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Artículo de investigación

Efecto de tres flavonoides de la soya sobre el intestino medio y la sobrevivencia de larvas de *Anticarsia gemmatalis* (Hübner) (Lepidoptera: Noctuidae)

Effect of three soybean flavonoids on the midgut and larval survival of Anticarsia gemmatalis (Hübner) (Lepidoptera: Noctuidae)

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RESUMEN

Introducción: La soya *Glyciine max* L. Merrill (Leguminosae: Papilionoidae) corresponde a uno de los principales cultivos en el Brasil, por lo tanto, constantemente se ve afectada por la presencia de plagas defoliadoras, como *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae). No obstante, algunas variedades de soya contienen compuestos químicos que pueden cumplir funciones de defensa contra estas, como los flavonoides. **Objetivo**: Este trabajo se propuso evaluar los efectos del consumo de plantas susceptibles, resistentes y de flavonoides sobre el intestino medio de las larvas de *Anticarsia gemmatalis*. **Metodología**: Las larvas de *Anticarsia gemmatalis* fueron alimentadas con hojas de plantas de soya susceptibles, resistentes, y una dieta artificial que contenía la misma concentración de flavonoides encontrados en la planta. Inicialmente, se realizaron análisis de sobrevivencia, tiempo de desarrollo y peso, y posteriormente se diseccionó el intestino medio de las larvas en el 5º instar de desarrollo y se realizaron análisis histopatológicos. Adicionalmente, hojas de soya se expusieron a defoliación por parte de *Anticarsia gemmatalis*, y se realizó extracción de metabolitos secundarios y análisis de flavonoides, daidzeina, quercetina y rutina, por cromatografía de alta performance (HPLC). **Resultados**: Las larvas alimentadas con la variedad de soya resistente tuvieron una menor ganancia de peso en comparación con la susceptible. Por lo otro lado, la sobrevivencia de *Anticarsia gemmatalis* se redujo en la dieta artificial que contenía los tres flavonoides en comparación con el control. Los análisis histopatológicos mostraron cambios morfológicos causados por la soya IAC-PL1 como, deformación de células columnares y reducción en el número de células regenerativas; en las larvas alimentadas con IAC-17 se observaron rupturas en la células columnares y deformación en las células del cáliz y en las alimentadas con la IAC-24 se generó desprendimiento del epitelio de la pared muscular y aumento en las vacuolas del citoplasma de las células columnares. Todas las dietas artificiales suplementadas con los flavonoides presentaron cambios morfológicos similares a los ocasionados por la soja resistente. Las plantas presentaron mayores concentraciones de los flavonoides analizados. **Conclusiones**: Las variedades de soya resistente al igual que las dietas suplementadas con los flavonoides daidzeina, quercetina y rutina, ocasionan daños

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en el intestino medio de las larvas de *Anticarsia gemmatalis*, originando alta mortalidad y menor ganancia de peso en comparación con el control. Los flavonoides estudiados, en conjunto, pueden encontrarse relacionados con la deficiencia en el desarrollo y el aumento de la mortalidad en *Anticarsia gemmatalis*.

Palabras clave: Histopatología, epitelio del mesenterón, intestino medio de insectos, sinergismo.

ABSTRACT

Morphological changes in *Anticarsia gemmatalis* Hübner caterpillar midgut, development and mortality feeding on soybean and artificial diets containing flavonoids were evaluated. Soybean flavonoid concentrations were measured, and artificial diets prepared to add synthetic flavonoids at same soybean proportions. *Anticarsia gemmatalis* caterpillars fed on susceptible and resistant soybean and artificial diets were submitted to histological analysis. Survival, development period and weight were accounted. Flavonoids daidzein, quercetin, and rutin were found in susceptible (IAC-PL1) and resistant (IAC-17, IAC-24) soybean. Only daidzein expressed high concentrations on resistant varieties. *Anticarsia gemmatalis* survival was lower feeding on resistant soybean and an artificial diet containing the three flavonoids. Caterpillars got less mass growth feeding on resistant than susceptible soybean. Artificial diets added with flavonoids did not have these effects and any treatment affected *Anticarsia gemmatalis* developing periods. Midgut cells were damaged, but not the peritrophic membrane and the muscular wall of the caterpillar midgut. The IAC-PL1 soybean deformed columnar cells and reduced the number of regenerative ones. The IAC-17 caused columnar cells rupturing and goblet cells deformation. The IAC-24 epithelium detachment from the muscular wall and increased vacuoles in the cytoplasm of the columnar cell. Artificial diets added with flavonoids caused similar changes in midgut morphology as those caused by resistant soybeans. Resistant soybean varieties damaged *Anticarsia gemmatalis* caterpillar's midgut, causing high mortality and decreased growth mass. Different daidzein, quercetin, and rutin concentrations, in these cultivars, may function synergistically against *Anticarsia gemmatalis* caterpillars as shown by the effects of artificial diet added with these three flavonoids.

