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Does mechanical damage on soybean induces the production of flavonoids?

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ABSTRACT

The response of plants to grazing includes the production of chemical defense compounds such as proteases inhibitors and secondary metabolites as flavonoids, which makes them less palatable to feeding and negatively affecting the physiology of insects. The aim of this study was to evaluate the phytochemical response of soybean cultivars (*Glycine max* (L.) Merrill) resistant (IAC-17, IAC-24) and susceptible (IAC-P1) to insects after mechanical damage. These cultivars were mechanically injured, and after 24 hours samples of these plants were analyzed by HPLC to identify and quantify flavonoids. The flavonoids daidzein, quercetin, and rutin were quantified, with the highest concentration of daidzin in soybean cultivars after mechanical damage. Rutin was biosynthesized by IAC-24. The cultivars IAC-PL1, IAC-17, and IAC-24 did not show a flavonoid response to mechanical damage. The soybean cultivars are not dependent on mechanical damage to produce flavonoids.

Key words: *Glycine max*, flavonoid, herbivory, mechanical damage, secondary metabolite.

INTRODUCTION

Secondary metabolites are chemical compounds that confer plant resistance to insects. Host plant resistance is an effect strategy used in pest control programs to minimize economic damage (Hoffmann-Campo et al. 2006, Pilon et al. 2009). These metabolites can also be produced by *Minthostachys mollis*, *Ocimum minimum* in response to mechanical damage (Zabaras and Wyllie 2001, Banchio et al. 2005, Piesik et al. 2006).

Plant response to insect attack can occur directly and/or indirectly. The direct defense against herbivory is the negative effect of secondary metabolites on insect physiology. The indirect defense induces the production of volatile compounds to attract predators (Arimura et al. 2005). The herbivores triggers a chemical signaling to plants to biosynthesize secondary metabolites

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through the shikimate pathway and acetylcoenzyme A (Winkel-Shirley 2001, Mello and Silva-Filho 2002).

Secondary metabolites, as flavonoids and phenolic acids are present in soybean leaves as rutin (Hoffmann-Campo et al. 2006, Stamp and Skrobola 1993, Piubelli et al. 2005, Salvador et al. 2010) daidzin, genistin (Piubelli et al. 2003), salicylic acid, 4-hydroxybenzoic, vanillic acid, 4-hydroxycinnamic acid, ferulic acid, caffeic acid, gentisic, syringic acid, genistein, naringenin, quercetin, and daidzein (Porter et al. 1986).

Flavonoids are essential to plant defense against microorganisms and insects (Dixon and Steele 1999, Simmonds 2001). The flavonoids kaempferol, genistein, quercetin, and naringenin have an antifeedant effect on *Coptotermes formosanus* (Isoptera: Rhinotermitidae), especially the last two (Ohmura et al. 2000), and the latter causing high mortality of this termite (Boué and Raina 2003). The incorporation of chlorogenic acid, quercetin, and rutin into the diet of caterpillars *Helicoverpa armigera* (Hübner), *Spodoptera litura* and *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) reduced larval weight, caused mortality and increased the pupae period of these insects (Gazzoni et al. 1997, Jadhav et al. 2012).

Mechanically injured plants produce volatile compounds as part of the defense system that can also attract natural enemies. The concentration of volatile compounds like eugenol, linalool, menthone, pulegone, and sesquiterpens increases in mechanically injury *M. mollis* and *O. minimum* (Zabaras and Wyllie 2001, Banchio et al. 2005, Piesik et al. 2006).

The increased expression of volatile compounds in plants injured are being studied, but the phytochemical flavonoid response in soybean plants needs to be better understood. Therefore, this study objected to investigate the phytochemical flavonoids response in soybean *Glycine max* (L.) Merrill cultivars resistant and susceptible to insects, after being undergo mechanical injuries.

MATERIALS AND METHODS

The experiments were performed at the Laboratory of Enzymology, Biochemistry of Proteins and Peptides, Instituto de Biotecnologia Aplicada a Agropecuária (BIOAGRO) and in the Laboratory of Biodiversity, Department of Biochemistry and Molecular Biology of the Universidade Federal de Viçosa, Minas Gerais State, Brazil.

