypsin inhibition was up to 100 times lower than exogenous inhibition, the strong effect of the benzamidine may have masked the damage-induced PIs over time.

Considering a desired insecticidal effect, artificially increased PIs in soybean leaves should have a greater impact on herbivore physiology and performance than endogenous plant PIs alone. Proteolytic activity in the midgut extract from *A. gemmatalis* larvae fed on soybean leaves was affected by the concentration of benzamidine sprayed on the plant ($F_{5\text{-}23} = 3.4$, $p = 0.03$), but time after spraying was not significant ($F_{3\text{-}23} = 1.3$, $p = 0.30$). Thus, effects of PIs were continued for 6 to 48 hours after spraying. The causal relation between benzamidine concentration and proteolytic activity was non-linear, following the polynomial equation $y = 0.29 - 0.83x + 0.97x^2$. Intermediate benzamidine concentrations, from 0.3 to 0.6 % w/v, showed better inhibitory effect on gut proteolysis than the highest concentration (Fig. 2). Therefore, sprayed benzamidine showed potential to adversely affect protein digestion in the velvetbean caterpillar, and doses higher than 0.3 – 0.6 % w/v may be, at first sight, unsuitable to improve protease inhibition.

Previous studies have also found that proteolytic activity in the midgut of *A. gemmatalis* is affected by the synthetic inhibitors benzamidine (Pilon et al. 2006), biz-benzamidine (Moreira et al. 2011) and berenil (Paixão et al. 2013). However, there are recurrent evidence that the velvetbean caterpillar may be adapted to compensate for higher doses of PIs, including the synthetic benzamidine. Similar to our non-linear results (Fig. 2), lower or intermediate concentrations of benzamidine decreased protein digestibility, but there was an

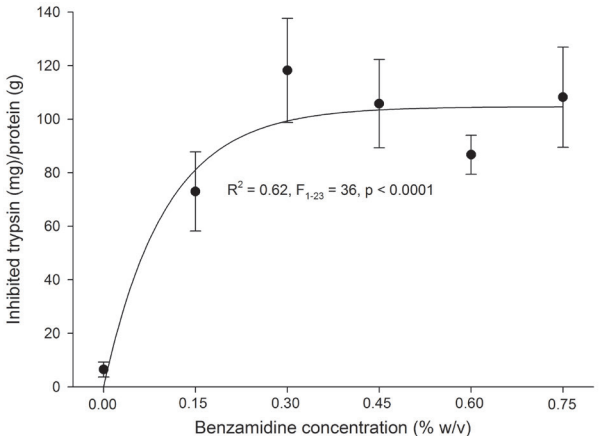

Figure 1 - Indirect determination of trypsin inhibitors in soybean leaves sprayed with benzamidine at different concentrations in presence of larvae of *Anticarsia gemmatalis*. Point and line indicate mean ± SE of four plants.

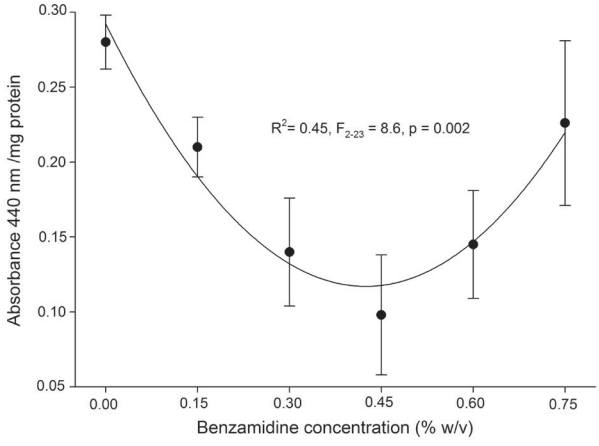

Figure 2 - Total proteolytic activity in the midgut extract from *Anticarsia gemmatalis* fed on soybean leaves sprayed with PI benzamidine at different concentrations. Point and line indicate mean ± SE of larvae in four plants.

