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# Opening Pandora's Box: Can Plant Breeding Influence Flavonoid Production and Biotic Resistance in Soybean Cultivars?

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**ABSTRACT:** The impact of plant breeding on flavonoid-mediated biotic resistance is unclear. In this study, we analyzed ten soybean cultivars subjected to herbivory by *Spodoptera cosmioides* using ultrahigh performance liquid chromatography−quadrupole time-of-flight−mass spectrometry (UHPLC−qTOF−MS) and chemometrics. The PI227682 cultivar exhibited elevated levels of *O*-methylated flavonoids, which correlated with strong resistance. In contrast, transgenic cultivars showed higher levels of glycosylated flavonoids, which attracted herbivores. Multivariate analysis revealed cultivar-specific metabolic reprogramming and identified formononetin and isorhamnetin glycosides as resistance biomarkers. Our findings highlight a trade-off: breeding for productivity may inadvertently reduce defense metabolites, thereby increasing pest susceptibility. This study provides actionable insights for developing flavonoid-enhanced cultivars, reducing reliance on pesticides, and promoting sustainable agriculture. Integrating metabolomics into breeding programs could bridge the gap between yield and biotic resilience, thereby addressing critical food security and environmental challenges.

**KEYWORDS:** *eco-metabolomics, soybean plants, secondary metabolites, UHPLC−qTOF−MS instrumentation*

## INTRODUCTION

Plant breeding has been instrumental in enhancing crop productivity, but its effects on secondary metabolism and pest resistance remain understudied.[1−4] While modern techniques such as genetic engineering have expanded trait manipulation,[5] unintended consequences such as reduced flavonoid diversity may compromise plant defenses.[6−8] This is particularly critical in soybean (*Glycine max*), where domestication has reduced genetic diversity by 50%, which could limit stress adaptation.[9]

Flavonoids constitute a key defensive arsenal, and their structural features determine their ecological roles.[10,11] These molecules have been detected in greater quantity and diversity in the These molecules have been detected in greater quantity and diversity in the leaves[12] and seeds[6] of wild soybean cultivars. The positive modulation of these metabolites is related to greater stress tolerance and environmental adaptability in the parent cultivars.[12]

Given the importance of flavonoids in defense and their prevalence in wild soybean cultivars, the structural plasticity of these molecules is crucial for plants' adaptation to different environmental pressures.[13] For example, *O*-methylated flavonoids exhibit increased lipophilicity and stability, enhancing insect deterrence.[13,14] Meanwhile, glycosylated forms can paradoxically attract herbivores via phagostimulant effects.[15] They can also increase resistance to *Anticarsia gemmatalis* in soybean plants.[16]

The structural diversification and subtle differences of flavonoids are complex and crucial to understanding their defensive roles. Advanced analytical tools, such as high-resolution qTOF mass spectrometry,[17] are required to study these characteristics. This technology provides sub-5 ppm mass accuracy and MS/MS fragmentation patterns, which allow discrimination of structural isomers. This capability is essential for comprehending flavonoid diversification[17,18] and is employed to map cultivar-specific metabolic responses to herbivory.

Our study addresses two critical knowledge gaps: (i) the impact of breeding-driven metabolic reprogramming on soybean resistance to *Spodoptera cosmioides*, and (ii) the ability of definable flavonoid biomarkers to predict resistance phenotypes. The timing is crucial, as global pesticide use is facing increasing restrictions[19] and climate change is altering pest dynamics.[10] Identifying natural resistance mechanisms is therefore essential. Our study demonstrates that modern metabolomics can bridge this gap by revealing how historical breeding decisions have shaped defensive chemistry in ways that modern science can optimize.

The implications of our investigation extend beyond fundamental science. By identifying a key resistance biomarker, we provide tangible targets for: (i) conserving wild soybean germplasm with valuable flavonoid profiles for defense, (ii) guiding marker-assisted breeding programs to efficiently select more resistant cultivars, and (iii) underpinning policies for the sustainable intensification of agriculture. Our results present a

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**Table 1. Description of the Source and Resistance Data for the Soybean Cultivars Used in This Study**

| source | plant introduction | genetic improvement | transgenics roundup Ready—with tolerance to the herbicide glyphosate | literature references |
|---|---|---|---|---|
| cultivar names (constitutive resistance to herbivores)[a] | PI227682 (HR) | UFUS Carajás (HR) | P98Y11RR (S) | Junior et al., 2015[54] |
| | | UFUS Impacta (R) | M8230RR (S) | Aguiar et al., 2022[15] |
| | | UFUS Milionária (R) | ANTA82RR (S) | |
| | | UFUS Xavante (R) | | |
| | | UFUS Capim Branco (S) | | |
| | | CD208 (S) | | |

[a]*Spodoptera cosmioides* insect feeding experiment; HR = highly resistant; R = resistant; S = susceptible.

pathway to sustain agricultural productivity while reducing the need for chemical inputs.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Standard solutions of caffeine and isoquercitrin (Sigma-Aldrich, St. Louis, USA) were prepared in water at concentrations of 25 mg·L$^{-1}$ and 40 mg·L$^{-1}$, respectively, and stored them at 8 °C. Methanol (LC−MS) was purchased from Honeywell (North Carolina, USA), and ultrapure water (Milli-Q system Millipore, Merck KGaA, Darmstadt, Germany) was used as the solvent. Formic acid (LC−MS, Fluka, Missouri, USA) was used as the mobile phase for liquid chromatography.

**Experimental System.** We grew soybean plants (see the ten cultivars evaluated in Table 1) in a greenhouse at the Department of Agricultural Sciences, São Paulo State University, Jaboticabal, São Paulo State, Brazil (S21°14′25″ and W48°17′21″), under the supervision of Professor Dr. Arlindo Leal Boiça Júnior. This study was registered in SisGen (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado) under the access registration number AFF721B. The greenhouse had a transparent screen. The treatment and control plants were exposed to the natural photoperiod between September and November 2017, usually 13 h of light and 11 h of dark, with an average temperature of 27 °C. The relative air humidity was 40 ± 20%.

Plants were grown in 3.0 L polyethylene vases containing a soil substrate of dystrophic red latosol,[20] sand and organic matter in a 3:1:1 ratio. Six vases of 10 plants each were prepared for each cultivar, for a total of 60 biological replicates per cultivar. The vases of soybean plants were kept in a greenhouse sealed with antiaphid fabric netting until they were used in the assays. 5 week-old vegetative stage plants were used.

The insects used in the experiments were obtained from a colony maintained for 26 generations at the Laboratory of Plant Resistance to Insects, also coordinated by Professor Dr. Arlindo Leal Boiça Júnior. We fed the insects with an artificial diet[21] and kept them in a room with a temperature of 25 ± 2 °C, relative humidity of 70 ± 10% and a 12 h light/dark photoperiod. We periodically added field-collected *S. cosmioides* caterpillars to maintain genetic variability in rearing. Fourth instar caterpillars were used for biological assays.