Keywords: Histopathology, midgut epithelium, insect intestinal epithelium, synergism

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Introducción

Brazil is the second soybean *Glycine max* (L.) Merrill (Leguminosae: Papilionoidae) producer and exporter in the world with 288,610,000,000 tons in 2017/2018 (CONAB, 2021). The "velvetbean caterpillar" *Anticarsia gemmatalis* (Hübner, 1818) (Lepidoptera: Noctuidae), is an important defoliating pest of this crop in America between other lepidopterans (de Freitas *et al*., 2011). This insect is present in all regions where this plant is cultivated and caterpillars damage from seedling to plants in the vegetative stage (Riffel *et al*., 2012; Panizzi, 2013).

However, the high quantity and frequency of insecticide application may reduce natural enemies in soybean crops, but it can affect the environment (Vianna *et al*., 2009). Soybean varieties with moderate insect resistance levels had been development (Piubelli *et al*., 2005; Kim *et al*., 2014) and are used associated with viral (Levy *et al*., 2011; Bernardi *et al*., 2012; Brito *et al*., 2015) or botanical insecticides (Nascimiento *et al*., 2003; Navickiene, 2007; Ribeiro *et al*., 2015) to solve this situation. This fact can reduce or eliminate the use of pesticides and improve the ecological sustainability of agricultural systems (de Freitas *et al*., 2011).

Many wild or cultivated soybean plants have some degree of insect resistance through secondary metabolites like flavonoids (Zhou *et al*., 2011; Silva *et al*., 2013; Tavares *et al*., 2014; Zavala *et al*., 2015). Flavonoids are found in different soybean parts and had been relationship with resistance against defoliating insects (Piubelli *et al*., 2005; John *et al*., 2013 and Magarelli *et al*., 2014). The flavonoids daidzein, quercetin, and rutin cause disturbances in the caterpillar midgut affecting lepidopteran development and biology (Jadhav *et al*., 2012; Silva *et al*., 2016).

Furthermore, *Anticarsia gemmatalis* midgut has a single-layered columnar epithelium with digestive, goblet, and regenerative cells likely other Lepidoptera (Chiang and Peng, 1986; Binder and Bowers 1994; Levy *et al*., 2008; Gomes *et al*., 2013). The peritrophic membrane separates the food bolus from the midgut epithelium protecting it against mechanical damage, but it is permeable to digestive enzymes and nutrients as well as a barrier against pathogens (Eisemann and Binnington, 1994; Terra 2001; Levy *et al*., 2009; Silva *et al*., 2016). Changes in caterpillar midgut morphology and mortality caused by flavonoids need to be better-studied and settle how these compounds affect the biology of lepidopteran (Gazzoni *et al*., 1997; Vandock *et al*., 2012).

Therefore, the flavonoids effects of susceptible (IAC-PL1) and resistant (IAC-17, IAC-24) soybean were accessed by picturing alterations on larval midgut morphology, development, and survival of *Anticarsia gemmatalis*

caterpillars and pupae fed on these cultivars and artificial diets added with synthetic flavonoids in equal proportions as found in soybean plants varieties. Thus, the objective of this work was to evaluate the morphological changes in the midgut of *Anticarsia gemmatalis* larvae, and its relationship with flavonoids.

Materials and Methods

Local. The experiments with artificial diets, biological assessments of *Anticarsia gemmatalis* caterpillars, and identification and quantification of flavonoids for the soybean varieties IAC-PL1, IAC-17, and IAC-24 were carried out at the Department of Biochemistry and Molecular Biology of the Universidade Federal de Viçosa (UFV) in Viçosa, Minas Gerais state, Brazil. The *Anticarsia gemmatalis* caterpillar midgut histology was analyzed in the Laboratory of Cell Biology, Molecular, and Microscopy Center of the General Biology Department of UFV.

Caterpillar rearing. *Anticarsia gemmatalis* (Hübner) adults were kept in cages (50 x 50 cm) with paper sheets for oviposition. Adults of this insect were fed on nutrient solution composed of honey (10.5 g), beer (350 mL), sucrose (60 g), ascorbic acid (1.05 g), nipagin (1.05 g), and water (1050 mL), embedded in cotton placed on the bottom of the cages in a Petri dish. *Anticarsia gemmatalis* egg masses were collected every three days and transferred to 500 mL plastic cups with a 2 cm circular hole in the cover closed with tulle screen. These cups were maintained at 25 ± 5 °C; 70 ± 10 % RH and 14:10 (d:n) hours photoperiod until hatching. The caterpillars were separated into groups of 20 individuals and placed in 500 mL plastic pots with about 10 g of artificial diet changed every three days. Pupae were placed in cages for an adult to emerge and copulate.

Preparing artificial diet. The artificial diet was composed of beans (4.8 g), brewer yeast (2.4 g), wheat germ (3.8 g), soy protein (3.8 g), casein (1.9 g), agar (1.34 g), and water (80 mL/100 g) mixed in an industrial blender and autoclaved at 1.5 kgf/cm^2 for 15 minutes and transferred to a blender. Ascorbic acid (6 g), sorbic acid (3 g), nipagin (methylparaben, 5 g), 40% formalin (6 mL) and vitamin solution (10 mL) (niacinamide1 mg, calcium pantothenate 1 mg, thiamine 0.25 mg, riboflavin 0.50 mg, pyridoxine 0.25 mg, folic acid 0.25 mg, biotin 0.02 mg and inositol 20 mg) and 1 L water were added and mixed until a homogenous consistency was obtained (Hoffman-Campo *et al*., 1985). At this point, for artificial diet used in experiments, several flavonoids previously weighted according to maximal concentrations found in resistant soybean cultivars were first diluted in 5 mL absolute ethanol (99.5%) and added to the mixture (diet used in control treatments were added with 5 mL ethanol without flavonoids). Then the liquid diets were left to cool in a germicidal chamber with ultraviolet light and stored at 4 °C.

