Effect of Natural and Artificial Diets on Protease Activity in the Midgut of Spodoptera cosmioides and Spodoptera eridania (Lepidoptera: Noctuidae) Larvae

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Effect of natural and artificial diets on protease activity in the midgut of *Spodoptera cosmioides* and *Spodoptera eridania* (Lepidoptera: Noctuidae) larvae

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Abstract

Spodoptera cosmioides Walker and *Spodoptera eridania* Stoll (both Lepidoptera: Noctuidae) are herbivorous insects affecting crop yield. Understanding midgut digestive enzyme properties in these caterpillars when feeding on different resources is important for control strategies of these agricultural pests. This study evaluated the activity of midgut digestive enzyme total proteases, trypsin, cysteine proteases, and chymotrypsin in *S. cosmioides* and *S. eridania* feeding on natural soybean and cotton leaves and artificial diets. The proteolytic activities of midgut digestive enzymes in *S. cosmioides* and *S. eridania* vary according to diet, suggesting adaptation of these caterpillars to different host plants in order to avoid the inhibitory effects of secondary metabolites through the overexpression of proteases. High activities occur for trypsin and total proteases in both insects indicating that these enzymes are potential targets for inhibition in pest control programs.

Key Words: enzymes; pests; chymotrypsin; soybean; trypsin

Resumo

Spodoptera cosmioides Walker e *Spodoptera eridania* Stoll (ambos Lepidoptera: Noctuidae) são insetos herbívoros que afetam o rendimento das culturas. A compreensão das propriedades das enzimas digestivas do intestino médio dessas lagartas ao se alimentarem de diferentes dietas é importante para o estabelecimento de estratégias de controle dessas pragas agrícolas. Este estudo avaliou a atividade de proteases totais, tripsina, quimotripsina e cisteíno-protease no intestino médio de *S. cosmioides* e *S. eridania* alimentadas com folhas de soja e algodão e em dietas artificiais. As atividades proteolíticas das enzimas digestivas do intestino médio em ambas as espécies variam de acordo com a dieta, sugerindo uma adaptação dessas lagartas à diferentes plantas hospedeiras para evitar os efeitos inibitórios dos metabólitos secundários com a superexpressão de proteases. Tripsina e proteases totais tem altas atividadesem ambos os insetos, indicando que essas enzimas são potenciais alvos para inibição em programas de controle de pragas.

Palavras Chaves: enzimas; pragas; quimotripsina; soja; tripsina

Spodoptera cosmioides Walker and *Spodoptera eridania* Stoll (both Lepidoptera: Noctuidae) are phytophagous insects that damage soybean and cotton crops (Souza et al. 2013). These caterpillars are controlled with synthetic insecticides, which have low efficacy due to induced tolerance by excessive use of these compounds (Diez-Rodríguez & Omoto 2001; Carvalho et al. 2013). Thus, chemical control may result in financial losses, unbalance the food web, and select for resistant strains, which may overlap with alternative pest control methods (Mills & Kean 2010; Vianna et al. 2011).

Production of defense proteins, such as protease inhibitors, is a strategy of some plants to avoid herbivory (Wasternack & Hause 2013). The protease inhibitors affect food digestion, reducing or inhibiting the protein synthesis required for insect growth, development, and reproduction (Meriño-Cabrera et al. 2018)

The Kunitz (Onesti et al. 1991) and Bowman-Birk inhibitors (Song & Suh 1998) are found in large amounts in soybeans, and gossypol is found in cotton (Meisner 1978; Souza et al. 2006), all inhibiting digestive trypsin and chymotrypsin (Liener 1994; Gariani & Leatherbarrow 1997).

Phytophagous insects have physiological adaptations to reduce the negative effects of the ingestion of protease inhibitors produced by plants (Moon et al. 2004; Patarroyo-Vargas et al. 2017; Meriño-Cabrera et al. 2018). These adaptations include increased levels of digestive proteases (Pilon et al. 2006, 2009; Scott et al. 2010; Meriño-Cabrera et al. 2018), and isoform synthesis, which do not bind to protease inhibitors, or bind and degrade protease inhibitors (Srinivasan et al. 2006; Zhang et al. 2010; Jamal et al. 2012).

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Midgut proteases have been studied for use in insect pest control due to their role in peptide bond hydrolysis and releasing of amino acids for insect growth, reproduction and survival (Mahdavi et al. 2013; Shi et al. 2013).