**Analysis of Inducible Resistance.** To evaluate the influence of biotic stress on metabolites, we infested plants with a fourth instar caterpillar of *S. cosmioides* on the third trifoliate from the top of the plants. We confined the caterpillars to each plant for 48 h in a 10 × 15 cm voile tissue cage. During this period, they consumed at least 50% of the leaf area. After 48 h, we removed the caterpillars and collected leaves from the second trifoliate. By collecting leaves above the infested leaves, we evaluated the systemic induction of resistance.

**Sample Preparation.** At the start of the resistance induction experiments, we collected leaves from the second trifoliate of soybean plants. For the metabolomic analyses, we used three vases, each containing ten plants. Samples from each pot were combined to create three analytical replicates for each experiment. The central leaf was immediately frozen in liquid nitrogen to quench the metabolism and stored for metabolite extraction. We lyophilized the soybean leaf samples under vacuum using an E Modulyo (Thermo Fisher Scientific, Massachusetts, USA). Finally, the lyophilized material was ground and sieved at 80 mesh.

**Preparation of Extracts.** This stage was carried out based on the previously presented optimized methods and analyses.[15] Thus, we extracted 25 mg of lyophilized plant material with 1.0 mL of a 50:50 (v·v$^{-1}$) water/methanol mixture in an 8.0 mL test tube using a 40 kHz ultrasonic bath (USC 1400 Unique, São Paulo, Brazil) for 5 min. We repeated this extraction process six times, hanging the solvent each time. After the last extraction, we centrifuged the combined supernatant (3200*g* for 1 min at 10 °C) and filtered it through a 0.22 μm PTFE membrane. We then diluted 10 μL of the extract with 230 μL water and 10 μL internal standard (caffeine 1.0 μg·mL$^{-1}$). Finally, triplicate samples ($n$ = 3) were analyzed by UHPLC−q-TOF−MS/MS (Agilent, 6545B LC/Q-TOF MS). Figure S1 (Supporting Information) illustrates the workflow.

**Quantification of Metabolites.** We selected isoquercitrin (quercetin 3-β-D-glucoside) as a reference control for the quantification of flavonoids detected in soy leaf samples.[22,23] Prior to quantification, we verified the efficiency of the extraction method and evaluated matrix effects. We spiked soy leaf samples (25 mg) with 10 μL of a 40 mg·L$^{-1}$ isoquercitrin solution and left them open for 5 h to evaporate the solvent. We then added 1.0 mL of the extraction mixture and proceeded with the extraction as described below.

To evaluate the extraction efficiency, we prepared seven standard isoquercitrin solutions in deionized water at concentrations of 10.0, 25.0, 50.0, 75.0, 100, 200, and 300 μg·L$^{-1}$. Each solution contained caffeine (1.00 μg·mL$^{-1}$) as an internal standard. To evaluate the matrix effect, calibration solutions were prepared with the sample matrix extract by adding 250 μL of the aqueous sample extract. All experiments were performed in triplicate. A calibration curve was constructed by plotting the ratio of analyte to internal standard area versus concentration. Linearity was evaluated using the coefficient of determination ($r^2$) and analysis of variance.

**Chromatographic Methods.** We performed target metabolite analysis using an ultrahigh performance liquid chromatography system (Agilent 1290, Agilent Technologies, CA, USA) equipped with a Phenyl-Hexyl Zorbax RRHD Eclipse Plus column (2.1 × 100 mm, 1.8 μm Agilent) as the stationary phase. We set the column oven and autosampler temperatures to 45 and 10 °C, respectively. We used a constant flow gradient (0.350 mL·min$^{-1}$) of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) under the following conditions 8−90% B (0−17 min) and 90% B (17−20 min) with a 4 min postrun. We injected 1.0 μL of sample.

We performed mass spectrometry (q-TOF MS) analyses using a quadruple time-of-flight tandem mass spectrometer (Agilent 6545 LC-Q-TOF MS) equipped with an electrospray ionization (ESI) source in positive ion mode. We set the capillary voltage to 3.5 kV, the source and desolvation temperatures to 350 °C, the desolvation gas flow to 11 L·min$^{-1}$, the cone gas flow to 8 L·min$^{-1}$, the fragmentor voltage to 250 V, the skimmer voltage to 65 V, the nozzle voltage to 1000 V, and the scan rate to 2 spectra·s$^{-1}$. We collected MS data in the mass range of 100−1000 Da using Mass Hunter Acquisition B.06.00 software (Agilent Technologies, CA, USA).