SOYBEAN CULTIVARS AND PHYTOCHEMICAL RESPONSE TO MECHANICAL DAMAGE

Seeds of the soybean cultivars IAC-PL1 (susceptible to insects) and IAC-17 and IAC-24 (resistant to insects) were obtained from the Instituto Agronômico de Campinas (IAC) of São Paulo State, Brazil, and cultivated until the V3 stage.

Three soybean plants were growner in pots with 4.0 kg of soil in the green house (temperature 25 °C $\pm$ 5, humidity 70% $\pm$ 10) and injured or not with a paper punch (6 holes of 0.5 mm per leaflet). The plants in V3 stage of development control and injured were cultured independently in plastic pots. After 24 hours, nine leaflets were removed from nine plants and were immediately frozen –80 ºC to phytochemical analysis.

OBTAINING THE LEAF EXTRACT

The extract of soybean leaves was prepared at 4 °C. The leaves were weighed (500 mg) and pulverized in liquid nitrogen with a mortar and pestle. The powder obtained was transferred to 15 ml plastic tubes with 5.0 ml of 80% methanol, kept in an ultrasound bath for 20 min, and centrifuged at 3.000 g for 5 min at 4 °C. The supernatants obtained were evaporatedina speed vacuum concentrator and the extracts dry were resuspended with 2 ml absolute methanol, filtered with a 0.45 µm polytetrafluoroethylene filter, and packed invials.

IDENTIFICATION AND QUANTIFICATION OF FLAVONOIDS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Aliquots (30 µl) were injected into the chromatograph Shimadzu Prominence System (pump LC-20 AD, Detector SPD-M20A, oven CTO-20A, Lab Solutions program) with a reverse phase column to detecting secondary metabolites (C-18, 4.6 mm internal diameter, 15 cm length, 4.8 µm particle diameter).

The extracts were eluted with a segmented gradiente solution of 2% acetic acid (A) and acetonitrile (B). The chromatographics trength of the mobile phase varied in proportion from 0-4 min, 5% B; 4-15 min 20% B; 15-20 min 35% B; 20-30 min 90% B; 30-45% B, with a temperature of 40 °C (CTO-20 A). The solvent flow was 0.6 ml/min and the absorption measured at 254 nm (diode array).

The flavonoids in the soybean leaf extracts were evaluated quantitatively by comparison with purchased standards (Sigma®) by injections of kaempferol, daidzein, daidzein, genistein, genistin, quercetin, and rutin in the HPLC. Calibration curves were made with injection concentrations of daidzein (1.2, 2.4, 3.6, 4.8 mg), quercetin (0.8, 1.6, 2.4, 3.2 mg), and rutin (0.8, 1.6, 2.4, 3.2 mg). Linear regression functions were obtained from the responses of these compounds, measured as peak area to calculate the concentration of flavonoids in the samples.

STATISTICAL ANALYSES

The experiment was conducted in a completely randomized design and the data analyzed with the R statistical program, considering 5% error. The experiment had treatments per cultivar, with three replications. The concentration of flavonoid were analyzed for normality with the Shapiro test, and the test of ANOVA and Tukey test were applied. The generalized linear model (GLM) was used to make the analysis ANOVA. Simplifying model was used with GLM.

RESULTS

IDENTIFICATION OF FLAVONOIDS IN SOYBEAN CULTIVARS

Soybean cultivars resistant (IAC-17 and 24) and susceptible (IAC-PL1) analyzed by liquid chromatography showed three peaks, identified as the compounds daidzein, quercetin, and rutin (Figure 1).

QUANTIFICATION OF FLAVONOIDS AND THE PHYTOCHEMICAL RESPONSE OF SOYBEAN CULTIVARS

The concentration of the flavonoids daidzein, quercetin, and rutin in the cultivar IAC-17 differed between them ($F_{2,6}$ = 62.20, $p<0.001$), with 0.207; 0.014 and 0.016 mg/g (w/w), respectively. Leaf extract of the IAC-24 showed a higher concentration of daidzein ($F_{2,6}$ = 481.93, $p<0.001$) than that of quercetin and rutin, 0.242; 0.011 and 0.033 mg/g (w/w), respectively (Figure 2).

The concentration of flavonoids of the IAC 24 (resistant) and IAC PL 1 (susceptible) cultivars showed differences only for the compounds rutin and daidzein ($F_{2,6}$ = 6.91, p= 0.027, $F_{2,6}$ = 12.23, p = 0.007, respectively), whereas quercetin had a similar value for the IAC-17, IAC-24, and IAC-PL1 ($F_{2,6}$= 2.22) (Figure 3).