increase in protein digestibility when bezamidine concentration in the diet was increased (Pilon et al. 2006). Such increases are the likely result of compensatory hyperproduction of proteases during chronic or higher doses of PIs ingestion (Jongsma and Bolter 1997, Bown et al. 2004). Thus, chronic ingestion of PIs does not eliminate protein digestion in the midgut, but the hyperproduction of digestive proteases may limit the bioavailability of essential amino acids for protein synthesis, impairing insect growth and development (Pilon et al. 2006, Moreira et al. 2011, Paixão et al. 2016). Consequently, despite this compensatory hyperproduction of proteases and the increase in protein digestibility, synthetic PIs may exhibit insecticidal activity in higher doses. Ultimately, doses higher than 0.3 – 0.6 % w/v (Fig. 2) may be, in reality, suitable to improve plant protection.

Mortality of *A. gemmatalis* larvae was affected by the presence of benzamidine sprayed on the plant ($F_{5\text{-}35} = 6.2$, $p < 0.001$); however, we found no difference between the doses. Any of the doses of benzamidine caused approximately 50 % of larval mortality. (Fig. 3a). Weight of last-instar of surviving larvae was not affected by the benzamidine sprayed on the host plant ($F_{5\text{-}35} = 0.9$, $p = 0.50$). Our results do not support the hypothesis that, in presence of the highest dose of benzamidine, a hyperproduction of digestive proteases (0.75 % w/v in Fig. 2) should limit availability of amino acids, protein synthesis and insect development. Thus, a hyperproduction of digestive proteases may have circumvented the effects of the benzamidine, which did not show an increased insecticidal activity. In spite of this, chemicals with low to medium lethality may still

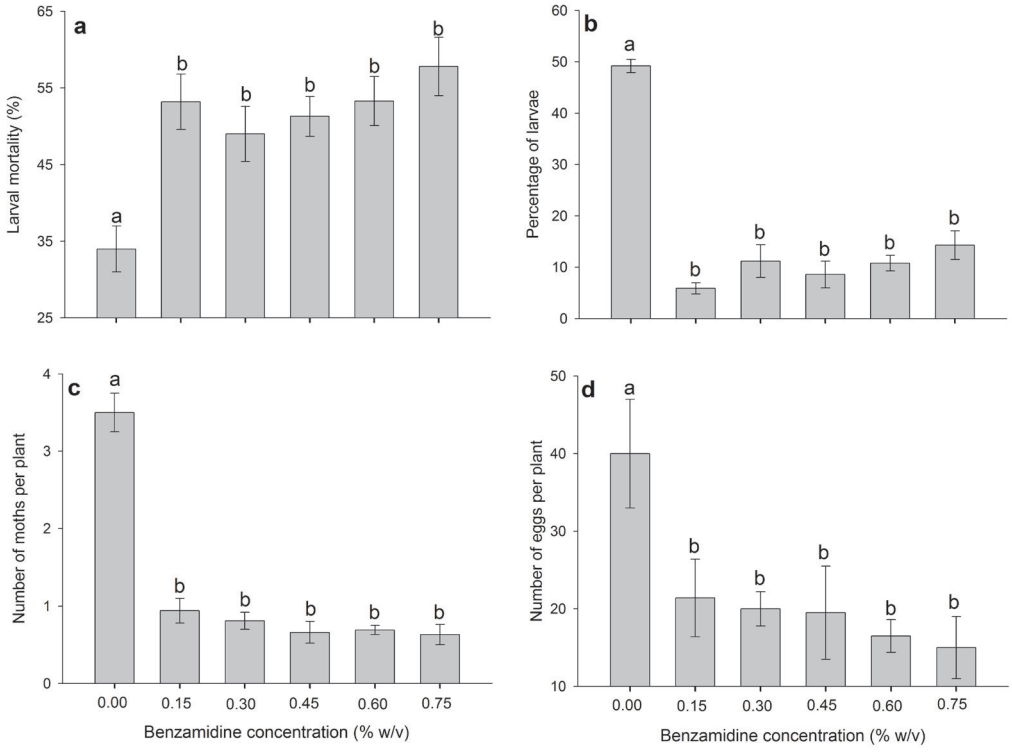

Figure 3 - Larval mortality (**a**), larval choice (**b**), preference of adult females (**c**) and eggs laid (**d**) by *Anticarsia gemmatalis* in soybean plants sprayed with PI benzamidine at different concentrations. Point and line indicate mean ± SE of six replicates in **a** and five in **b**-**d**. Different letters between concentrations indicate significant differences by the Holm–Sidak HSD test ($p < 0.05$).

show deterrent effects on insect oviposition and feeding.