**Table 2. Compounds Detected in *Glycine max* Leaf Extract**

| | compound annotation | precursor ions detected | | molecular formula | error (ppm) | major fragments | retention time (min) |
|---|---|---|---|---|---|---|---|
| | | $(M + H)^+$ | $(M + Na)^+$ | | | | |
| QGR | quercetin-3-*O*-glucosyl-rutinoside | | 795.1948 | $C_{33}H_{40}O_{21}$ | 0.86 | 465, 347, 303, 279, 153, 117, 107 | 6.6 |
| KGA | Kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-galactopyranoside | 756.2107 | 779.2 | $C_{33}H_{40}O_{20}$ | 0.91 | 495, 449, 287 | 7.3 |
| KGL | kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-glucopyranoside | 756.2103 | 779.1996 | $C_{33}H_{40}O_{20}$ | 1.31 | 497, 449, 287 | 7.36 |
| KDGA | kaempferol-3-*O*-digalactopyranoside | 610.1522 | 633.1413 | $C_{27}H_{30}O_{16}$ | 2.51 | 287 | 7.49 |
| IRG1 | isorhamnetin glycoside | 787.2299 | 809.2102 | $C_{34}H_{42}O_{21}$ | 3.08 | 641, 479, 317, 302 | 7.54 |
| KDGL | kaempferol-3-*O*-diglucopyranoside | 610.1525 | 633.1422 | $C_{27}H_{30}O_{16}$ | 1.32 | 287 | 7.58 |
| KRGA | kaempferol-3-*O*-*β*-D-(2,6-di-*O*-*α*-L-rhamnopyranosyl) galactopyronoside | 740.215 | 763.204 | $C_{33}H_{40}O_{19}$ | 2.06 | 287 | 7.69 |
| IRG2 | isorhamnetin glycoside | | 663.1517 | $C_{28}H_{32}O_{17}$ | 2.45 | 317, 302 | 7.75 |
| 3MQ | isorhamnetin (3-*O*-methylquercetin) | 317.0646 | | $C_{15}H_{10}O_7$ | 3.12 | 302, 274, 229 | 8.02 |
| IRS1 | isorhamnetin glycoside (soyanin) | | 793.2159 | $C_{34}H_{42}O_{20}$ | 0.66 | 479, 317 | 8.02 |
| IRS2 | isorhamnetin glycoside (soyanin) | | 793.2107 | $C_{34}H_{42}O_{20}$ | 0.66 | 479, 317 | 8.23 |
| GGG | genistein-7-*O*-glucoside (genistin) | | 455.096 | $C_{21}H_{20}O_{10}$ | 2.13 | 271 | 8.27 |
| KRG | kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1 → 6)-*β*-D-galactopyronoside | 594.1582 | 617.1475 | $C_{27}H_{30}O_{15}$ | 0.56 | 287 | 8.69 |
| KGT | kaempferol-3-*O*-galactoside (Trifolin) | | 471.0884 | $C_{21}H_{20}O_{11}$ | 3.29 | 287, 165, 153, 121 | 8.79 |
| KRGL | kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1 → 6)-*β*-D-glucopyronoside | 594.1577 | 617.1472 | $C_{27}H_{30}O_{15}$ | 1.16 | 287 | 9.06 |
| KGA | kaempferol 3-*O*-glucoside (astragalin) | | 471.0884 | $C_{21}H_{20}O_{11}$ | 3.42 | 287 | 9.08 |
| IRGA | isorhamnetin-3-*O*-(6″-*O*-rhamnosyl)galactoside (isorhamnetin-3-O-robinobioside) | 625.1768 | 647.158 | $C_{28}H_{32}O_{16}$ | 0,38 | 599, 479, 364, 317 | 9.19 |
| IGC | isorhamnetin-3-*O*-galactoside (cacticin) | | 501.0984 | $C_{22}H_{22}O_{12}$ | 3.76 | 317 | 9.23 |
| GLY | glycitein | 285.0754 | | $C_{16}H_{12}O_5$ | 0.84 | 270, 242, 128, 115, 107 | 9.3 |
| IGE | isorhamnetin-3-*O*-glucoside | | 501.0989 | $C_{22}H_{22}O_{12}$ | 3.37 | 317 | 9.3 |
| IRGL | isorhamnetin-3-*O*-(6″-*O*-rhamnosyl)glucoside (isorhamnetin-3-*O*-rutinoside) | 625.1768 | 647.1579 | $C_{28}H_{32}O_{16}$ | 1.09 | 599, 479, 364, 317 | 9.32 |
| AGC | apigenin-7-*O*-glucoside (cosmosiin) | | 455.0938 | $C_{21}H_{20}O_{10}$ | | 271 | 9.73 |
| BAI | baicalein | 271.0597 | | $C_{15}H_{10}O_5$ | 1.11 | 141, 123, 115, 105 | 10.01 |
| 6MG | 6″-*O*-malonylgenistin | 518.1053 | 541.0941 | $C_{24}H_{22}O_{13}$ | 1.75 | 271 | 10.02 |
| CHR | chrysin | 255.0651 | | $C_{15}H_{10}O_4$ | 0.3 | 115, 103 | 10.11 |
| DAI | daidzein | 255.0655 | | $C_{15}H_{10}O_4$ | −0.27 | 227, 199, 152, 128, 115, 107, 103 | 10.35 |
| BIO | biochanin A | 285.0751 | | $C_{16}H_{12}O_5$ | 1.96 | 270, 253, 213 | 10.6 |
| NAR | naringenin | 273.0752 | | $C_{15}H_{12}O_5$ | 1.51 | 153, 147 | 10.82 |
| IAA | isorhamnetin acetylglucoside or acetylgalatoside | | 543.1112 | $C_{24}H_{24}O_{13}$ | 0.24 | | 10.83 |
| LUT | luteolin | 287.0547 | | $C_{15}H_{10}O_6$ | 0.88 | 133, 128, 107 | 10.93 |
| GOA | genistein or apigenin glucoside | | 519.1129 | $C_{24}H_{22}O_{13}$ | 1.05 | 271 | 10.98 |
| TMG | tectorigenin-7-*O*-(6″-*O*-malonyl)glucoside (6″-*O*-malonyltectoridin) | 549.1224 | | $C_{25}H_{24}O_{14}$ | 2.55 | 301, 286 | 11.1 |
| API | apigenin | 271.0598 | | $C_{15}H_{10}O_5$ | 0.99 | 197, 187, 153, 131, 115, 109 | 11.42 |
| MOS | mosloflavone | 299.0913 | 321.0727 | $C_{17}H_{14}O_5$ | 0.64 | 284 | 12.01 |
| GEN | genistein | 271.06 | | $C_{15}H_{10}O_5$ | 0.49 | 243, 159, 152, 115, 107 | 12.09 |
| TEC | tectorigenin | 301.0704 | | $C_{16}H_{12}O_6$ | 0.72 | 286 | 12.29 |
| FOR | formononetin | | 269.0805 | $C_{16}H_12O_4$ | 1.57 | 253, 226, 213 | 12.90 |
| AFR | afrormosin | 299.0912 | 321.0731 | $C_{17}H_{14}O_5$ | −0.17 | 284, 141, 128, 121, 117 | 13.37 |

Table 3. Linearity of the Method and Recoveries[a]

| analyte | equation | $r^2$ | recovery (%) sample 1 | sample 2 | sample 3 | average | RSD (%) |
|---|---|---|---|---|---|---|---|
| isoquercitrin | $y = 2.6274x + 0.0360$ | 0.996 | 97.45 | 96.88 | 95.61 | 96.65 | 0.97 |

[a]RSD = relative standard deviation.

We continuously monitored system stability by analyzing quality control samples, and internal standard solutions. We assessed the accuracy of the injection system by evaluating the area values of the internal standard in all samples analyzed. Details on how we conducted the data analysis can be found in the "Data Analysis" section of the Supporting Information.

## RESULTS AND DISCUSSION

**Flavonoid Identification and Quantification.** We found about 1500 molecular features. We used the Mass Hunter Qualitative Analysis Workflows B.08.00 software. 38 flavonoid metabolites were identified at levels 2 and 3.[24] These were identified after a detailed analysis of the mass spectra, including experimental $m/z$ values, MS/MS analysis, error (ppm), molecular formula, and reference data (Table 2).

The metabolites fall into four main subgroups: flavones (15.8%), flavanones (2.63%), isoflavones (21.1%), and glycosides associated with kaempferol (23.7%), quercetin (2.63%), isorhamnetin (23.7%), apigenin (2.63%), genistein (2.63%), and tectorigenin (2.63%). Among these subgroups, we also identified molecules with *O*-methylation in the hydroxyl groups. Notable differences were observed between plant introduction, genetic improvements, and transgenic cultivars, such as the addition of glycoside molecules and *O*-methylation.

