

Post-ingestive effects of flavonoids in the midgut epithelium of *Anticarsia gemmatalis* (Hübner 1818) (Lepidoptera: Erebidae) larvae

Efeitos pós-ingestivos de flavonóides no epitélio do intestino médio de larvas de Anticarsia gemmatalis (Hübner 1818) (Lepidoptera: Erebidae)

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Abstract

An alternative control to soybean caterpillar has been the use of insect resistant plants that present phenolic flavonoids. The midgut is the main access pathway of food and chemical substances ingested. The present study examined morphological alterations in the midgut epithelium of the soybean caterpillar, after the ingestion of soybean genotypes containing the flavonoids rutin and genistin. The caterpillars and genotypes (BRS 257 - control, BR 16, Dowling, PI 229358, IAC 100 and PI 227687) were obtained from the insect rearing facility at the Embrapa Soja. The midguts were collected, fixed in Karnovsky, processed and analyzed under a light microscope. All treatments caused alterations in the midgut epithelial cells. These alterations in the columnar cells were more assiduous than the on other cell types, showing an increase of cytoplasmic protrusions and vacuoles. The goblet cells showed few changes for all genotypes tested, while the regenerative cells presented alterations mainly for the Dowling and PI 227687 treatments. The peritrophic membrane was absent for genotypes IAC100 and PI 227687. We conclude that the Dowling and PI 227687 genotypes were effective and promoted great morphological alterations in the midgut of the soybean caterpillars, being able to be very effective for the control of this plague.

Keyword: Soybean caterpillar. Morphology. Digestive tract. Rutin and genistin.

Resumo

Um controle alternativo para a lagarta da soja tem sido o uso de plantas resistentes a insetos que contém flavonóides fenólicos. O intestino médio é a principal via de acesso do alimento e substâncias químicas ingeridas. O presente estudo examinou as alterações morfológicas no epitélio do intestino médio da lagarta da soja, após a ingestão de genótipos de soja contendo os flavonóides rutina e genistina. As lagartas e os genótipos (BRS 257 - controle, BR 16, Dowling, PI 229358, IAC 100 e PI 227687) foram obtidos do laboratório de criação de insetos da Embrapa Soja. Os intestinos médios foram coletados, fixados em Karnovsky, processados e analisados ao microscópio de luz. Todos os tratamentos causaram alterações nas células epiteliais do intestino médio. As alterações foram mais assíduas nas células colunares do que nos demais tipos celulares. Essas apresentaram grande quantidade de protrusões citoplasmáticas e de vacúolos. As células caliciformes apresentaram poucas alterações para todos os genótipos testados, enquanto as células regenerativas apresentaram alterações principalmente nos tratamentos Dowling e PI 227687. A membrana peritrofica estava ausente para os genótipos IAC100 e PI 227687. Conclui-se que os genótipos Dowling e PI 227687 foram bastante efetivos e promoveram grandes alterações morfológicas no intestino médio das lagartas da soja, podendo ser bastante eficaz para o controle desta praga.

Palavras chave: Lagarta da soja. Morfologia. Tubo digestivo. Rutina e genistina.

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Introduction

Insect pests are one of the main yield reducing factors in different cultivated plant species. The soybean caterpillar, *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Erebidae), is considered the main foliage feeding pest of soybean in Brazil, causing great damage to this crop. To reduce the costs of production and negative environmental impacts in Brazil, efforts have been made to develop alternative methods to control this caterpillar and other soybean insect-pests (HOFFMANN-CAMPO; HARBONE; MCCAFFERY, 2001). Genotypes resistant to insects are considered an ideal method of control, since the insect population can be reduced, but not causing considerable damage in the field and to environment. Furthermore, it doesn't require adopting sophisticated technology for the producers and with low production costs (LARA, 1991).

Resistance to insects is mainly due to chemical substances (allelochemicals) present in the host plants, such as alkaloids, flavonoids, terpenoids, sterols, etc. (KUBO; HANKE, 1986). In soybean, the probable defense substances are the flavonoids that are generally more abundant in pest-resistant genotypes. These flavonoids form a group of natural substances that have various biological activities (HOFFMANN-CAMPO, 1995).