Inhibition of serine proteases (E.C. 3.4.21), the main digestive enzymes in Lepidoptera, decrease the total digestive activity in these insects by up to 95% (Pilon et al. 2006). In this sense, protease inhibitors in natural and artificial diets are potential compounds to control these pests (Gatehouse 1999; Kidd 2000; Pilon et al. 2006; Rosell et al. 2008; Moreira et al. 2011). The total spectrum of midgut proteases and adaptation of insects to modify the activity of their proteases in different diets should be studied to allow the use of these compounds in pest management (Jongsma & Bolter 1997; Visôtto et al. 2009a, b).

Given that the production and activities of digestive enzymes in *S. eridania* and *S. cosmioides* change according to diet (Kotkar et al. 2012; Sarate et al. 2012), this study evaluated the digestive enzyme activity in these species fed on a natural diet rich in protease inhibitors (soybean and cotton leaves) and an artificial diet free of inhibitors.

Materials and Methods

INSECTS

Spodoptera cosmioides and *S. eridania* caterpillars were obtained from mass rearing in the Insect-Plant Interaction Laboratory of the Department of Entomology of the Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

Adults were kept in cages fed with honey (10.5 g), beer (350 mL), sucrose (60 g), ascorbic acid (1.05 g), nipagine (1.05 g), and water (1,000 mL). *Spodoptera cosmioides* and *S. eridania* oviposition began after 3 d. Old females and eggs were collected every 2 d and maintained at 27.5 °C, 75% relative humidity, and a 14:10 h (L:D) photoperiod.

Hatched caterpillars were transferred to containers (500 mL) and fed on artificial diet (Greene et al. 1976), soybean leaves (TMG 1264 RR, V3 stage) or cotton leaves (FM 910, V4 stage) until the fifth instar, when the midguts were dissected for enzyme assays.

MIDGUT EXTRACT AND PROTEIN DETERMINATION

The midguts of 10 *S. cosmioides* and 10 *S. eridania* caterpillars were macerated with 1 mL 1 mM HCl at 4 °C and centrifuged at 10,000 g for 10 min at 4 °C (Paixão et al. 2013).

Total protein concentration in triplicate of the midgut extracts was determined according to Bradford methods (1976) using bovine serum albumin as a standard.

DETERMINATION OF AMIDASIC AND ESTERASE ACTIVITIES OF TRYPSIN

The amidasic trypsin activity was determined using the chromogenic substrate N-benzoyl-L-arginyl-p-nitroanilide (L-BApNA 2 mM) in 0.1 M Tris-HCl buffer, pH 8.2 containing 20 mM $CaCl_2$ (25 °C) (Erlanger et al. 1961). The control had enzyme substrate buffer without midgut extract. The product formation was calculated at 410 nm for 2.5 min, using molar extinction coefficient of 8800 (M^{-1} cm^{-1}).

The esterase trypsin activity was determined using 0.1 mM N-α-p-tosyl-L-arginine methyl ester (L-TAME) substrate in 0.1M Tris-HCl pH 8.2 buffer, containing 20 mM $CaCl_2$ (25 °C) (Hummel 1959). The product formation was calculated at 247 nm for 2.5 min using 540 M^{-1} cm^{-1} molar extinction coefficient. All analyses were performed in technical triplicate.

DETERMINATION OF THE AMIDASIC AND ESTERASE ACTIVITIES OF CHYMOTRYPSIN

The amidasic chymotrypsin activity was determined with 1.2 mM N-Benzoyl-L-tyrosine-p-nitroanilide (L-BTpNA) substrate in 0.1M Tris-HCl buffer pH 8.2 with 20 mM $CaCl_2$ (25 °C). For the control, the midgut extract was omitted from the reaction. The product formation was calculated at 410 nm for 2.5 min using 8 800 (M^{-1} cm^{-1}) molar extinction coefficient.

The esterase chymotrypsin activity was determined with 0.1 mM N-Acetyl-L-tyrosine ethyl ester monohydrate (ATEE) substrate in 0.1M Tris-HCl pH 8.2 buffer containing 20 mM $CaCl_2$. The control contained substrate and buffer without midgut extract. The product formation was calculated at 247 nm for 2.5 min. All analyses were performed in technical triplicate.

DETERMINATION OF CYSTEINE PROTEASE ACTIVITY

The activity of cysteine proteases was determined according to Mendonça et al. (2011). Briefly, the reaction mixture had 500 µL 0.5 mM L-BapNA substrate (25 °C), 500 µL of 0.1 M Tris-HCl buffer pH 8.2, containing 20 mM $CaCl_2$ and 5 mM Dithiothreitol (DTT), 100 µL of 1 mM benzamidineand 10 µL of the midgut extract. For control, the midgut extract was omitted from the mixture. The product formation was calculated at 410 nm for 2.5 min using 8800 M^{-1} cm^{-1} molar extinction coefficient. The analyses were performed in technical triplicate.