Plants with higher levels of methylated flavonoids are more resistant to herbivores through several mechanisms. Methylation increases the lipophilicity and stability of flavonoids,[14] reduces their degradation by herbivore enzymes, and improves membrane disruption.[25] Additionally, methylation may improve the membrane permeability of these compounds, facilitating their interactions with microorganisms and enhancing their antimicrobial activity.[25]

We have identified naringenin, daidzein, genistein, and apigenin among the molecules associated with resistance to lepidopteran caterpillars in soybean plants. In contrast, researchers have associated glycosylated molecules of kaempferol and isorhamnetin with induced resistance to *Caliothrips phaseoli*.[26] This suggests that flavonoids and their derivatives may affect different insect species in different ways. We found these molecules in each cultivar. See Tables S1 and S2 in the Supporting Information for details. We did not find the molecule rutin in the selected soybean cultivars. Rutin is a type of flavonoid that is glycosylated. It is commonly used to study how plants defend themselves against herbivores.[27] This molecule absorbs UV−B radiation and helps reduce reactive oxygen species in plants. This reduces cell damage caused by oxidative stress.[28,29]

Based on the constructed library, we used isoquercitrin (quercetin 3-$\beta$-D-glucoside) as a reference standard to compare the relative amounts of metabolites produced in each cultivar. We investigated how the choice of solvent for the calibration curve affects analyte quantification. This is critical due to the complexity of the plant extract matrix. Matrix effects can alter the response of analytes in chromatographic analyses. Although the exact mechanisms remain unclear, this is a widely accepted concept in the field.[30,31]

Matrix components can coelute with the analytes. This interferes with mass spectrometry ionization.[30,31] Matrices may also contain chelating agents that can bind analytes and render them unavailable. Nonvolatile components can accumulate in the chromatographic system and in the mass spectrometer. This accumulation results in the formation of new active sites.[30] Matrix effects can affect analytical signals, either enhancing or attenuating their strength. These effects can cause recovery rates to be very high (above 100%) or very low. This can affect accuracy and precision.[30,31] We investigated these effects in two ways: with a matrix extract (matrix-matching) and with methanol Figure S1 (Supporting Information) shows the results.

We observed a nonlinearity in the pure organic solvent calibration curve. This indicates that the ionization efficiency varies over the concentration range (Table 3). Solvent composition and matrix components can affect ion formation and stability. This can affect sample nebulization at the ionization source. As a result, stable spray formation and ion generation efficiency may be affected.[32]

A linear detector response was observed. The analyte signal in the matrix extract calibration curve increases over at the selected concentration range (Table 3). Several mechanisms may contribute to this: (i) co-ionization of matrix molecules with the analyte, forming adducts or clusters that increase signal strength at the $m/z$ of the analyte or in other spectral regions; (ii) matrix components facilitating proton or electron transfer to the analyte, increasing ionization efficiency; and (iii) matrix components competing with the analyte for active sites during nebulization, resulting in increased analyte transfer to the mass analyzer.[30,31]

We calculated isoquercitrin recovery using a matrix-matched calibration curve.[33] The mean recovery rate was 96.65% with a relative standard deviation (RSD) of 0.97% (Table 3). Our extraction method and the matrix-matched standard curve are effective. They work well for the analysis of flavonoids and their derivatives in the samples. Therefore, we used the matrix extract calibration curve. It improved the detection of varying concentrations of the reference analyte and reduced discrepancies due to matrix effects. Table 3 shows the calibration curve and the $r^2$ value. We used an internal standard. This corrected for variations due to matrix effects and the injection system. Finally, we diluted the samples to reduce matrix effects and increase analytical sensitivity. We evaluated the calibration curve and also verified the consistency of the injection system. The internal standard response was analyzed in 60 assays. The RSD of the internal standard was less than 3.6%. The data indicate that metabolite detection was stable. Variations in metabolite levels are likely to reflect changes in the biological system.

**Metabolite Profiling and Clustering.** We looked at the molecules in Table 2 and the quantified molecules in Tables S1 and S2. We then performed a detailed multivariate analysis using the molecules listed in Tables S1 and S2. This helped us to identify trends and clusters in major classes of metabolites. We examined how soybean plants defend themselves and

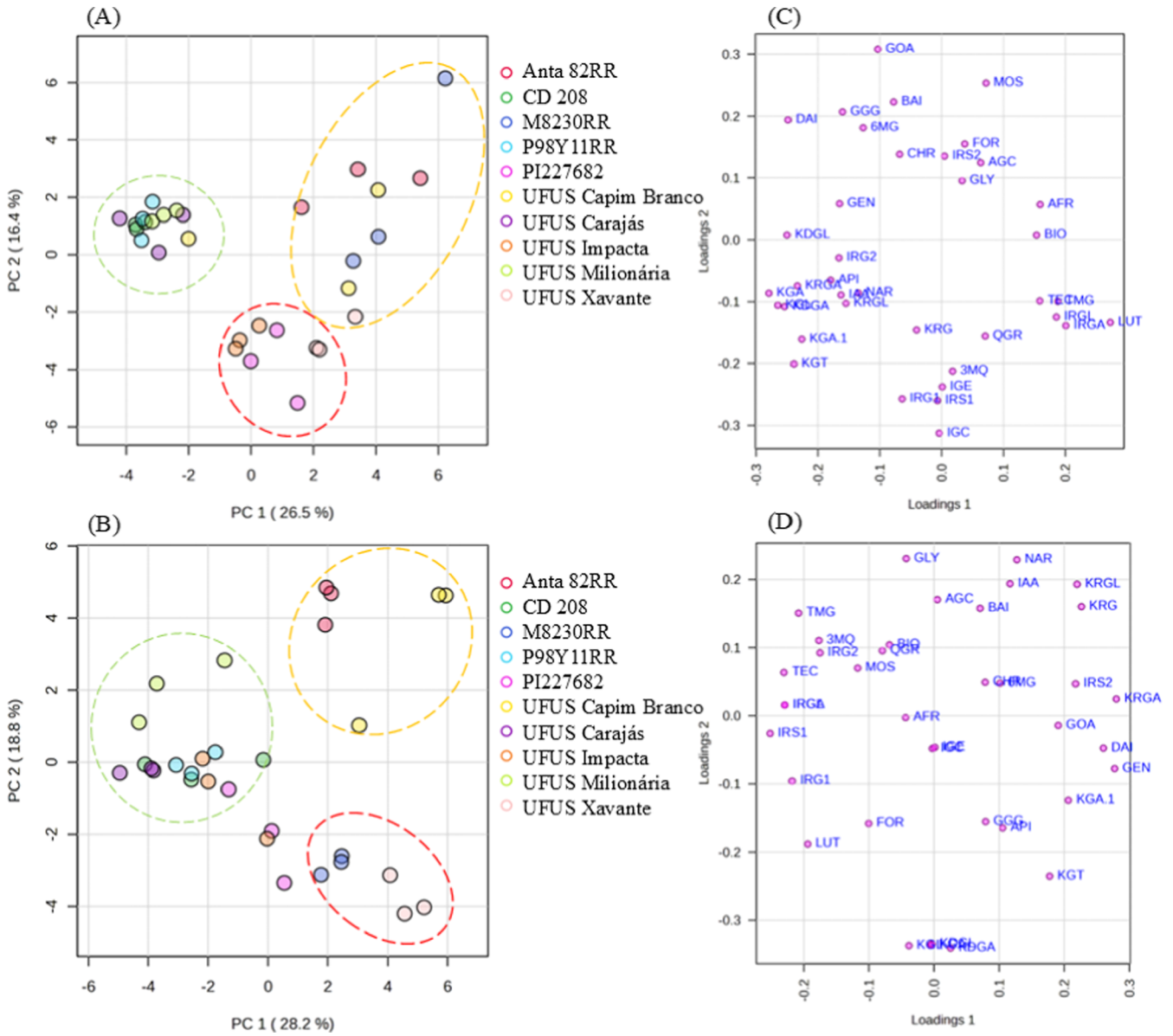


**Figure 1.** First and second component scores (A,B) and loadings (C,D) of samples and variables. They are arranged according to the influence of the relative areas of the chromatographic signals found in the leaves of the cultivars Anta82RR, CD208, M8230RR, P98Y11RR, PI227682, UFUS Capim branco, UFUS Carajás, UFUS Impacta, UFUS Milionária and UFUS Xavante (codes of the variables listed in Table 2). Compound acronyms are shown in Table 2.