Tests of food preferences on *A. gemmatalis* were developed and the researchers observed that the genotypes BR82-12547, IAC74-2832, PI 227687 and PI 274454 were the most rejected by the caterpillars (HOFFMANN-CAMPO; MAZZARIN; LUSTOSA, 1994). Later seven flavonoids were identified in PI 227687 (HOFFMANN-CAMPO, 1995), a genotype widely used in breeding programs as source of resistance to insects, indicating toxicity in this cultivar and could explain the rejection by the insect. Among the phenolic compounds identified, the flavonoid rutin and the isoflavonoid genistin are recognized as playing an important role in the defense of soybean to lepidopteran insects. Previous studies of these substances have shown an antibiotic and/or non-edible effect in larvae of various insect pests (HOFFMANN-CAMPO, 1995; HOFFMANN-CAMPO; HARBONE; MCCAFFERY, 2001; STAMP; SKROBOLA, 1993).

The action of these substances begins when the caterpillar ingests the soybean leaves; the digestion occurs on the digestive tract, especially on the midgut, the principal region of the absorption of food and

chemical-biological substances (CHAPMAN, 2013; TERRA; FERREIRA, 1991). Levy et al. (2004, 2009) and Levy (2005), have studied the internal morphology of digestive tract of the *A. gemmatalis* for years, especially with insects infected with nucleopolyhedrovirus *AgMNPV*, but there are no reports of the acting of flavonoids in midgut epithelium. Thus, the objective of the present study was to investigate the alterations caused by soybean genotypes with different rutin and genistin concentrations on the midgut epithelium of these insects.

Material and Methods

Resistant soybean plants tested in this experiment were the genotypes BRS 257 (control), BR 16, Dowling, PI 229358, IAC 100 and PI 227687 (table 1). They were produced under greenhouse conditions (mean temperature $23 \pm 2^\circ\text{C}$, mean relative humidity 78%) at the Empresa Brasileira de Pesquisa Agropecuária - Embrapa Soja, Londrina, Paraná State, Brazil.

Table 1 - Genealogy of the soybean genotypes used and the rutin and genistin concentrations.

Treatments Genotypes	Genealogy	Concentration (mg/g $\times 10^{-2}$)	
		Rutin	Genistin
BRS 257	BR93-32109 x BR94-23396	0.0	0.0
BR 16	D69-1310-M58 (D49-772 x D49-2491) x 'Davis'	0.0	0.16
Dowling	Semmes x PI 200492	0.0	4.29
PI 229358	Tóquio, Japão	2.12	1.36
IAC 100	'IAC 12' x IAC 78-2318	9.72	1.42
PI 227687	Okinawa, Japão	36.82	1.22

Source: Valle e Lourenção (2002).

The leaves were collected, washed in 5% sodium hypochlorite solution, rinsed in distilled water and kept in an acrylic box containing *A. gemmatalis* eggs. The eggs were also supplied by the entomology laboratory at Embrapa Soja. To obtain the eggs, the larvae were maintained in the laboratory on an artificial diet (HOFFMANN-CAMPO; OLIVEIRA; MOSCARDI, 1985), under controlled temperature ($25-27^\circ\text{C}$), photoperiod (14L/10D) and 80% relative humidity.

After hatching, the larvae were observed daily until 2th instar when they were placed in 6 acrylic boxes containing 25 larvae/box. The larvae were continuously fed with leaves until the 4th instars (10 - 12 days of development) and the leaves were substituted every 12 hours there is no lack of food to the insects. In 4th instar the larvae were dissected in insect saline solution (ISS- 1.80g de NaCl; 1.88g de KCl; 0.16g de CaCl₂; 0.004g de NaHCO₃; distilled water-q.s.p. 100ml).

The midguts (25 per treatment) were fixed in glutaraldehyde (2.5%) and paraformaldehyde (4.0%) solution (0.1M phosphate buffer, pH 7.3) for 6h, dehydrated in ethylic alcohol solutions and embedded in glycol methacrylate resin (Historesin®-Leica), according to the manufacturer instructions.

