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Profile of Secondary Metabolite Compounds in Soybean (*Glycine max* L.) Leaves under Different Shading Treatments

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This study aims to assess the impact of shading treatment variations on soybean leaf metabolites. The research was conducted as follows: (i) planting several soybean varieties under different shading intensities; (ii) extracting metabolic substances from soybean leaves through maceration; (iii) identifying the extracted substances using High-Performance Liquid Chromatography (HPLC); and (iv) analyzing changes in primary metabolic substances. This study applied different shading treatments to various local soybean varieties: Anjasmoro, Mutiara 1, Denasa 1, Denasa 2, Dena 1, and Dena 2. The results indicate that among the different varieties and shading treatments, the highest isoflavone content was observed in Dena 2 with shading treatment on producing plants (1.820 g), followed by Anjasmoro with shading treatment on non-producing plants (1.788 g), and Dena 2 with no shading treatment (1.780 g). In addition, the statistical analysis revealed that shading treatments show significant differences only in extract mass content treated using fermentation on day 1 (p-value = 0.011) and day 3 (p-value = 0.039). These findings provide valuable insights for the scientific cultivation, management, and large-scale breeding of soybeans, specifically offering guidance on optimal shading levels for improved growth and metabolite production.
Keywords: Photosynthetic efficiency, isoflavone accumulation, crop management, metabolic profiling, shading effects.

INTRODUCTION

As an essential ecological component, light exerts a significant influence on the growth and development of plants. The effect of light as a source of environmental cues on plants may lead to diverse adaptations in their physiological, morphogenetic, and metabolic processes (Liu *et al.*, 2018a; Liu *et al.*, 2018b; Huang *et al.*, 2021). Hence, lighting conditions are frequently regarded as an environmental element highly associated with plant growth and secondary metabolic processes (Murthy *et al.*, 2024). Additionally, plants of the same species have different light environmental requirements at different growth phases, and farmers frequently implement a variety of technical measures to modify light intensity to meet these needs (Su *et al.*, 2024). Changes in light due to shading profoundly affect plant photosynthesis, plant growth, morphology, anatomy, various aspects of cellular physiology and biochemistry, flowering time, and plant productivity (Zhang *et al.*, 2022). Under insufficient light conditions, plant growth is disrupted due to a lack of ATP and the energy supply needed for photosynthesis (Valladares and Niinemets, 2018; Niinemets, 2020). Additionally, plant responses to light deficiency or shading are related to physiological, biochemical, anatomical, and leaf morphology processes (Valladares and Niinemets, 2018). In this context, light intensity can be adjusted by shading which can reduce active photosynthesis radiation, influencing photosynthesis and photomorphogenesis (Dennis *et al.*, 2020; Liu *et al.*, 2020; Xu *et al.*, 2020).

Soybean cultivation using shading techniques results in competition among plants for nutrients, water, and light. The shading reduces light intensity, which hinders the growth of soybean plants. As a result, soybeans possess specific adaptive characteristics to grow and produce effectively. Previous studies have proven that plants can adapt to their surrounding light conditions by increasing leaf area, reducing leaf thickness, maximizing photosynthetic pigments, and optimizing mineral absorption (Chmura *et al.*, 2017). Earlier research also demonstrated a relationship between secondary metabolites and light intensity. Under sufficient nutrient conditions, shading techniques can inhibit the photosynthesis process and reduce the carbon/nutrient (C/N) ratio. This leads

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to a decrease in carbon-containing compounds, such as flavonoids and terpene phenols (Cai *et al.*, 2009). Additionally, the accumulation of anthocyanins, proanthocyanidins, and sucrose in black soybean seeds can be influenced by shading techniques, both spatially and temporally (Dennis *et al.*, 2020). In this context, secondary metabolites are more sensitive to environmental conditions than primary metabolites, as they play a role in maintaining plant stress resistance (Erb and Kliebenstein, 2020).

This study applied several shading treatments, including shading under non-producing coconut trees versus producing coconut trees. The soybean plants used in this study were from various varieties. The objective was to analyze the influence of different shading intensities on soybean leaf metabolites. To achieve this goal, we identified several metabolite components in soybean leaves, then the metabolic substances were classified, and trends in the changes of primary metabolic substances under different shading intensities were determined. Eventually, the overall objective of this study is to obtain a data profile of secondary metabolites produced by soybean plants during the shading adaptation process.

MATERIALS AND METHODS

***Materials*:** The materials used included soybean seeds (*Glycine max* L.) of the following varieties: Anjasmoro, Mutiara 1, Denasa 1, Denasa 2, Dena 1, and Dena 2. Additionally, a fertilizer mixture consisting of 25% manure (Setia Tani, Tangerang, Indonesia) and 75% water hyacinth compost (obtained from local community in Medan Labuhan, Indonesia) was used. The materials employed for cultivating the soybeans in small polybags included soil, rice husks, and a small quantity of charcoal obtained from PT Bukit Asam Tbk, Indonesia. Ethanol 70% was also utilized as material.

***Instrumentation*:** The instruments used in this study included the following: High-Performance Liquid Chromatography (HPLC; Rigol L3000), a centrifuge (swing type model CD-50 SR Tomy Seiko), a rotary evaporator (IKA, RV 3 V), and an oven. HPLC is particularly well-suited for separating, identifying, and quantifying complex mixtures of metabolites, which is essential for understanding the biochemical changes in soybeans (Board, 2013; Wilson, 2011). Unlike other techniques, HPLC can handle complex and diverse samples, providing high-resolution results that clearly distinguish between individual metabolites. Additionally, HPLC can quantify the exact amounts of each metabolite. However, it has several limitations. The time required for each HPLC run can limit sample throughput, making it time-consuming to process many samples. Furthermore, HPLC may not detect all metabolites, particularly those in very low concentrations or those not interacting well with the column's stationary phase.