analyzed their natural and induced responses. This helped us to find similarities and differences. Figure 1A shows the PCA plot for control plants. Figure 1B shows the PCA plot for herbivore-damaged plants.

Figure 1A shows a clear group of the cultivars CD208, P98Y11RR, UFUS Carajás, and UFUS Milionária. Several compounds cause this variation. These include luteolin, mosloflavone, daidzein, biochanin A, afrormosin, isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-rutinoside, 6″-*O*-malonyltectoridin, kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-galactopyranoside, kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-glucopyranoside, kaempferol-3-*O*-digalactopyranoside, kaempferol-3-*O*-diglucopyranoside, isorhamnetin 3-*O*-galactoside, isorhamnetin glycoside (soyanin), and another unknown isorhamnetin glycoside (see Figure 1C).

Figure 2 shows the variation of these molecules in each cultivar. Figures 2A,B show similar changes for two compounds: kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-galactopyranoside and kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-glucopyranoside. Both cultivars, CD208, P98Y11RR, UFUS Carajás, and UFUS Milionária, have higher levels. Kaempferol-3-*O*-digalactopyranoside and kaempferol-3-*O*-diglucopyranoside also show similar patterns (Figure 2C,D). Kaempferol molecules are essential for plant health. They aid in defense by regulating salicylic acid levels.[34] However, conjugated secondary metabolites are generally less reactive than aglycones. They serve as storage or transport forms.[35]

We cannot now make a direct link between glycosylated kaempferol and plant defense now. The cultivars PI227682, UFUS Xavante, and M8230RR had more of two compounds.

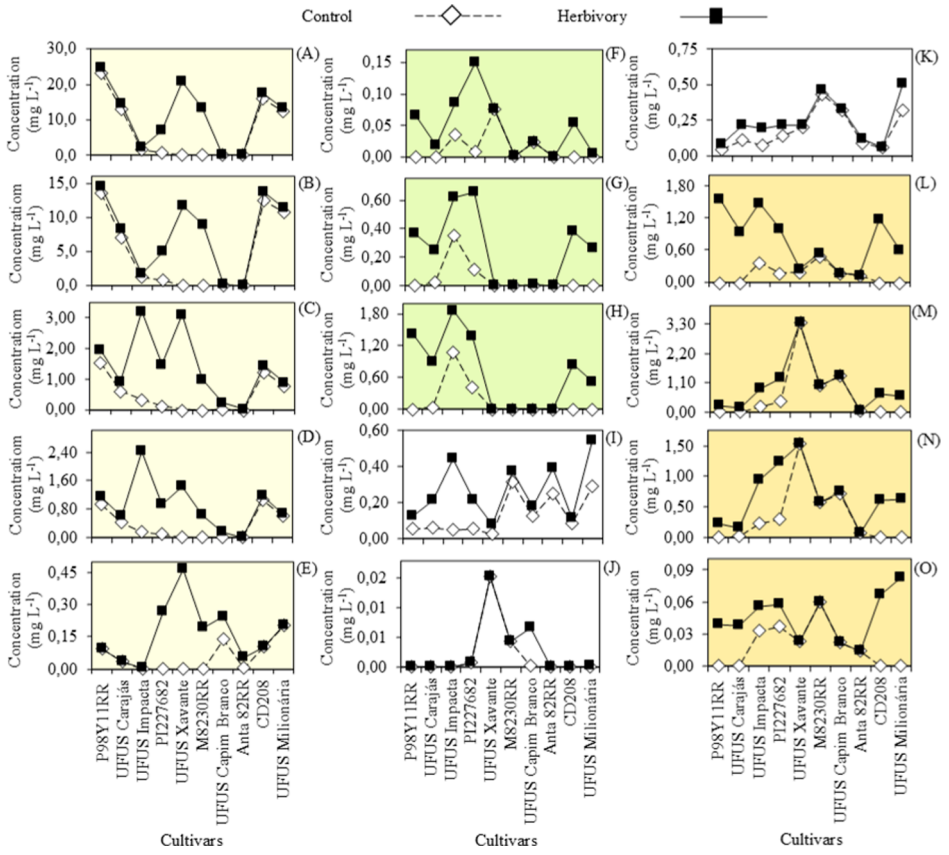


**Figure 2.** Variation in the content of KGAG (A), KGL (B), KDGA (C), KDGL (D), DAI (E), IGC (F), IRS1 (G), IRG1 (H), MOS (I), BIO (J), AFR (K), LUT (L), IRGA (M), IRGL (N), and TMG (O) with and without herbivory induction in the ten cultivars evaluated. Compound acronyms are shown in Table 2.

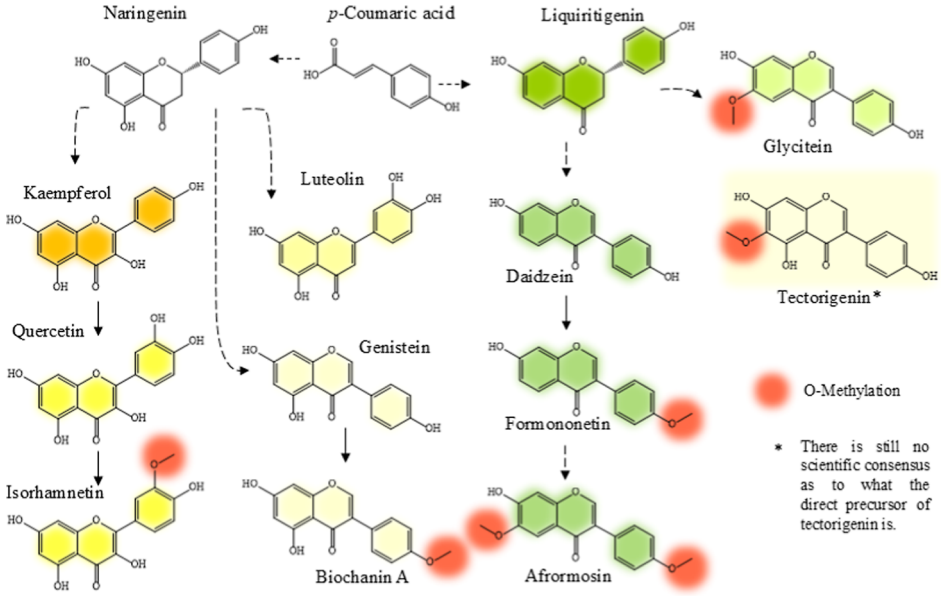


**Figure 3.** Summary of the biosynthesis of flavonoids that showed significant variation in the leaves of the soybean cultivars evaluated. We highlight the region of the molecules in which there is *O*-methylation.