The material was cut with glass knives in a rotary microtome (Leica RM 2145) and the sections with 3µm were stained with Haematoxylin-Eosin and analyzed in under a light microscope (Axiophot, Zeiss) at the Laboratório de Insetos/Departamento de Histologia/ Universidade Estadual de Londrina.

Results

The qualitative degree of the alterations caused by rutin and genistin in midgut of *A. gemmatalis* are shown in Table 2. In larvae treated with BRS 257 (control), the midgut wall presented an epithelial layer, covered by a peritrophic membrane and surrounded by a muscular layer (Fig. 1A).

All the genotypes tested caused damages in the epithelial cells. The Dowling and PI 227687 genotypes caused the greatest alterations (Figs. 1C and 1F) in all midgut collected. The insects that fed PI 229358, BR 16 and IAC 100 (Figs. 1D, 1B and 1E) also showed the tube with morphological alterations, but these were punctual and more scarce. The treatments promoted morphological changes in columnar cells (Co), with intensely disorganized on the striated border (S) (Figs. 1C, 1D and 1F); great cytoplasmic vacuolization (V) (Figs. 1B, 1C, 1D, 1E and 1F) and sometimes with total cell detachment in the direction of the intestinal lumen (L) (Figs. 1C, 1D and 1E).

In goblet cells (Gc), all the treatments showed damages, especially Dowling and PI 227687, when we could detect cell voluminous and with an apparent increase in the quantity (Figs. 1C and 1F). Alterations qualitative were also observed in the regenerative cells (Rc) in insects fed with the BR16, Dowling and PI 227687 genotypes (Figs. 1B, 1C and 1F).

The peritrophic membrane (Pm) showed changes in all treatments, as fragmentation and distance of epithelium observed in BR16, Dowling e PI229358 (Figs. 1B, 1C e 1D), or total absence as detected in IAC100 and PI227687 (Figs. 1E e 1F).

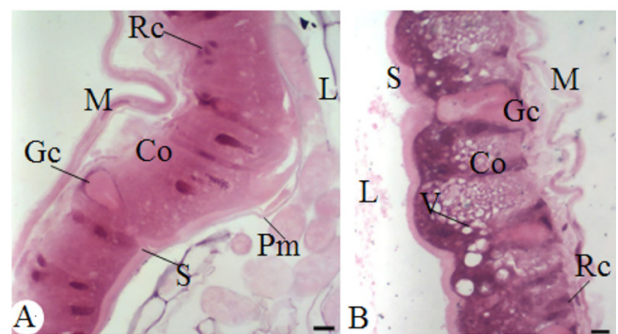
Table 2 - Qualitative assessment of the alterations caused by rutin (R) and genistin (G) in the midgut epithelium of *A. gemmatalis* larvae. Flavonoids concentrations in mg/g x10⁻².

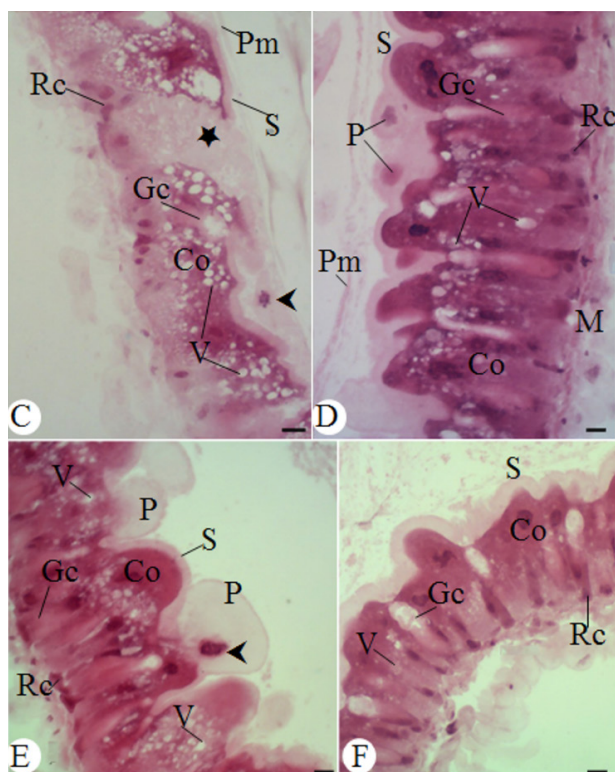
Structure	Genotypes					
	BRS257 R: 0 G:0	BR16 R: 0 G:0.16	Dowling R: 0 G:4.29	PI229358 R: 2.12 G: 1.36	IAC100 R:9.72 G: 1.42	PI227687 R: 36.82 G:1.22
Peritrophic membrane	-	+	+	+	+++	+++
Striated border	-	-	+++	+	+	+++
Columnar cells						
Cytoplasm cell	-	++	+++	++	++	++
Protrusions	-	+	+++	+++	+++	++
Goblet cells	-	++	+++	++	++	+++
Regenerative cells	-	+	++	+	+	++