Larval choice was affected by the presence of benzamidine sprayed on the plants ($F_{5\text{-}29} = 51.7$, $p < 0.001$); however, we found no difference between the doses (Fig. 3b). In the same way, moth preference and oviposition preference were both affected by the benzamidine sprayed on the soybean plants (respectively $F_{5\text{-}29} = 56.8$, $p < 0.001$ and $F_{5\text{-}29} = 9.2$, $p < 0.001$), but we found no difference between the doses (Fig. 3c-d). The pattern of larval mortality (Fig. 3a) was inversely corresponding to the pattern consistently found in herbivore preference (Fig. 3b-d). There is no evolutionary history modulating this strong negative relationship between preference and performance for synthetic PIs. However, larvae and adults of *A. gemmatalis* were efficient in identifying and avoiding even the smallest concentrations of the synthetic benzamidine. The mechanisms of insect recognition and rejection of this chemical are unknown, so we do not know whether the functional nature of the benzamidine, as a potent PI, was important to stimulate the insect. Regardless of this, low doses of benzamidine increased mortality and hindered insect choice and oviposition as well as higher doses. Since many synthetic inhibitors are usually expensive, heave doses of benzamidine may be dispensable to protect soybean against *A. gemmatalis* attack. Our results highlight the potential of synthetic inhibitors for insecticidal and deterrent purposes in insect pest management.

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REFERENCES

BORTOLOTTO OC, POMARI-FERNANDES A, BUENO RCOF, BUENO AF, KRUZ YKS, QUEIROZ AP, SANZOVO A AND FERREIRA RB. 2015. The use of soybean integrated pest management in Brazil: a review. Agron Sci Biotechnol 1: 25-32.

BOWN DP, WILKINSON HS AND GATEHOUSE JA. 2004. Regulation of expression of genes encoding digestive proteases in the gut of a polyphagous lepidopteran larva in response to dietary protease inhibitors. Physiol Entomol 29: 278-290.

BRADFORD MM. 1976. A rapid and sensitive method for the quantification of microgram quantities of proteins utilizing the principle of protein dye binding. Anal Biochem 72: 248-254.

BRANDON DL, BATES AH AND FRIEDMAN M. 1993. Antigenicity of soybean protease inhibitors. In: Troll W and Kennedy AR (Eds), Proteinase inhibitors as cancer chemopreventive agents. New York: Plenum, p. 107-129.

CHAUDHARY B. 2013. Plant domestication and resistance to herbivory. Int J Plant Genom 2013: 572784.

CHEN MS. 2008. Inducible direct plant defense against insect herbivores: A review. Insect Sci 15: 101-114.

CHEN YH, GOLS R AND BENREY B. 2015. Crop domestication and its impact on naturally selected trophic interactions. Annu Rev Entomol 60**:** 35-58.

FARMER EE AND RYAN CA. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. Plant Cell 4: 129-134.

FORTUNATO FS, OLIVEIRA MGA, BRUMANO MHN, SILVA CHO, GUEDES RNC AND MOREIRA MA. 2007. Lipoxygenase-induced defense of soybean varieties to the attack of the velvetbean caterpillar (*Anticarsia gemmatalis* Hübner). J Pest Sci 80: 241-247.

FORTUNATO FS, OLIVEIRA MGA, BRUMANO MHN, ZANÚNCIO JC, OLIVEIRA JA, PILON AM, ALMEIDA FT, SEDIYAMA CS AND MOREIRA MA. 2004. Effect of the *Anticarsia gemmatalis* injury on the lipoxigenases activity from the soybean leaves. Biosci J 20: 37-46.