These compounds are kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-galactopyranoside and kaempferol-3-*O*-*β*-D-glucopyranosyl (1 → 2)-*O*-[*α*-L-rhamnopyranosyl (1 → 6)]-*β*-D-galactopyranoside. This increase occurred in response to herbivory by *S. cosmioides*. Herbivory increased kaempferol-3-*O*-digalactopyranoside and kaempferol-3-*O*-diglucopyranoside levels in the UFUS Impacta cultivar. This suggests that these compounds may contribute to plant defense. These results suggest that different cultivars use different defense strategies.

We found daidzein associated with the largest group of cultivars: CD208, P98Y11RR, UFUS Carajás, and UFUS

Milionária. We measured daidzein in UFUS Capim Branco and its leaves under control conditions. Its profile was similar to that of molecules derived from kaempferol. Daidzein helps plants to defend themselves against herbivores. It acts as a defense response by increasing the production of defense compounds. It also interacts with cell signaling, changing the way cells express defense genes.[36] This leads to a faster and stronger response when herbivores attack.[37]

The cultivars CD208, P98Y11RR, UFUS Carajás, UFUS Impacta, and UFUS Milionária initially increased daidzein biosynthesis. However, they did not show any significant changes in daidzein content in the presence of herbivores (Figure 2E). Cultivars PI227682, UFUS Xavante, and M8230RR showed large increases in daidzein biosynthesis. Their levels reached up to three times the "basal" levels found in CD208, P98Y11RR, UFUS Carajás, UFUS Impacta, and UFUS Milionária. See Tables S1 and S2 in the Supporting Information for details. The persistence of herbivore-induced metabolites in leaves is complex. Higher daidzein levels in some cultivars may enhance herbivore defense or increase plant metabolism.[36] Thus, future studies should clarify how daidzein biosynthesis functions and its relationship to herbivore resistance.

PCA analysis showed that cultivars PI227682 and UFUS Impacta differed from the others. They showed a greater increase in isorhamnetin and its glycosides. These include isorhamnetin-3-*O*-galactoside, isorhamnetin-3-*O*-glucoside, and an unknown isorhamnetin glycoside under control conditions. See Figure 2F−H for details. These cultivars produce more phenolic compounds, such as isorhamnetin. This suggests that they have a unique metabolic profile. They may also have better defense mechanisms.

Isorhamnetin is a 3′-methoxylated form of quercetin (Figure 3). It has antioxidant properties and stimulates the production of phytoalexins. These substances help plants resist pathogens and herbivores. Isorhamnetin helps cowpeas resist aphids.[38] It also helps soybean cultivars resist the velvet bean caterpillar (*A. gemmatalis*).[39] PI227682 and UFUS Impacta can produce more isorhamnetin. This ability may help them survive better in regions with many herbivores. The Anta82RR cultivar does not contain isorhamnetin or its glycosides. This shows that soybean genotypes have a wide range in their ability to produce phenolic compounds. Breeding programs can take advantage of this genetic variability. It helps to develop cultivars that are more resistant to pests and diseases.

We found that the cultivars Anta 82RR, M8230RR, UFUS Capim Branco, UFUS Xavante, UFUS Impacta, and PI227682 had higher levels of mosloflavone, biochanin A, afromosin, luteolin, isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-rutinoside, and 6″-*O*-malonyltectoridin. The PCA analysis (Figure 1C) showed this. All related metabolites have an *O*-methylated aglycone structure, except for luteolin. This is an interesting detail. The PCA analysis showed that the cultivars Anta 82RR, M8230RR, UFUS Capim Branco, UFUS Xavante, UFUS Impacta, and PI227682 induced more methoxylated flavonoids. These include mosloflavone, biochanin A, afromosin, and tectorigenin. These cultivars also had high levels of isorhamnetin glycosides. These include isorhamnetin 3-*O*-robinobioside and isorhamnetin 3-*O*-rutinoside.

Methylation is a common modification of flavonoids. It increases stability and supports various biological activities, including defense mechanisms.[40] Researchers have not done a comprehensive study of mosloflavone. However, they have found that it may help soybean plants resist herbivores.[41] Cultivars such as Anta 82RR, M8230RR, UFUS Capim Branco, CD 208, and UFUS Milionária showed mosloflavone levels greater than 0.08 $mg{\cdot}L^{-1}$ under in normal conditions (see Figure 3I). However, only UFUS Impacta showed a significant increase in this metabolite.

We have identified biochanin A, afromosin, and tectorigenin as keys to resistance in soy plants.[14,42] Methylation of these molecules contributes to the production of defense-related metabolites.[14] For example, biochanin A is derived from the 4′-*O*-methylation of genistein and is key to the isoflavonoid biosynthetic pathway (Figure 3). This pathway leads to several metabolites that aid in defense.[43] In addition, methoxyisoflavones may enhance plant interactions with beneficial microorganisms.[14] Figure 2J,K show clear differences in biochanin A and afromosin levels between cultivars under standard growing conditions. However, only UFUS Capim Branco showed a significant increase in biochanin A levels after herbivory.

Genetic diversity helps plants adapt to different environments. This explains why different cultivars produce different amounts of methoxyflavones. Cultivars that increase these molecules could help improve defenses against herbivores and other biological threats.[37] Isorhamnetin glycosides protect plants. They do this by scavenging free radicals and reducing oxidative damage from herbivores. One study compared aphid resistance in different chemotypes of a species. The results showed that those with more isorhamnetin had better resistance. This suggests a direct link between isorhamnetin and the resistance mechanism.[38]

Figure 2L−O show that the cultivars CD208, P98Y11RR, UFUS Carajás, and UFUS Milionária do not contain luteolin, isorhamnetin-3-*O*-robinobioside, isorhamnetin-3-*O*-rutinoside, or 6″-*O*-malonyltectoridin when grown under control conditions. This absence suggests that these metabolites may be related to certain defense mechanisms. However, not all cultivars are able to produce them. In contrast, varying concentrations of luteolin were always present in the other cultivars (Figure 2L). Luteolin is a flavone that helps plants defend themselves against herbivores. It enhances systemic acquired resistance and directly repels insects.[44] These metabolites can influence how different plant cultivars cope with stress. This includes stress from pests and environmental conditions.[45]

Figure 2 shows a variety of phenolic compounds. It highlights methoxyflavones and flavonoid glycosides. This shows that each soybean cultivar has a unique metabolic profile. This metabolic variability shows that cultivars have developed unique defense strategies. These strategies adapt to environmental conditions and herbivore and pathogen pressure. Plants gain an advantage by storing defense-related metabolites. This allows them to respond more quickly and efficiently to future attacks. The lack of changes in metabolites after biotic stress may indicate other defense strategies. This could mean that new metabolites are being produced or that other pathways are being activated.[37]

Previous studies show that different cultivars produce different defense metabolites. They also have different responses to stress.[26,38,43] Our results confirm these findings. They emphasize the importance of genetic diversity and environmental conditions. These factors are key in assessing how plants defend themselves. We conclude that the response of soybean to herbivory is complex and varies among cultivars.