Soybean varieties. Seeds of the susceptible IAC-PL1 and resistant IAC-17 and IAC-24 varieties were obtained from the Agronomic Institute of Campinas (São Paulo, Brazil). Variety IAC-PL1 is the product of a Japanese introduction which the grain has high protein concentration, but low productivity (578 ± 34 kg.ha^{-1}) (Lourenção *et al*., 2010) and has been demonstrated to be susceptible to pathogens and insects and used as the control for scientific research (Silva *et al*., 2010; Souza 2013). Varieties IAC-17 and IAC-24 are the product of different crossings (D 72-9601-1 x 'IAC 8' and IAC80-1177 x IAC83-288, respectively) and present high productivity (2953 kg.ha^{-1} for IAC-17 and 3480 kg.ha-1 for IAC-24) (Miranda *et al*., 2003; Silva *et al*., 2010), resistant to pests and pathogens of high genotype and phenotype similarity (Souza *et al*., 2016; Souza *et al*., 2017). Seeds of each variety were previously sprouted on a wet towel paper into germination chamber and then planted in pots containing 2 kg of the fertilized substrate inside a greenhouse (25 °C ± 5, 70% ± 10 RH) isolated in cages to avoid pest damage, pesticides free and daily irrigated. The planting was staggered to have the required availability of plant vegetative stages. Nine plants per variety were selected to access flavonoid concentrations and the rest to feed *Anticarsia gemmatalis* caterpillars for experiments.

***Anticarsia gemmatalis* (Hübner) caterpillar development and mortality.**

Plant experiment: A mean of 155 *Anticarsia gemmatalis* caterpillars were used for each soybean cultivar. Ten first instar caterpillars from mass-rearing were put in trifoliate plant leaves at vegetative stages between V4 to V6. Leaves were covered with plastic cages of 150 mL closed with rubber bands. After three days, every plastic cage was opened and cleaned for caterpillars to be accounted, weighted, and instar determined. Then, repeated every two days until pupae formation. When necessary, each caterpillar group were put in a new leaf from the same or different plant. Once the caterpillars reached the third instar were separated in groups of three individuals per cage.

Artificial diets experiment: Five different artificial diets treatments were prepared and the control. Control consists of an artificial diet without flavonoids. First treatment, diet containing with daidzein, second treatment with quercetin and third with rutin. The other treatments were diets with a mix of flavonoids. Fifth treatment diet containing quercetin with rutin and a sixth treatment diet containing the three flavonoids. Every treatment had five blocks with twelve caterpillars each kept in 300 mL plastic cups with 10 g of diet changed every two days. Caterpillars were accounted, weighted and instar determined every two days until pupae formation. Experiments were conducted in a laboratory at 25 ± ºC, 60 ± 10 % RH and photoperiods of 10:14 L to D.

Histology of the midgut of the caterpillar *Anticarsia gemmatalis* (Hübner). Eight *Anticarsia gemmatalis* caterpillars per plant and artificial diet treatment were dissected in 125 mM NaCl, and four midguts per treatment were transferred to Zamboni fixative solution. Then the samples were dehydrated in a graded ethanol series (70%, 80%, 90%, and 95%) and embedded in JB-4 historesin.

Stained sections 3 µm thick with haematoxylin and eosin were observed in a light microscope and pictures taken. The control for the treatments was the artificial diet without flavonoids.

Flavonoids identification and quantification. Plants were exposed to damage by *Anticarsia gemmatalis* herbivory to access the high flavonoid concentrations in plants. The third trifoliate leaf from bottom to the top of each plant in the vegetative stage 3 (V3) was collected, wrapped in foil packages, frozen with liquid nitrogen, and stored at -80 °C for chemical compound analysis. Flavonoids were determined by high-performance liquid chromatography (HPLC) in the Biochemistry and Molecular Biology Department of UFV. The leaflets of the soybean plants of each variety were weighed (500 mg) and macerated with liquid nitrogen and transferred to 15 mL plastic tubes containing 5.0 mL of 80% methanol. This solution was submitted to ultrasound for 20 minutes and centrifuged at 3,000 g for 5 min at 4 °C. The extracts were evaporated in speed vacuum and resuspended in 2 mL of absolute methanol, filtered with PTFE lter 0.45 µm and stored vials. The soybean leaf extracts were eluted with a segmented gradient of acetic acid and 2% acetonitrile solution. An aliquot of 30 µL of leaf extracts from each soybean variety was placed into a chromatograph (Shimadzu Prominence LC pump 20 AD, detector SPD M 20 A, CTO oven 20 A, LabSolutions program) with a reverse-phase column to detect secondary metabolites (C-18, 4.6 mm internal diameter, 150 mm length, 4.8 µm particle diameter). Chromatographic force of the mobile phase ranged from 0 to 4 min 5% acetonitrile; 4 to 15 min 20% acetonitrile; 15 to 20 min 35% acetonitrile; 20 to 30 min 90% acetonitrile; 30 to 40 minutes 5% acetonitrile; and the column temperature was 40 °C (CTO-20A). The solvent was 0.6 mL/min and absorption measured at 254 nm (DAD). The flavonoids concentration was ascertained by comparison with standards kaempferol, daidzein, daidzin, genistein, genistin, rutin, and quercetin. Curves were calibrated by injection of daidzein (1.2; 2.4; 3.6; 4.8 µg), quercetin (0.8; 1.6; 2.4; 3.2 µg), and rutin (0.8; 1.6; 2.4; 3.2 µg) concentrations. Linear regression curves were derived from the concentration peak area of these compounds and concentrations of these flavonoids in the samples calculated. A complete randomised design was applied for statistical analysis.