FÜRSTENBERG-HÄGG J, ZAGROBELNY M AND BAK S. 2013. Plant defense against insect herbivores. Int J Mol Sci 14: 10242-10297.

HOFFMANN-CAMPO CB, OLIVEIRA EB AND MOSCARDI F. 1985. Criação massal da lagarta da soja (*Anticarsia gemmatalis*). Embrapa Soja, Documentos 10, 23 p.

ISHAAYA I AND HOROWITZ AR. 2009. Biorational control of arthropod pests: application and resistance management. Dordrecht, Springer, 408 p.

JONGSMA MA AND BOLTER C. 1997. The adaptation of insects to plant protease inhibitors. J Insect Physiol 43: 885-895.

KAKADE ML, RACKIS JJ, MCGHEE JE AND PUSKI G. 1974. Determination of trypsin inhibitor activity of soy products: a collaborative analysis of an improved procedure. Cereal Chem J 51: 376-382.

MITCHELL C, BRENNAN RM, GRAHAM J AND KARLEY AJ. 2016. Plant defense against herbivorous pests: exploiting resistance and tolerance traits for sustainable crop protection. Front Plant Sci 7: 1132.

MOREIRA LF, CAMPOS WG, RIBEIRO FR, GUEDES RNC AND OLIVEIRA MGA. 2011. Survival and developmental impairment induced by the trypsin inhibitor bis-benzamidine in the velvetbean caterpillar (*Anticarsia gemmatalis*). Crop Prot 30: 1285-1290.

NICHOLSON GM. 2007. Fighting the global pest problem. Toxicon 49: 413-422.

OHTA H, IDA S, MIKAMI B AND MORITA Y. 1986. Changes in lypoxigenase components of rice seedling during germination. Plant Cell Physiol 22: 911-918.

PAIXÃO GNP, LOURENÇÃO AL, SILVA CR, CORDEIRO G, BARROS RAB, OLIVEIRA JA, VISÔTTO LE AND OLIVEIRA MGA. 2016. Active response of soybean to defoliator *Anticarsia gemmatalis* Hübner: strategies to overcome protease inhibitor production. Idesia 34: 69-75.

PAIXÃO GNP, LOURENÇÃO AL, SILVA CR, MENDONÇA EG, SILVA PL, OLIVEIRA JA, ZANUNCIO JC AND OLIVEIRA MGA. 2013. Biochemical responses of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) in soybean cultivars sprayed with the protease inhibitor berenil. J Agri Food Chem 61: 8034-8038.

PILON AM, OLIVEIRA MGA AND GUEDES RNC. 2006. Protein digestibility, protease activity and post-embryonic development of the velvetbean caterpillar (*Anticarsia gemmatalis*) exposed to the trypsin-inhibitor, benzamidine. Pestic Biochem Physiol 86: 23-29.

RYAN CA. 1990. Proteinase inhibitors in plants: genes improving defenses against insects and pathogens. Annu Rev Phytopathol 28: 425-449.

SMITH PK, KROHN RI, HERMANSON GT AND MALLIA AK. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 15: 76-85.

TOMARELLI RM, CHARNEY J AND HARDING ML. 1949. The use of azoalbumin as a substrate in the colorimetric determination or peptic and tryptic activity. J Lab Clin Med 34: 428-433.

WAR AR, PAULRAJ MG, BUHROO AA, HUSSAIN B, IGNACIMUTH US AND SHARMA HC. 2012. Mechanisms of plant defense against insect herbivores. Plant Signaling Behav 7: 1306-1320.

XAVIER LP, OLIVEIRA MGA, GUEDES RNC, SANTOS AV AND SIMONE SG. 2005. Trypsin-like activity of membrane-bound midgut proteases from *Anticarsia gemmatalis* (Lepidoptera: Noctuidae). Eur J Entomol 102: 147-153.