Concentrations of daidzein, quecertin, and rutin from IAC-PL1, IAC-17, and IAC-24 with or without mechanical damage were similar (daidzein: $F_{1,4}$ = 0.07, $p>0.05$, quercetin: $F_{1,4}$ = 1.56, $p>0.05$, rutin: $F_{1,4}$ = 3, $p>0.05$) after 24 hours of injury (Table I).

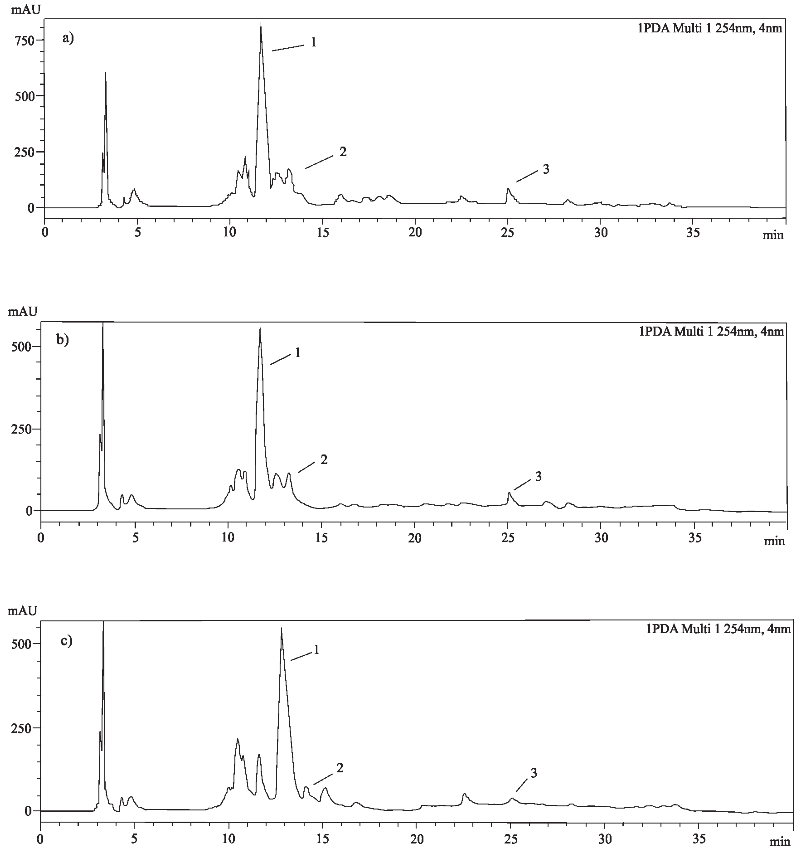

Figure 1 - Chromatograms of the soybean cultivars IAC-17 (**a**), IAC-24 (**b**) and IAC-PL1 (**c**) without mechanical damage. The daidzein compounds (1), retention time - RT 11.6 min, rutin (2), RT 13.7 min and quercetin (3), TR25 min were monitored under the chromatographic conditions flow 0.06 ml/min, 254 nm, column 4.5 x 15 cm (ODS - C 18), oven 40 °C, gradient phase (**a**) water acidified with acetic acid and (**b**) acetonitrile HPLC.