Legend: Key (-) no alteration; (+) slight alteration; (++) moderate alteration; (+++) strong alteration.

Source: Authors.

Figure 1 - Photomicrographs of the *A. gemmatalis* midgut showing alterations from the following treatments: BRS 257 (control) (A); BR 16 (B); Dowling (C); PI 229358 (D); IAC 100 (E); PI 227687 (F).





Legend: Columnar cells (Co), cytoplasmic vacuoles (V), striated border (S), cytoplasmic protrusions (P) sometimes with nucleus (★), goblet cells (Gc), regenerative cells (Rc), muscular layer (M), peritrophic membrane (Pm), lumen (L). Note total cell detachment (▶) to lumen. Bar = 10µm.

Source: Authors

Discussion

In larvae treated with BRS 257 (control), the midgut wall presented an epithelial layer, covered by a peritrophic membrane and surrounded by a muscular layer, similar to described by several authors in others Lepidopteran species (CHAPMAN, 2013; LEVY et al., 2004; LEVY et al., 2009; SOUSA et al., 2009).

Apical cytoplasmic protrusion released to the lumen was characteristic in all the treatments, but there was an apparent increase of these structures according to concentration of genistin and rutin when compared with the control. In midgut of *A. gemmatalis* infected by *AgMNPV*, Levy (2005) observed the same cellular degradation followed by cell regeneration, allowing the recovery of epithelial tissue against infection by the virus. Washburn, Wong e Volkman (2001) also noted these cytoplasmic columnar portions which considered it as a mechanism of resistance against infection by

viruses. Hakim, Baldwin e Smagghe (2010) informs that the protuberances may be associated with cell death, once when the midgut is infected with microbial agents, the columnar cells swell and form apical protuberances releasing their entire contents into the lumen. Another aspect was related by Cristofolletti, Ribeiro and Terra (2001) and Pinheiro, Quagio-Grassiotto and Gregório (2008), where the protrusions would be responsible by apocrine secretion of digestive enzymes into the lumen.

Similar aspects have been reported by several authors for various insect species including *Helicoverpa armigera* (BROOKS et al., 2002); *Aedes aegypti* (ARRUDA; OLIVEIRA; SILVA, 2008); *Apis mellifera* (KAKAMAND; MAHMOUD; AMIN, 2008); *Spodoptera frugiperda* (CORREIA et al., 2009); *Alabama argilaceae* (SOUSA et al., 2009), *Plutella xylostella* (RIBEIRO et al., 2013) and *Spodoptera exigua* (ADAMSKI et al., 2016)

The alterations detected in cytoplasm of columnar cells could be reflected in the digestive enzyme synthesis and secretion and nutrient absorption, since these cells presented organelles like rough endoplasmic reticulum, pinocytotic vesicle, Golgi complex, mitochondria and secretion granules (LEVY, 2005; ADAMSKI et al., 2016).

The striated border and the basal cytoplasmic region of this cell type were intensely altered in the Dowling treatment that only has the genistin isoflavonoid. Damages in the striated border and consequently on the glycoprotein lining of the glycocalyx would also be a crucial factor in their adhesion, recognition capacity and absorption of nutrients. The isoflavonoid genistin (7-O-β-D-glycoside) was observed in lower concentration in most of leaf extracts of the genotypes that are used by insects control program (PIUBELLI et al., 2005). Salvador (2008) studied the interaction of rutin and genistin, and observed higher mortality, reduction of initial and pupa weights, lower weight gain and growth in *A. gemmatalis*, besides causing greater morphological changes in midgut. Diets and genotypes containing only genistin did not present antinutritional effect on the insect or any major morphological changes.