DETERMINATION OF TOTAL PROTEASE ACTIVITY

The activity of total proteases was determined with 2% azocasein substrate in 0.1 M Tris-HCl buffer pH 8.2 containing 20 mM $CaCl_2$ (37 °C) (w/v) (Tomarelli et al. 1949). The reaction mixture had 50 µL of substrate and 60 µL of midgut extract and was incubated at 37 °C for 30 min. The reaction was blocked with 240 µL of 10% trichloroacetic acid (w/v).

Samples were then homogenized, maintained in ice for 15 min, and centrifuged at 8,000× g for 5 min at 25 °C. The supernatant (240 µL) was transferred to 280 µL of 1M NaOH and analyzed at 440 nm in a spectrophotometer.

STATISTICS

The experiment was conducted in a completely randomized design with 3 technical replicates from a pool of 10 caterpillars. The presuppositions of normality and homoscedasticity were verified with SAS residue analysis (PROC MIXED followed by PROC UNIVARIATE and PROC GPLOT) (SAS 2013). The means obtained for each enzyme analysis were compared using *t* test at 5% level of significance, protected by ANOVA. Data were processed with SAS software version 9.1 (SAS 2013).

Results

The analysis of the effect of the different diets on proteolytic activity in the *S. cosmioides* midgut showed that trypsin activity changed according larval diet (Fig. 1A, B), with those fed on cotton showing esterase and amidasic trypsin activity of 2,098.2 ± 654.4 and 86.4 ± 40.5 μmol s^{-1} per μg of protein, respectively, higher than those fed on soybean leaves and artificial diet.

For chymotrypsin enzyme, larvae fed on soybean leaves presented activity of 39.0 ± 13.0 μmol s^{-1} per μg of protein for esterase chymotrypsin (Fig. 1C), higher than for larvae fed on cotton and artificial diet ($P \leq 0.05$). Amidasic chymotrypsin activity was higher in larvae fed on