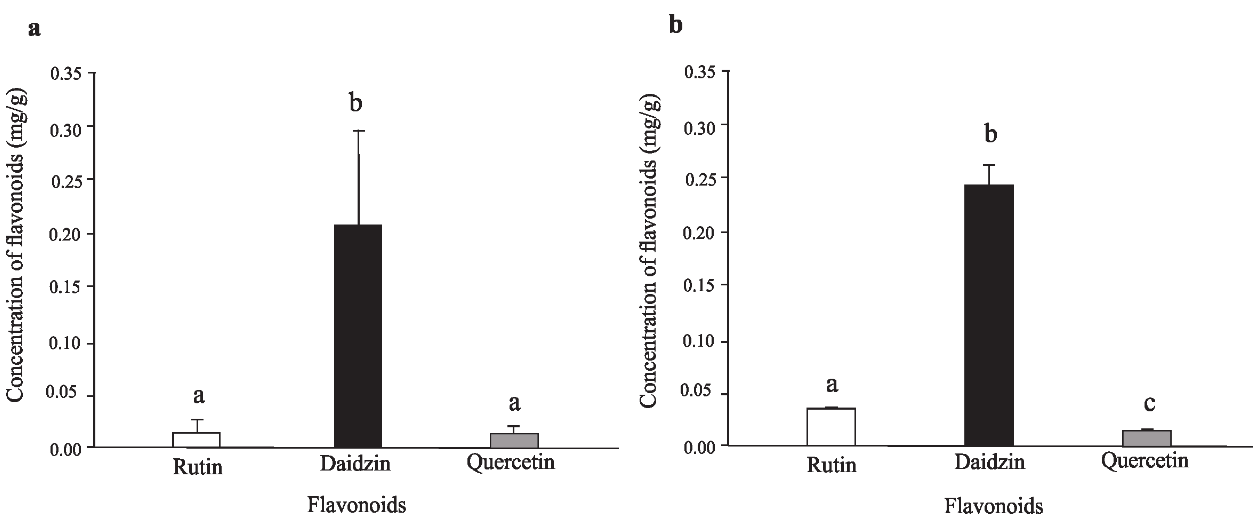

Figure 2 - Flavonoid concentrations in leaf extracts of the insect resistant soybean cultivars IAC-17 (**a**) and IAC-24 (**b**) mechanically damaged. Means followed by the same letter are similar between them by ANOVA (IAC-17 p<0.001 and IAC-24 p<0.001).

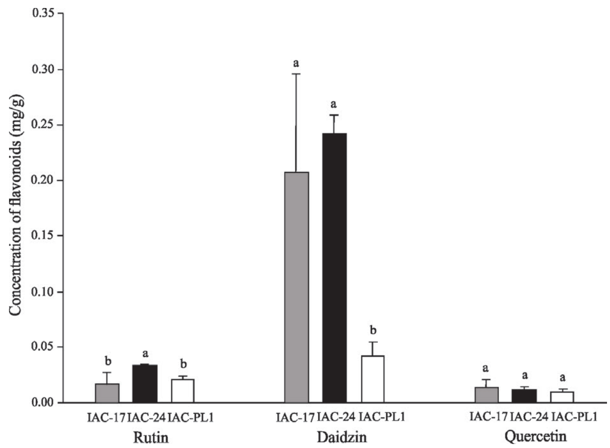

Figure 3 - Flavonoids concentrations in the insect resistant soybean cultivars IAC-17 and IAC-24 and susceptible IAC-PL1 24h after, mechanically damaged. Means followed by the same letter are similar between them by Tukey test ($p<0.01$) (Rutin $p = 0.027$, Daidzein $p =0.007$, Quercetin $p> 0.05$).

DISCUSSION

Flavonoids have diverse biological functions protecting plant against abiotic and biotic factors (Pourcel et al. 2007) including damage by ultraviolet light (Takahashi and Ohnishi 2004) and against microorganisms and insects and they are also responsible for flower colors (Yao et al. 2004, Griesbach 2005).

The quercetin and rutin, wich were detected in lower concentrations in extracts of resistant soybean plants in this work, can cause adverse reactions in pests. Extracts of *Hybanthus parviflorus* contained rutin which had an insecticidal effecton *Ceratitis capitata* Wiedemann, 1824 (Diptera: Tephritidae) flies (Broussalis et al. 2010). Rutin found in pods soybean (Hoffmann-Campo et al. 2001, Piubelli et al. 2005) is used in plant breeding programs as a resistance source to defoliating insects. This compound causes antibiotics and/or anti-nutrients in chewing insects (Stamp and Skrobola 1993), including *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) (Hoffmann-Campo et al. 2006). *Ficus sarmentosa* var. *henryi* plant extracts containing quercetin showed an insecticidal effect on *Musca domestica* Linnaeus, 1758 (Diptera: Muscidae) and *Aedes albopictus* Skuse, 1894 (Diptera: Culicidae) (Xue-gui et al. 2011). The concentrations of rutinin soybean cultivars resistant to insects ranged from 0.212 to 3.682 mg/g (Piubelli et al. 2005). The concentration of this compound (0.033 mg/g) in the cultivar IAC-24 was lower than these values. The daidzein had greatest quantity in soybean plants, but the three chemical compounds may act synergistically against herbivory (Malarvannan et al. 2008). The addition of quercetin and rutin in the diet caused mortality of *Helicoverpa armigera* caterpillar (Hübner), *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae), and *Anticarsia gemmatalis* Hübner, 1818 (Lepidoptera: Noctuidae) (Jadhav et al. 2012, Gazzoni et al. 1997).