Data analysis. Data normality was verified with the Shapiro test and homogeneity of variances with the Bartlett test. Analysis of variance (ANOVA) was applied to determine differences between flavonoid concentration found in soybean cultivars, caterpillars weight and larvae period. Means were compared by Tukey HSD test ($p < 0.05$). Caterpillar survival was estimated by the Kaplan-Meier method, and curves were compared with Log-Rank Test at 95% confidence interval. Statistical analyses were performed using the statistical program RGui 3.4.3.(R Development Core Team (2017).

Results

Caterpillar survival. Kaplan-Meier method showed that the final percentage of caterpillar survival fed on soybean cultivar was 78% for IAC-PL1; 61% for IAC17; and 66% for IAC-24. The highest death rates were observed during day seven on IAC-PL1, day 7 and 15 on IAC-17 and day15 for the IAC-24 (Figure 1a). Log-Rank test showed significant differences in survival caterpillars between soybean varieties (X2= 11.9; d.f.= 2; p= 0.0075), those being between susceptible variety IAC-PL1 and resistant IAC-17 and 24 (X2= 10,2; d.f.= 1; p= 0.0013 and X2= 5.4; d.f.= 1; p= 0.02; respectively), but not between resistant ones (X2= 0.7; d.f.= 1; p= 0.42).

Experiment with flavonoids in artificial diets did not show differences between the total treatments (X2= 6.4; d.f.= 5; p= 0.265) and only the treatment which include the three flavonoids in the diet had significant difference with control diet (X2= 3.9; d.f.= 1; p= 0.0479). Caterpillars fed on a diet containing daidzein, quercetin and rutin had an ultimate survival of 60% against 76% of control diet (Figure 1b). This survival was close to treatments with soybean varieties IAC-17 and IAC-24. The diet with only quercetin was the second to bring mortality to *Anticarsia gemmatalis* caterpillars with 66% survival, followed by diets with only daidzein (71%), quercetin and rutin (76%) and only rutin (78%).

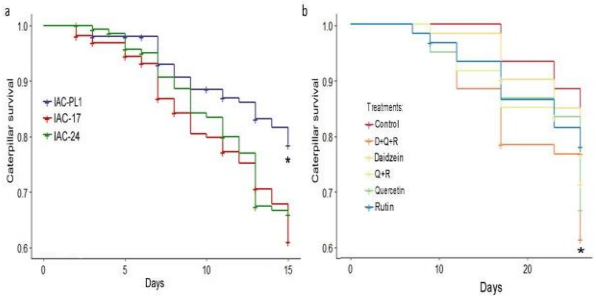

Figure 1 Survival of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) caterpillars and pupae by Kaplan-Meier method: (a) fed on leaves of susceptible (IAC-PL1) and resistant (IAC-17 and IAC-24) soybean (curve mark with asterisc "*" differ from others); (b) fed on artificial diets added with flavonoids (curves mark with asterisc "*" differ from control). Significance differences were determined by Log-Rank test at 95% confidence interval.

Caterpillar development. Neither soybean varieties nor artificial diets had effects on caterpillar periods (data not shown). Resistant soybeans have a negative effect on the weight of *Anticarsia gemmatalis* caterpillars. Those fed on IAC-17 soybean got less mass growth on caterpillars instar 4th and 5th, followed by IAC-24 and compared to susceptible IAC-PL1, from which caterpillars gain more mass growth. The caterpillars weight fed on artificial diets shows differences only at fourth instar between diets

containing only quercetin and diets containing quercetin and rutin (Table 1).

Table 1 Weight (means and standard errors) of *Anticarsia gemmatalis* (Noctuidae: Lepidoptera) caterpillars and pupae feed on: susceptible (IAC-PL1) and resistant (IAC-17, IAC-24) soybean cultivars (d.f= 2;15); and on artificial diets (d.f.= 5;20). Control (without flavonoids); added with only Daidzein, Quercetin or Rutin; added with a mixture of Quercetin and Rutin (Q+R); and a mixture of Daidzein, Quercetin and Rutin (D+Q+R). Means indicated with the same letter do not differ between treatments by Tuckey HSD test ($p < 0.05$).