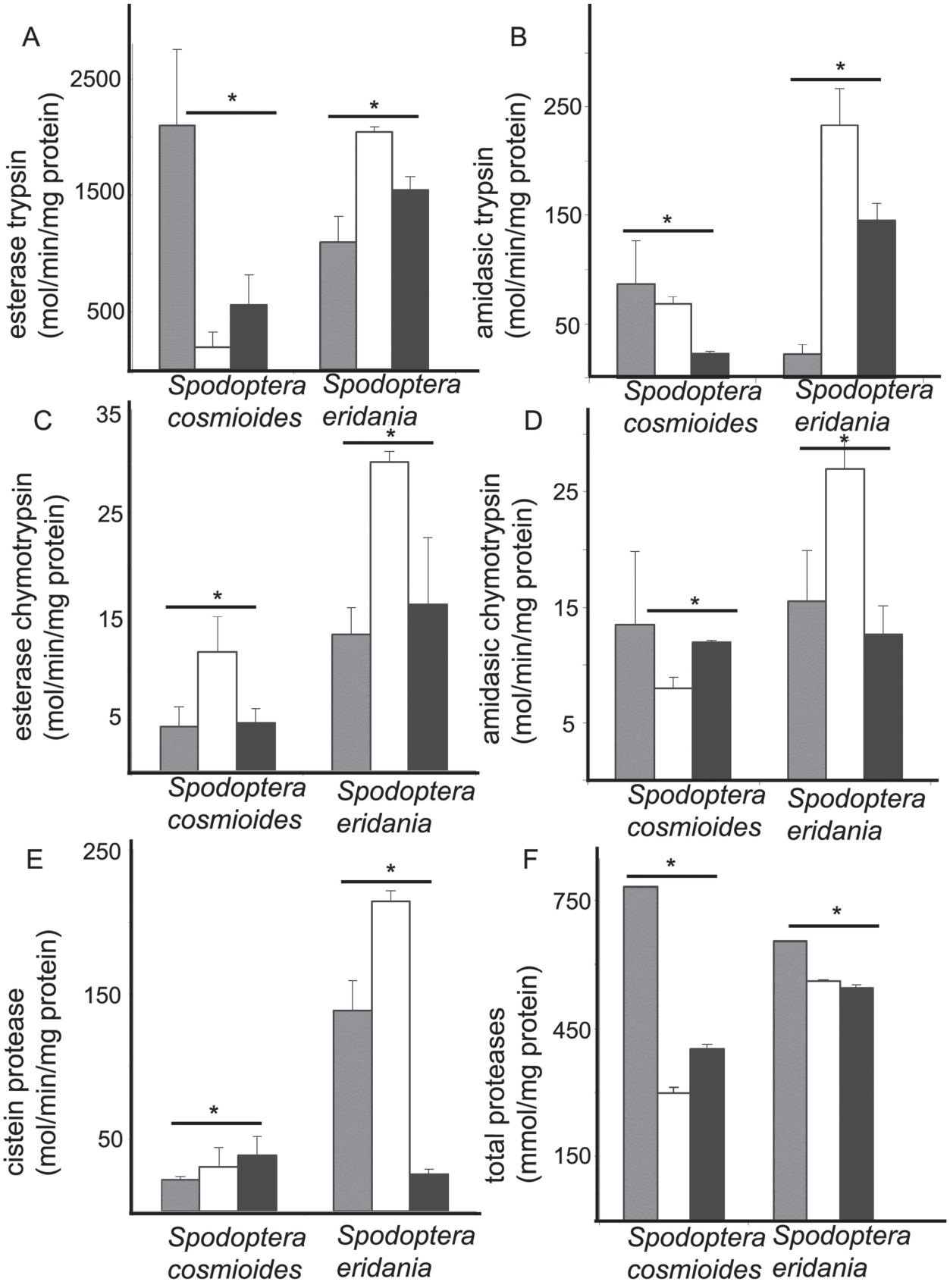

Fig. 1. Proteolytic activity in the midgut of *Spodoptera cosmioides* and *Spodoptera eridania* (Lepidoptera: Noctuidae) larvae fed on cotton, soybean, and artificial diets. (A) Amydasic trypsin. (B) Esterase trypsin. (C) Esterase chymotrypsin. (D) Amydasic chymotrypsin. (E) Cysteine protease. (F) Total protease. Gray bars: larvae fed with cotton; white bars: larvae fed with artificial diet; black bars: larvae fed with soybean. Bars with asterisks differ ($P > 0.05$) by the Fisher minimum difference test protected by analysis of variance.

cotton leaves (13.4 ± 6.4 μmol s^{-1} per μg of protein) than with soybean leaves and artificial diet (Fig. 1D).

In relation to cysteine protease activity, the higher activity was found in caterpillars fed on artificial diet ($P \leq 0.05$), with 11.5 ± 3.4 μmol s^{-1} per μg of protein (Fig. 1E).

The higher content of total proteases was found in the midgut of larvae fed on cotton leaves (784.2 ± 0.7 µg per µL) compared with the other treatments ($P \leq 0.05$) (Fig. 1F).

Spodoptera eridania showed higher trypsin activity in larvae fed on an artificial diet, ($P \leq 0.05$), with esterase and amidasic trypsin activities of 2,040.7 ± 42.9 and 233.3 ±33.8 μmol s^{-1} per μg of protein, respectively, followed by soybean and cotton leaves (Fig. 1A, B).

In the analyses of the chymotrypsin enzymes, the larvae fed on the artificial diet showed activity of 214.2 ± 7.2 μmol s^{-1} per μg of protein for esterase chymotrypsin and 27.0 ± 2.5 μmol s^{-1} per μg of protein for amidasic, followed by cotton and soybean leaves (Fig. 1C, D).

For cysteine protease, higher activity was found in caterpillars fed on artificial diet in comparison with cotton and soybean diets, with 30.0 ± 1.0 μmol s^{-1} per μg of protein (Fig. 1E).

The higher content of total proteases was found in the midgut of larvae fed on cotton leaves (654.7 ± 2.7 µg per µL) compared to the other diets ($P \leq 0.05$) (Fig. 1F).

The activity of serine proteases (trypsin and chymotrypsin), cysteine proteases, and total proteases varied according to insect species ($P < 0.05$) (Table 1). In addition, these enzymes, except for amidasic chymotrypsin, varied according to diet type (Table 1).

Discussion

The high serine protease activity in *S. cosmioides* larvae fed on cotton leaves suggests adaptation of this species to this plant. Cotton plants produce gossypol, which plays a role as protease inhibitor (Lara 1991; Calhoun 1994) and insects may bypass inhibitory effects overexpressing proteases (Jongsma et al. 1994; Mosolov & Valueva 2008; Moreira et al. 2011). *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) larvae fed on plants expressing trypsin inhibitors, present hyperproduction of this enzyme as an adaptation to protease inhibitors (De Leo et al. 1998).