The concentration of daidzein in the cultivar IAC-24 was approximately 6.3 times higher than that of the quercetin and rutin, which is not observed in the IAC-17. These compounds can affect the physiology of insects, although the mode of action of this phytoestrogens needs to be better studied. Soybean seeds injured by stink bug produced a higher quantity of daidzein and genistein isoflavones, which reduced food quality for insects (Piubelli et al. 2003).

The concentration of flavonoids rutin was 2.5 times higher in the IAC-24 cultivar than in the IAC-17 for (F, $p = 0.027$). The concentration of daidzein was 5.7 times higher than the lowest level of cultivar IAC-PL1, susceptible to insects. These concentrations of flavonoids can give rise in these cultivars to increased resistance to insects because these compounds affect the physiology of these organisms (Boué and Raina 2003, Jadhav et al. 2012). Furthermore, it has been shown that in genetically modified tobacco plants increased synthesis of rutin has an insecticidal effect on larvae *Spodoptera litura* Fabricius, 1775 (Lepidoptera: Noctuidae) and *Helicoverpa armigera* Hübner,

TABLE I
Concentration of flavonoids rutin, daidzein and quercetin (mg/g) in insect resistant and susceptible soybeans with and without mechanical damage.

Cultivate	Treatment	Flavonoids (mg/g)		
		Rutin	Daidzin	Quercetin
IAC-PL1	w[1]/ injury	0.015 $\pm$ 0.003	0.045 $\pm$ 0.003	0.007 $\pm$ 0.001
	wi[2]/ injury	0.020 $\pm$ 0.004	0.042 $\pm$ 0.013	0.009 $\pm$ 0.003
IAC-17	w[1]/ injury	0.025 $\pm$ 0.012	0.182 $\pm$ 0.087	0.017 $\pm$ 0.006
	wi[2]/ injury	0.016 $\pm$ 0.011	0.207 $\pm$ 0.089	0.014 $\pm$ 0.007
IAC-24	w[1]/ injury	0.039 $\pm$ 0.013	0.263 $\pm$ 0.032	0.012 $\pm$ 0.001
	wi[2]/ injury	0.033 $\pm$ 0.001	0.242 $\pm$ 0.017	0.011 $\pm$ 0.003

w[1]: without, wi[2]: with. The values did not differ by ANOVA, $p>0.05$.

1805 (Lepidoptera: Noctuidae), which are important agricultural pests (Pandey et al. 2012).

The cultivars IAC-PL1, IAC-17, and IAC-24 did not show a phytochemical response related to increased flavonoids after 24h of mechanical damage. However, mechanically damaged plants tend to express a phytochemical response (Zabaras and Wyllie 2001, Banchio et al. 2005, Piesik et al. 2006), as observed for wheat leaves, where there was an increase in the release of the secondary metabolites linalool (3,7-dimethyl-1,6-octadien-3-ol) and linalool oxide (5-ethenyltetrahydro-2-furanmethanol) (Piesik et al. 2006). Synthesized compounds due to mechanical damage tend to have lower levels after a certain time (Banchio et al. 2005). The absence of a chemical plant response of plants by producing flavonoids after mechanical damage may be related to the absence of the elicitors ß-glucosidase and volicitin from insects (Alborn et al. 1997). These compounds in contact with the cell membrane of plant can trigger a cascade of signaling and consequently activating ion flows in the plasma membrane, changes in the cytoplasmic calcium concentration, active oxygen generation, and protein phosphorylation (De Bruxelles and Robert 2001). It also induces the jasmonic acid production, responsible for triggering genes in the shikimate and acetyl coenzyme A metabolic pathways, both synthesizers of secondary metabolites (Winkel-Shirley 2001, Mello and Silva-Filho 2002). These metabolites, such as volatiles and terpenes, are produced by mosses in response to herbivory by Coleoptera (Piesik et al. 2011a, 2014). Microorganisms also induce volatile production incorn roots and leaves (Piesik et al. 2011b).

The production of chemical compounds in soybean leaves indicates the need of further studies to understand the flavonoid biosynthesis in these plants. The physiological response or its absence in soybean plants after abiotic stress confirms the importance of studying the potential of flavonoids as part of plant defense against herbivory.

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