	Caterpillar instars																Pupa			
	L2				L3				L4				L5							
	Mean	S.E.	F	P	Mean	S.E.	F	p	Mean	S.E.	F	p	Mean	S.E.	F	p	Mean	S.E.	F	p
Soybean																				
IAC-PL1	6.13^{a}	1.52	2.59	0.11	57.2^{a}	10.5	1.28	0.31	176.4^{a}	9.25	3.36	0.05	294.9^{a}	22.1	12.4	<0.01	175.1^{a}	23.9	0.02	0.98
IAC-17	6.34^{a}	0.87			38.3^{a}	9.9			133.8^{b}	12.3			180.9^{b}	14.6			172.0^{a}	2.83		
IAC-24	3.38^{a}	0.16			57.3^{a}	10.4			157.7^{c}	8.61			200.9^{c}	7.88			172.0^{a}	3.29		
Flavonoid																				
Control	3.41^{a}	0.28	1.83	0.14	12.6^{a}	0.76	1.28	0.31	60.9^{ab}	4.66	2.48	0.05	261.7^{a}	13.4	0.59	0.70	225.4^{a}	4.84	0.67	0.65
Daidzein	3.68^{a}	0.36			11.8^{a}	0.82			57.1^{ab}	4.04			271.5^{a}	9.61			222.9^{a}	4.14		
D+Q+R	4.42^{a}	0.59			14.9^{a}	1.94			62.6^{ab}	8.87			285.9^{a}	13.0			224.4^{a}	6.63		
Quercetin	3.32^{a}	0.25			13.8^{a}	1.10			67.6^{b}	2.53			276.9^{a}	14.6			221.3^{a}	5.80		
Q+R	3.32^{a}	0.25			10.8^{a}	1.28			43.8^{a}	4.89			260.1^{a}	15.7			229.6^{a}	10.2		
Rutin	3.11^{a}	0.1			12.5^{a}	1.16			53.4^{ab}	4.86			263.1^{a}	11.9			235.5^{a}	4.75		

Morphology changes in caterpillar midgut by diet consumption. In all treatments, the peritrophic membrane did not have apparent fragmentation. In *Anticarsia gemmatalis* caterpillar midgut fed on an artificial diet without flavonoids had an intact muscle wall, peritrophic membrane and a simple columnar epithelium with striated border evident in the apical portion. Goblet cells were less numerous, with a central cavity and attended nucleus. Regenerative cells were small round or oval (Figure 2a and Figure 3a). Digestive cells were numerous and columnar with basophilic granular cytoplasm, with an elongated nucleus in the cell apical third. The apical surface of digestive cells showed an acidophilic region, the striated border. Small cytoplasmic protrusions were released into the gut lumen.

The midgut of *Anticarsia gemmatalis* caterpillars feed on leaves from the insect susceptible soybean IAC-PL1 did not show important changes in the muscle layer, only partial detachment of the intestinal epithelial cells and a low regenerative cell count (Figure 2b). Caterpillars fed on leaves of the insect-resistant soybean IAC-17 had a high number of regenerative cells, discontinuities in the epithelium and disruption of digestive cells due to number and growth of goblet cells, which cavities were dilated losing their calyx form turning shapeless and disorganised in appearance (Figure 2c). The midgut of *Anticarsia gemmatalis* caterpillars feed on leaves of the insect-resistant soybean IAC-24 showed epithelium detachment from the basement membrane, including regenerative cells and many vacuoles in the cytoplasm (Figure 2d).

Digestive cells had nuclei with the predominance of condensed chromatin (Figure 2d-c). Soybean varieties did not affect the peritrophic membrane of *Anticarsia gemmatalis* caterpillars.

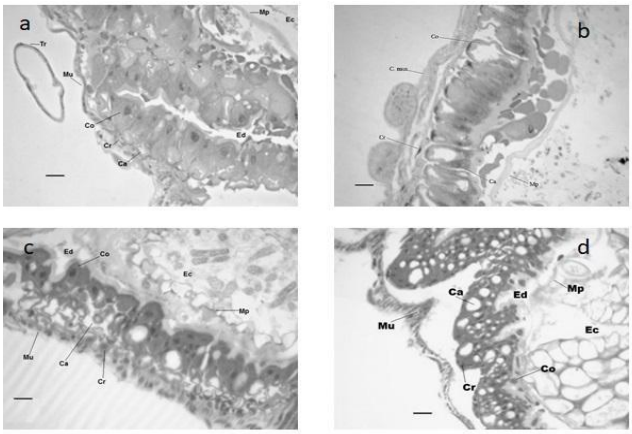

Figure 2 Light micrographs (20x) of the midgut of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) caterpillar fed on: artificial diet without flavonoids (a); susceptible soybean varieties IAC PL1 (b), resistant soybean varieties IAC-17 (c) and resistant soybean varieties IAC-24 (d). Mu = muscle layer; Co = columnar cell; Cr = regenerative cell; Ca = goblet cell; Ed = endoperitrophic space; Ec = ectoperitrophic space; Mp = peritrophic membrane; Tr = tracheola; Bars = 20 µm.