The higher serine protease activity in *S. eridania* larvae fed on artificial diet may be due to this diet containing high protein content and some starch without compounds that decrease the activity of this enzyme (Mendonça et al. 2009). The esterase trypsin activity in *S. eridania* fed on soybean leaves is 3 times higher than *S. cosmioides* fed on the same diet, suggesting an increase in the expression of serine protease genes, favoring their polyphagous feeding habits. *Spodoptera frugiperda* is non-sensitive to soybean Kunitz trypsin inhibitor (SKTI) and soybean Bowman-Birk inhibitor (SBBI) due to an evolutionary mechanism favoring its highly polyphagous nature (de Oliveira et al. 2013).

Our findings show higher esterase activity of serine proteases (trypsin) compared to amidasic activity in both species studied, suggesting high affinity for L-TAME substrate (Oliveira et al. 2005). Proteases have acylation with slow acyl-enzyme formation and deacylation with rapid product formation during amidase activity, the latter being slow during esterase activity. The acylation with formation of acyl-enzyme is the main step in the hydrolysis reaction of amide substrates by trypsin enzymes, whereas deacylation is the main step of the ester substrate hydrolysis with product formation (Inagami 1972; Fastrez & Fersht 1973; Xavier et al. 2005).

The esterase chymotrypsin activity increases in *S. cosmioides* fed on soybean, suggesting higher soybean consumption compared with *S. eridania* (Bortolotto et al. 2015) and serine protease trypsin sensitivity

Table 1. Source of variation, degrees of freedom, and analysis of variance (ANOVA) for specific activities of proteolytic enzymes of *Spodoptera cosmioides* and *Spodoptera eridania* (Lepidoptera: Noctuidae).

		Amidasic trypsin		Esterase trypsin		Amidasic chymotrypsin		Esterase chymotrypsin		Cysteine proteases		Total proteases	
Source of variation	DF	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Insect	1	15.87	0.0018	5.82	0.0328	6.48	0.0256	95.4	0.0001	22.8	0.0004	263.87	0.0001
Diet	1	9.43	0.0035	1.86	0.1972	1.12	0.3585	27.9	0.0001	7.61	0.0073	1006.83	0.0001
Residual	12												

DF – degrees of freedom

to inhibitors in soybean plants. This compensatory feeding is a consequence of the absence of amino acids available for protein synthesis, increasing other serine proteases such as chymotrypsin (Scriber & Slansky 1981; Simpson & Simpson 1990) for insect growth (Broadway & Duffey 1986; Ryan 1990).

The higher cysteine protease activity for both species fed on artificial diet may be due to this diet not containing substances that affect the activity of this enzyme. However, survival of *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidade) is low with artificial diet plus a combination of trypsin and cysteine inhibitors, proving the importance of this enzyme for digestion in Lepidoptera even with low activity (Senthilkumar et al. 2010).

Cysteine proteases have low activity in the midgut of the larvae studied here, confirming that serine proteases are the most active proteolytic enzymes in Lepidoptera (Kipgen & Aggarwal 2014; Meriño-Cabrera et al. 2018).

The high total protease content in the midgut of *S. cosmioides* and *S. eridania* may be explained by the efficient use of plant proteins and artificial diet by both species. This indicates that the level of free amino acids and low molecular weight soluble proteins are sufficient for the development of *S. cosmioides* and *S. eridania* larvae, as reported for *Cameraria ohridella* (Deschka & Dimic) (Lepidoptera: Gracillariidae) (Stygar et al. 2010).

It has been claimed that the profile of midgut digestive enzymes is specific according to insect order (Terra & Ferreira 1994; Fialho et al. 2012; 2013). Our findings suggest that the variation in protease activity in the 2 species studied may be associated with their feeding habits. Nevertheless, whether changes in the amino acid composition or mechanism of action of these enzymes occurs due to evolutionary divergence or selective pressure exerted by different feeding habits remains uncertain.

Our findings show that diet-biased protease activity (serine and cysteine proteases and total proteases) presents high plasticity, suggesting that diet affects the amount of specific digestive proteases available for digestion, which may be related to de novo synthesis or post-translational activation of proteinases (Bolter & Jongsma 1995). In addition, this diet-biased variability may be explained by the rapid change in digestive proteolytic metabolism in these insects following ingestion of proteinase inhibitors from plants (Bolter & Jongsma 1995; Overney et al. 1997). Digestive proteases in animals are affected by both the amount and type of the proteins ingested (Lhoste et al. 1993; Noriega et al. 1994).

Overall, this study shows that the variability of proteolytic activity according to insect species may reflect different adaptations of these pests to the nutritional composition of plants with protease inhibitors.

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