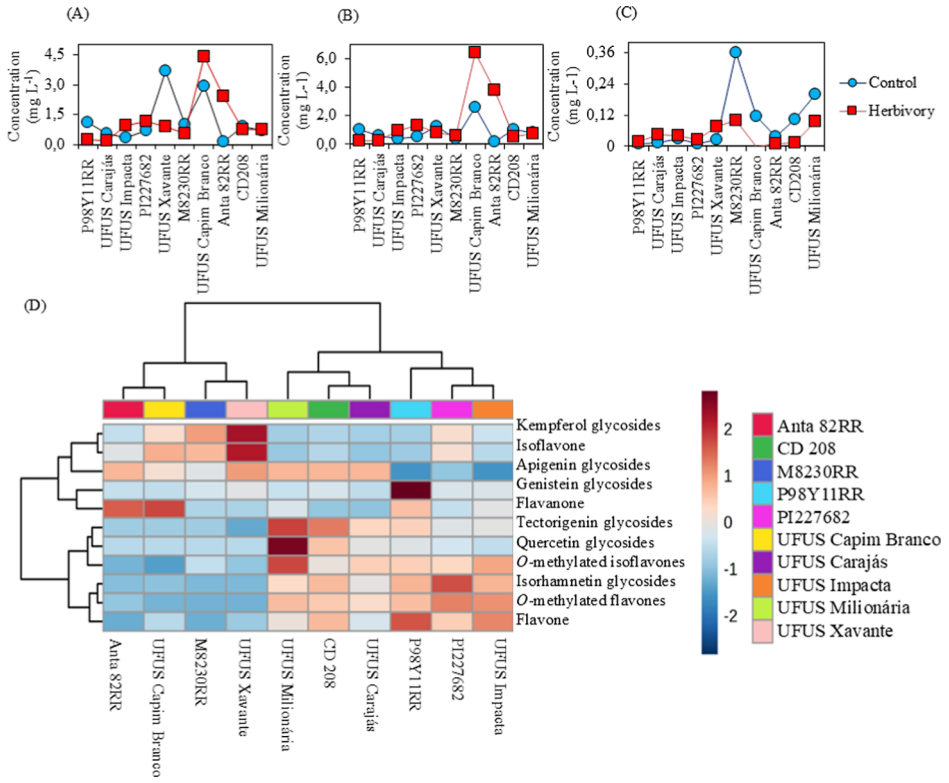


**Figure 4.** Variation in the content of KRGA (A), HRGL (B), and FOR (C) with and without herbivory induction in the ten cultivars evaluated. (D) Heatmap. Samples are represented by columns and metabolites by rows. The abundance of each metabolite is represented by a specific color. Up-regulated and down-regulated metabolites are represented by different shades of red and blue, respectively. Compound acronyms are shown in Table 2.

Multivariate analysis identified several metabolites involved in defense mechanisms. Among them, isoflavonoids and their derivatives stand out. We need to look at which metabolites are associated with the most and least affected clusters of cultivars. This will help us to understand the defense strategies after *S. cosmioides* induces resistance. The following topic describes this analysis in detail.

**Soybean Leaf-Induced Resistance Response.** The PCA analysis in Figure 1B revealed changes in the cultivar groups after herbivory was induced. Cultivars with similar metabolic profiles showed different responses to herbivory. Cultivar M8230RR, grouped with UFUS Capim Branco and Anta 82RR, showed a unique metabolic profile after induction. This profile showed a lower production of two compounds: kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1 → 6)-*β*-D-galactopyranoside and kaempferol-3-*O*-*α*-L-rhamnopyranosyl (1 → 6)-*β*-D-glucopyronoside (Figure 1D). This reorganization illustrates the different responses of each cultivar to herbivory, highlighting the complex defense mechanisms and genetic diversity among the genotypes.

Figure 4A,B show that herbivores consuming UFUS Capim Branco and Anta 82RR produced more kaempferol glycosides. Other cultivars did not show significant changes in glycosylated kaempferol levels after herbivory. The biosynthesis of these compounds was at least twice as high in UFUS Capim Branco and Anta 82RR as in compared to the other cultivars. These compounds may contribute to the protection of these specific cultivars. It is unclear how the production of kaempferol glycosides is related to resistance to *S. cosmioides*.

The relationship between metabolite production and herbivore resistance is complex and can vary depending on the compounds, herbivores, and environmental factors involved. Research shows that some glycosylated phenolic compounds may repel or attract herbivores. This depends on the species and the amount used.[15,38,46] These findings suggest that soybean cultivars have different responses to herbivory. Also, the role of specific metabolites may be more complex than we thought.

Glycosylation can alter the bioactivity of flavonoids, and some glycosides are known to stimulate feeding in other herbivores.[47] However, without behavioral assays (e.g., dual-choice tests), we cannot conclude that these metabolites directly increase susceptibility. Future studies should test whether glycosylated flavonoids function as attractants or if their accumulation correlates with other metabolic trade-offs.

Despite producing more kaempferol glycosides, these cultivars were still considered susceptible to *S. cosmioides*. Plant defense mechanisms are complex. They involve a cascade of metabolites. These metabolites work together, so they're not tied to one type. Glycosylated kaempferol molecules play a key role in plant defense. They can inhibit indole-3-acetic acid (IAA). Such inhibition increases IAA levels and facilitates the production of other defense-related compounds.[48]

Herbivory induction targeted a specific group of cultivars: CD208, P98Y11RR, PI227682, UFUS Carajás, UFUS Impacta, and UFUS Milionária. This induction was associated with more luteolin, formononetin, isorhamnetin-3-*O*-rutinoside, narcissin, and soyanin (see Figure 1D). Formononetin is a methoxyflavone that helps soybeans defend themselves. It was found to be significantly increased in these cultivars (Figure 4C). Formononetin helps plants in two ways.[49] First, it protects against herbivores. Second, it promotes mycorrhiza-

tion, which strengthens the root system. This makes plants more resistant to stress.[50] Studies show that mycorrhization helps plants resist herbivores and improves their health.[51]

These results show that herbivory can lead to a variety of plant responses. Plants produce defensive compounds and alter their relationships with supportive microorganisms. Analysis of the heat map based on the identified compound classes helps us to visualize this information. To do this, we imported the different metabolites into the database according to their chemical classes. Herbivory induction altered several metabolites. These include kaempferol glycosides, methoxyflavones, and luteolin. This suggests that insects activate different pathways when they attack.