Larvae of *Anticarsia gemmatalis* fed on an artificial diet with flavonoids (Daidzein, Quercetin and Rutin) showed histopathological changes in the midgut epithelium larval (Figure 3b-f), mainly present high vacuolization in the cytoplasm, dislocated and deformed nuclei, elongation of columnar epithelial cells, destruction of the brush border of columnar epithelial cells and cell fragments in the

endoperitrophic space. Control larvae showed an epithelium with digestive cells having a well-developed brush border and the nucleus with decondensed chromatin. The goblet cells were less, with abundance vacuoles (Figure 3a).

Larva after feeding on a diet with one flavonoid, showed aspects like control, with the difference (especially under Daidzein treatment) of having an abundance of vacuoles in the digestive cells and cell fragments in the endoperitrophic space (Figure 3b-d). The treatment with Rutin and Quercetin showed globet cells with an increase in size and with a deformed, compacted and dislocated nucleus, loss of the brush border and cell fragments released in the midgut lumen (Figure 3e). Treatment with three flavonoids damaged columnar cells, blebbing cells and attachments in the basal membrane. High vacuolization, autophagic bodies, and apocrine vesicles containing amorphous cytoplasmic content. Also, many cell fragments were dispersed in the endoperitrophic space, which indicates an essential cell's death. Goblet cells with changed morphology, spherical, and reduced plasma membrane projection (Figure 3f).

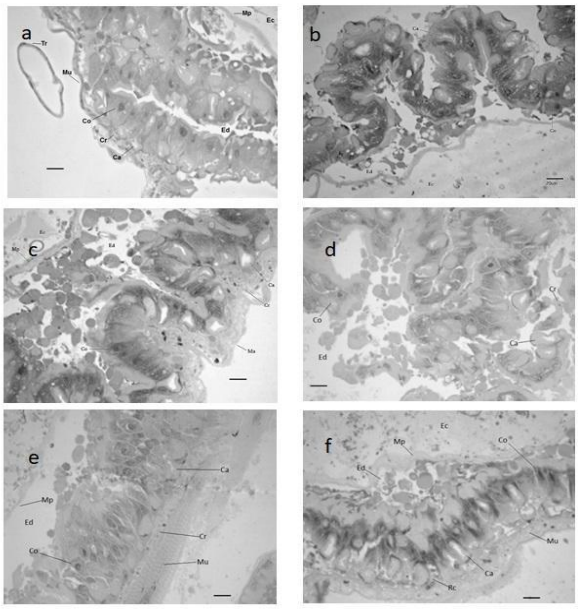

Figure 3 Light micrographs (20x) of the midgut of *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) caterpillar fed on: artificial diet without flavonoids (a), artificial diet with Quercetin (b), artificial diet with Rutin (c), artificial diet with Daedzein (d), artificial diet with Quercetin + Rutin (e) and artificial diet with Quercetin + Rutin + Daidzein (f). Mu = muscle layer; Co = columnar cell; Cr = regenerative cell; Ca = goblet cell; Ed = endoperitrophic space; Ec = ectoperitrophic space; Mp = peritrophic membrane; Tr = tracheola; Bars = 20 µm.

Flavonoid concentrations of soybean varieties. Three flavonoid compounds, daidzein, quercetin and rutin were determined from nine plants of each of the three soybean varieties IAC-PL1, IAC-17 and IAC-24. One-way ANOVA for a completely randomised design indicated significant differences for flavonoids concentrations between soybean varieties [Daidzein ($F_{2,24} = 45.455$; $p = 6.891 \times 10^{-09}$); Quercetin ($F_{2.24} = 17.247$; $p = 2.276 \times 10^{-05}$); Rutin ($F_{2.24} = 12.09$; $p = 0.0002334$)] (Figure 4). Daidzein had high concentrations in the three soybean varieties, mainly in the IAC-17 and IAC-24, while quercetin and rutin had low and similar concentrations in the three soybean varieties. The average values of these flavonoids in the susceptible variety IAC-PL1 were 0.423 mg/g daidzein; 0.087 mg/g quercetin; and 0.20 mg/g of rutin. The IAC-17 and IAC-24 resistant varieties had 2.07 mg/g and 2.40 mg/g of daidzein; 0.177 mg/g and 0.107 mg/g of quercetin; and 0.130 mg/g and 0.330 mg/g rutin, respectively.