Cytoplasmic vacuolization in apical and middle portion of columnar cells was constant in all the treatments, including BR16, considered by Piubelli et al. (2005) as susceptible to *A. gemmatalis* larvae, since it presents genistin in low concentration. Similar alterations were described by other authors,

(ADAMSKI et al., 2016; ARRUDA et al., 2008) who studied the cytotoxic effects of substances extracted from plants. Kakamand, Mahmoud and Amin (2008) also detected alteration by using other chemical controlled such as deltamethrin, malathion and thiamethoxam while Ribeiro et al. (2013) detected alteration by using commercial formulations of *Bacillus thuringiensis*.

According to Appel (1993), the columnar cells alteration could be associated to the process of activating phenolic compounds by oxidation, forming free hydroxyl radicals with toxic action, which would rupture the cell membrane and consequently disturb the insect metabolism.

The goblet cells, although less studied than the columnar, are important cellular type responsible to transport of ions to the midgut lumen. The K⁺ -pump is now known to be composed of two independent counterparts: a vacuolar H⁺ -ATPase (V-ATPase) responsible for the establishment of an H⁺ gradient and a K⁺ /H⁺ exchanger (WIECZOREK et al., 2009). According to Harvey et al., (2010), the stimulation of nutrient uptake by the presence of a V-H⁺-ATPase has subsequently been shown to be a common pattern found in other insect models a characteristic that is not shared by vertebrates. In our work, all the treatments showed damages in goblet cell, especially Dowling and PI 227687, when we could detect cell voluminous and with an apparent increase in the quantity. This aspect could change the midgut alkalization, and consequently deficiency in food digestion.

Alterations qualitative were also observed in the regenerative cells in insects fed with the BR16, Dowling and PI 227687 genotypes. The most differential aspects noted was that the apparent increase in number was detected especially in genotypes that contained only genistin in their constitution (Dowling). In the future, cell counts studies will be conducted to corroborate our visual observations on the goblet and regenerative cells.

The regenerative cells were shown in cells groups, called regeneration nests, and could be related to the epithelial cell renovation process that consists of substituting older or damaged cells (BILLINGSLEY; LEHANE, 1996; FRANÇA, 2006; ROST-ROSKOWSKA; VILIMOVA; CHAJEC, 2010). This intensive mitotic division is an important way to *A. gemmatalis* larvae in defends itself of compounds like rutin and genistin, as observed by França, (2006) and

Rost-Roszkowska, Vilimova and Chajec (2010), who worked with different controlling agents.

Another significant result noted was the fragmentation the peritrophic membrane of midgut of larvae fed with BR16, Dowling and PI229358 genotypes and/or its absence in those fed with the IAC 100 and PI 227687 genotypes. This membrane consists of a fine network composed of chitin, glycosaminoglycans, glycoproteins and proteins (LEVY et al., 2004; LEVY et al., 2009; TERRA, 2001). It has a role in preventing mechanical injuries to the MI epithelial cells and responsible for the compartmentalization of digestion and protection against microorganisms and toxins ingested along with food (TERRA, 2001). Thus, the rupture of this structure could be associated with the intense disorganization of the columnar cells and consequently your synthesis.

Conclusions

In this study we can conclude, through morphological analysis, that there were great alterations in the epithelium of *A. gemmatalis* larvae in all the treatments tested. We also observed that these alterations occurred principally in the columnar cells which showed intense vacuolization and increase in protrusions released towards the lumen, and in goblet cells which increase in number. The regenerative cells and peritrophic membrane were also modified in some genotypes tested, indicating that the compounds rutin and genistin can affect the digestion and development in *A. gemmatalis* larvae, even leading to death. Generally, among the genotypes tested, those that caused the greatest alterations were Dowling and PI 227687. These data may be utilized as an additional tool to control this pest in the soybean, either as recommended cultivars or for genetical enhancement.

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