Figure 4D shows that herbivory increased the levels of many *O*-methylated metabolites. These include glycosylated and aglycone forms. The cultivars that showed this change are CD208, P98Y11RR, PI227682, UFUS Carajás, UFUS Impacta, and UFUS Milionária.

Although our study provides robust chromatographic data, using samples that were initially collected and subjected to an metabolomic quenching by $N_2$ liquid, it has some limitations. We used in vitro extraction and UHPLC−qTOF−MS analysis, which may not accurately reflect all metabolic changes in the real world. To the best of our knowledge, no single protocol can investigate a full chemical profile. It is necessary to build and overlap the pieces of information according to new discoveries. Future research should test these findings in field trials under different conditions, such as drought or temperature shifts, to evaluate flavonoid stability. Additionally, enzymatic assays or genetic experiments could help confirm the role of key biomarkers. Finally, since factors such as soil microbes, seasons, and herbivores can alter flavonoid profiles,[10,37] further studies should ensure that these metabolic patterns remain consistent across various farming systems.

**Identification of Resistance Biomarkers in Soybean.** Identifying resistance biomarkers is essential to developing more sustainable and pest-resistant soybean varieties. Understanding the molecular mechanisms of resistance allows for genetic improvements that increase the production of defense compounds, thereby reducing pesticide use and environmental damage.[52]

In this section, we examined how each cultivar responds to herbivory. Our goal was to identify biomarkers of soybean resistance. We used partial least-squares discriminant analysis (PLS-DA) to analyze the data. We analyzed variable importance in projection (VIP) scores. This analysis revealed the key metabolites that distinguished our results. See Figures S2−S5 in the Supporting Information for more details. We considered variables with a VIP score of 1 or higher to be important in the PLS-DA model. Table 4 lists these variables.

Each cultivar showed a wide range of responses to herbivory by *S. cosmioides*. However, a common trend emerged: the modulation of kaempferol glycosides, as detailed in Table 4. We compared three types of cultivars: susceptible, resistant, and highly resistant (see Table 1). We found a relationship between glycosylated kaempferol derivatives and herbivory resistance. Susceptible cultivars have higher levels of these compounds. In contrast, resistant and highly resistant cultivars have lower concentrations. In addition, resistant cultivars appear to prioritize the biosynthesis of other defense-related molecules. Adding sugars (glycosylation) to flavonoids changes their properties. This can change the way they interact with

**Table 4. Compounds with VIP Scores ≥ 1**[a]

| Compound annotation* | P98Y11RR | UFUS Carajas | UFUS Impacta | PI227682 | UFUS Xavante | M8230RR | UFUS Capim Branco | Anta 82RR | CD208 | UFUS Milionaria | High resistance | Resistance | Susceptible |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LUT | | | | | | | | | | | | | |
| GLY | | | | | | | | | | | | | |
| BIO | | | | | | | | | | | | | |
| GEN | | | | | | | | | | | | | |
| KGA | | | | | | | | | | | | | |
| KGL | | | | | | | | | | | | | |
| KDGA | | | | | | | | | | | | | |
| KDGL | | | | | | | | | | | | | |
| KRGA | | | | | | | | | | | | | |
| KRG | | | | | | | | | | | | | |
| KRGL | | | | | | | | | | | | | |
| KGA | | | | | | | | | | | | | |
| IRGA | | | | | | | | | | | | | |
| GGG | | | | | | | | | | | | | |
| AGC | | | | | | | | | | | | | |

[a]Blue color = decrease in concentration; red color = increase in concentration; yellow color = no change in concentration. *Compound acronyms are shown in Table 2.

insect sensory receptors. This may also reduce their bioactivity.[47]

Similar flavonoid-mediated resistance mechanisms have been observed in other crops. For instance, maize[13] and rice[53] modeled the production of *O*-methylated flavonoids as resistance mechanisms against fungal pathogens and *Meloidogyne graminicola*, respectively. This consistency across species suggests that flavonoid biomarkers could establish universal standards for evaluating crop resistance. Regulatory bodies may want to consider cross-category guidelines for flavonoid-enhanced cultivars, especially in regions with similar pest issues. However, species-specific metabolic pathways necessitate a tailored approach to risk assessment.

Our results also suggest that the resistance of some soybean cultivars to *S. cosmioides* is not due to increased production of defense compounds. We have found that reduced production of certain metabolites, such as glycosylated kaempferol, contributes to resistance. These metabolites may attract insects or increase leaf consumption. Herbivore resistance involves many factors. It includes the production of repellents substances and the reduction of attractants. Reducing the production of metabolites makes cultivars less attractive to herbivores, thereby increasing their resistance and protecting them from predators.

The discovery of resistance biomarkers, such as formononetin and isorhamnetin glycosides, is paving the way for the development of soybean varieties that are more resistant to herbivores. The relationship between the production of defense metabolites and herbivore resistance is complex. It involves multiple factors. Further research is needed to elucidate the molecular mechanisms involved. Understanding these mechanisms is essential for developing more effective and sustainable pest management strategies.

Our findings have significant implications for food regulation and risk assessment. Identifying flavonoid biomarkers, such as formononetin and isorhamnetin glycosides, creates targets for breeding programs aiming to enhance the biotic resistance of

soybean cultivars. This could reduce the need for synthetic pesticides, aligning with the global shift toward sustainable agriculture.[19]

Additionally, the differential accumulation of flavonoids (e.g., glycosylated versus *O*-methylated forms) in transgenic versus traditional cultivars reveals potential trade-offs between yield and defense chemistry. Regulatory frameworks could leverage these insights to evaluate the ecological risks of genetically modified crops and ensure that metabolic trade-offs do not inadvertently increase susceptibility to pests. Future studies should explore the in vivo and in vitro validation of these and other flavonoid structural variants to refine resistance strategies. Furthermore, flavonoid profiling could serve as a tool for certifying crop resilience, informing labeling practices, and guiding integrated pest management policies.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.5c00235.

> Workflow required for the execution of this research (Figure S1). Analytical curves prepared (Figure S2). Quantification of the compounds extracted from the leaves of *G. max* for the cultivars P98T11RR, UFUS Carajás, UFUS Impacta, PI227682 and UFUS Xavante (Table S1) and for the cultivars M8230RR, UFUS Capim Branco, Anta 82RR, CD 208 and UFUS Milionária (Table S2). PLS-DA variable importance projection (VIP) scores for P98Y11RR, UFUS Carajás, and UFUS Impacta (Figure S3), PI227682, UFUS Xavante, and M8239RR (Figure S4), UFUS Capim Branco, Anta 82RR, and CD 208 (Figure S5), and UFUS Milionária (Figure S6) (PDF)

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