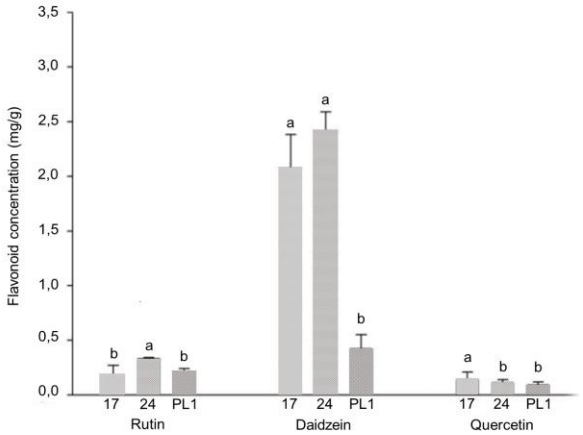

Figure 4. Flavonoids concentration in soybean *Glycine max* (Leguminosae: Papilionoidae) resistant IAC-17 (17), IAC-24 (24) and susceptible IAC-PL1 (PL1) to insects. Mean bars (± SEM; n= 9) between soybean varieties for each flavonoid with the same letter do not differ by Tukey HSD test ($p < 0.05$)

Discussion

Anticarsia gemmatalis (Hübner) (Lepidoptera: Noctuidae) caterpillars are important soybean pests and are important development natural control pesticides (Almeida *et al*., 2014). The higher mortality of *Anticarsia gemmatalis* caterpillars fed on the resistant soybean varieties IAC-17 and IAC-24, than for those fed on the susceptible IAC-PL1 may be due to damage in the caterpillar midgut. It was probably caused by deleterious effects of flavonoids acting synergistically, as it was commented on results of survival caterpillars fed on artificial diets containing these compounds. This high mortality of *Anticarsia gemmatalis* also agrees with similar results on the resistant soybean variety IAC-100.7 Artificial diets with daidzein, quercetin and rutin also caused significant mortality of *Anticarsia gemmatalis* caterpillars as previously demonstrated in similar experiments combining quercetin and rutin (Gazzoni *et al*., 1997; Salvador *et al*., 2010). Also causing mortality on *Sitophilus oryzae* (Linnaeus) (Coleoptera: Curculionidae) and *Rhyzopertha dominica* (Fabricius)

(Coleoptera: Bostrichidae) larvae (Nenaah 2013). Consumption of resistant soybean varieties, Consumption of resistant soybean varieties, especially IAC-17, negatively affects the mass growth of *Anticarsia gemmatalis* caterpillars in fourth and fifth instar. Artificial diets containing flavonoids did not affect larval stage duration nor caterpillar and pupal weight, like that of *P. guildinii* adults fed on IAC-17 and IAC-24 varieties (Silva *et al*., 2013).

Compounds of secondary metabolism such as flavonoids are produced in response to biotic stress against herbivores (Anshul *et al*., 2013; Nenaah 2013; Céspedes *et al*., 2014). Vandock (2012), Salvador (2010) and Silva (2016) demonstrated that the flavonoids compounds affect morphological and physiologically of the caterpillar midgut cell, like we demonstrated in this study. High concentrations of the daidzein in the resistant soybean varieties IAC-17 and IAC-24 may be responsible for their resistance to *Anticarsia gemmatalis* and two Hemiptera: Pentatomidaes, *Piezodorus guildinii* (Westwood) and *Nezara viridula* (Linnaeus) (Zavala *et al*., 2015). This compound is a vital antibiosis agent for insects (Zhou *et al*., 2011). Similar concentrations of quercetin and rutin in the three soybean varieties support their frequent expression in leaves of this plant (Zavala *et al*., 2015), whereas daidzein has been found in high concentrations in soybean seeds (Kim *et al*., 2014; Zavala *et al*., 2015). These compounds are part of plant constitutive defences (Zvala *et al*., 2015) and function synergistically against herbivorous insects (Barbehenn and Kochmanski, 2013; War *et al*., 2013). Daidzein expressed in the wild soybean *Glycine soja* Sieb et Zucc (Leguminosae: Papilionoidae) inhibits the growth of *Spodoptera litura* (Fabricius) (Lepidoptera: Noctuidae) caterpillars (Zhou *et al*., 2011). However, the development, fertility, and survival of *Lymantria dispar* (Linnaeus) (Lepidoptera: Lymantriidae) caterpillars were not affected when fed on artificial diets with different daidzein concentrations. Which may be due to this herbivore being a generalist and naturally exposed to this flavonoid (Karowe and Radi, 2011). Quercetin and rutin are generally better studied and were effective against *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) (Broussalis *et al*., 2010), *Musca domestica* Linnaeus (Diptera: Muscidae), *Aedes albopictus* Skuse (Diptera: Culicidae) (Wang *et al*., 2011) larvae and *S. litura*, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) (Jadhav *et al*., 2012; Pandey *et al*., 2012) and *Anticarsia gemmatalis* caterpillars (Gazzoni *et al*., 1997). Rutin has been demonstrated as a dangerous flavonoid for caterpillars because of its anti-nutritional effects (Jadhav *et al*., 2012; Silva *et al*., 2016), possibly more than daidzein. However, our results point to a synergistic effect of flavonoids combination and a significant effect of daidzein due to higher concentrations in the soybean varieties tested. Like Hay *et al*., (2020) showed, the soybean flavonoid compounds have synergism, in this work, was represented for the intestinal damages in cells of the caterpillars with the artificial diet supplement with three flavonoids, and the survival that was close to survival in treatments with resistant soybean varieties (that have these flavonoids and another not evaluated in this work). Despite, artificial diets containing daidzein do not affect the survival and development of *L. dispar* caterpillars (Barbehenn and Kochmanski, 2013). However, it may be a defence against phytophagous hemipterans (Piubelli *et al*., 2005). Rutin concentrations in the soybean varieties tested. Artificial diet did not prolong the *Anticarsia gemmatalis* larval stage, contrary to that of this species, consuming 0.45, 0.91, and 1.82mg/g of this compound in artificial diets (Salvador *et al*., 2010). Survival analysis indicated that a significant proportion of variation in *Anticarsia gemmatalis* survival during our trials could be attributed to the slow-action mode of these flavonoids (Grella *et al*., 2019). Other cause could be that the supplementation was doing in sublethal doses, or the plant has more compounds that participate in the resistance, the presence of cell elimination into the lumen, changes in the brush border, vacuolation/loss of cytoplasmic material, and presence/height of the brush border are indicating that happened an easily reversible lesion (Grella *et al*., 2019). General histomorphology of the midgut consists of two layers of muscle which includes inner circular and outer longitudinal and the epithelial layer consisting of columnar cells, goblet cells and regenerative cells and peritrophic membrane. The midgut morphology of *Anticarsia gemmatalis* caterpillars fed on an artificial diet without flavonoids agrees with descriptions for this lepidopteran fed on similar diets without flavonoids or other compounds that may alter cell morphology (Chiang *et al*., 1986; Salvador *et al*., 2014). The presence of flavonoids causes the histological variations in the epithelial layer, which may lead to digestive and food absorption disorders or even death. *Anticarsia gemmatalis* (Hübner) caterpillar midgut morphological alterations such as detachments of the epithelium, vacuolization, increase of goblet cell compartment, and tissue discontinuity for the resistant soybean varieties may be due to a synergistic action of daidzein, quercetin and rutin flavonoids, as similar reported for L. dispar (Barbehenn and Kochmanski, 2013). This fact may be due to oxidative stress changing glutathione redox balance in the caterpillar midgut. Similar changes in the midgut morphology were also reported for *Anticarsia gemmatalis* (Knaak and Fiuza 2005) and *Alabama argillacea* (Hübner) (Lepidoptera: Noctuidae) caterpillars (Sousa *et al*., 2010) infected with *Bacillus thuringensis* (Berliner) (Bacillales: Bacillaceae) and multiple nuclear polyhedrosis virus (Hay *et al*., 2020). Also, extracts seeds of Azadirachta indica, A. Juss (Sapindales: Meliaceae). Furthermore, a leaf of *Clerodendrum infortunatum* L. and *Eupatorium odoratum* L. in artificial diets generated similar effects in the midgut of *Anticarsia gemmatalis* caterpillar (Almeida *et al*., 2014) and *Orthaga exvinacea* Hampson (Lepidoptera: Pyralidae) (Ranjini and Nambiar, 2015) respectively. Vacuolization of the columnar and goblet cell cytoplasm may be due to the increased ion because these

cells are responsible for the active transportation of potassium ions from hemolymph into the gut lumen (Chiang *et al*., 1986). These detoxication vacuoles neutralize the deleterious effects of flavonoids (Sousa *et al*., 2010). The increase in the number of autophagic vacuoles in midgut digestive cells of *Anticarsia gemmatalis* larvae treated with flavonoids indicates that these cells undergo cytoplasmic reorganization. Autophagy is a primary proteolytic system for delivering cytoplasmic constituents to the lysosome for degradation with the help of hydrolytic enzymes, which has a crucial role in cellular energy mobilization and homeostasis. A possible explanation for apoptosis in midgut cells here observed might be because these cells cannot recover the damage caused by flavonoids leading to the activation of the apoptotic pathway, such as reported before for Fiat *et al*., (2018) treated *Anticarsia gemmatalis* with tebufenozide. The lack of damage to the peritrophic membrane by resistant cultivars may be explained by the presence of several layers with more chitin and a thicker epithelium (Silva *et al*., 2016) on *Anticarsia gemmatalis* caterpillars. The soybean caterpillar used in this study has a dark phenotype, dominant in dense populations, and is resistant to multiple nuclear polyhedrosis virus (Silva *et al*., 2016). Maybe, for this reason, the peritrophic membrane was not damaged. However, other undetected changes occur at the cellular and biochemistry level, related to the onset of toxin-induced apoptosis, like the enzyme V-ATPase (ATPV-ATPase vacuolar complex that sits on the apical membrane of the goblet cells. The interference in V-ATPase significant the alterations in the transport of H+/K+, consequently destabilizing the ionic balance and the pH of the midgut and could result in a negative trade-off with growth due to nutritional stress (Sousa *et al*., 2010), further work is needed to test this hypothesis.

The resistant soybean varieties IAC-17 and IAC-24 affected survivor, mass growth and the midgut morphology of *Anticarsia gemmatalis* caterpillars, mainly the digestive and goblet cells. Epithelium discontinuities, detachment of the muscle wall and vacuolization of digestive cell cytoplasm show the toxic effects of daidzein, quercetin, and rutin flavonoids functioning synergistically and causing mortality in caterpillars of *Anticarsia gemmatalis*. Concentrations of these flavonoids in different combinations on artificial diets points and strengthens the flavonoids synergy hypothesis for the development of soybean varieties and even botanical insecticides with increased resistance to herbivore insects.

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