***Soybean planting*:** The soybean planting method is based on Widiastuti and Latifah (2016). The soybean seeds (*Glycine max* L.) used were Anjasmoro, Mutiara 1, Denasa 1, Denasa 2, Dena 1, and Dena 2 varieties. Fertilizers were applied with a mixture of 25% manure and 75% water hyacinth compost, and the watering frequency was once a day. Water hyacinth compost is preferred over manure for soybean growth because it provides essential nutrients like nitrogen, phosphorus, and potassium. Its slow-release nature helps a steady nutrient supply to soybeans throughout their growth cycle (Ogbuehi and Ibe, 2021).

These soybean varieties had a daily water requirement ranging from 440 to 550 mL per plant, which is equivalent to 5.37 to 5.95 mm per day. The water use efficiency is estimated to be 3.49 to 5.60 g/mm (Makarim *et al.*, 2017). The temperature for optimal growth was maintained between 25-32 °C. Soybean pods form optimally at temperatures ranging from 26.6-32.0 °C (Widiastuti and Latifah, 2016). In the initial 7 days of growth, the plants were cultivated using artificial planting media, which is a combination of soil, rice husks, and a small amount of charcoal placed in small polybags. After 7 days, the plants were transferred to slightly larger polybags. At the 14-day mark, stakes were added to support the soybean plant stems and encourage upright growth. Harvesting was done after 70-90 days when the soybean plants were ready. Leaf samples from the soybean plants were then prepared for isoflavone testing.

***Shading treatment on soybean leaves*:** The treatments consisted of shading levels (S) as the primary treatment, with three treatment levels, namely no shading (S_0), shading on non-producing coconut plants (S_1), and shading on producing coconut plants (S_2). The main reason for choosing coconut plants for shading is their ability to provide ample shade coverage (Tscharntke *et al.*, 2011). Coconut palms naturally grow tall with wide canopies means offering significant shade.

In addition, soybean plant varieties (V) as the second treatment have six varieties: Anjasmoro (V_1), Mutiara 1 (V_2), Denasa 1 (V_3), Denasa 2 (V_4), Dena 1 (V_5), and Dena 2 (V_6). In this study, 18 treatment combinations (3 × 6) with two repetitions were applied. So, the total was 36 research plots × 9 plants per plot = 324 plants, of which two were destructive samples.

***Isoflavones extraction via maceration method*:** This study employed maceration because it provides a simple and efficient means of extracting isoflavones from soybean leaves. It is simple, particularly suitable for smaller setups, and gentle on heat-sensitive compounds like isoflavones (Abubakar and Haque, 2020). However, it can be slow and might not yield as much as newer methods (Geow *et al.*, 2021). Additionally, it necessitates a substantial amount of solvent and lacks specificity in targeting particular compounds.

Initially, 100 g of young leaf samples underwent no fermentation and were ground into a powder. The powdered samples were then macerated in 250 mL of 70% ethanol for

24 hours, filtered, and the resulting filtrate was collected (Purba *et al.*, 2024). Subsequently, the same procedure was repeated for fermentation days 1, 2, 3, and 4. Fermentation was conducted at a temperature range of 25-35 °C. This study applied fermentation method because different fermentation durations can result in different levels of enzymatic activity and microbial breakdown of plant cell walls, which can impact the release of target compounds like isoflavones (Lasinskas *et al.*, 2020). During fermentation, enzymes secreted can break down bonds between flavonoids and proteins or fats, potentially enhancing their release (Xu *et al.*, 2023).

This study used ethanol as the solvent because, besides having polarity close to that of methanol, ethanol is relatively non-toxic. The residue was mixed with 100 mL of 70% ethanol, macerated for 24 hours, filtered, and the filtrate was collected. The second residue was re-filtered after being mixed with 100 mL of 70% ethanol. The filtrate from the maceration was then concentrated using a rotary evaporator, resulting in a concentrated extract. The concentrated extract was heated for 30 minutes at 50 °C to produce an ethanol extract. This extract was then placed in an oven at 40 °C to evaporate any remaining solvent before being weighed to determine the extraction yield. The HPLC was then used to identify the isoflavones in the ethanol extract.

Isoflavones identification: The identification of isoflavones was carried out through HPLC instrument conditioning and sample solution preparation. The sample solution was prepared by taking 1 mg of ethanol extract obtained from the extraction, and each was dissolved in 10 mL of ethanol. The solution was then centrifuged, and 20 µL was taken using an injection tool. The sample was then injected into the HPLC after the conditioning was completed. The HPLC chromatogram was analyzed using a comparison of standard isoflavone chromatograms consisting of daidzein, genistein, and glycitein.

RESULTS

Isoflavones extraction results: The extraction results of isoflavone from soybean leaves across several varieties under different shading treatments are presented in Table 1.

According to Table 1, the variety with the highest extraction amount on the fourth day under the no shading treatment (S_0) is Denasa 1 (S_0V_3), with 5.741 g from 100 g of the sample. Meanwhile, under the shading treatments for non-producing (S_1) and producing plants (S_2), the largest extract yields on the fourth day are found in the Dena 2 varieties (S_1V_6 and S_2V_6), specifically 5.726 and 5.623 g, respectively, from 100 g of the sample.

Table 1. Isoflavone extract from soybean leaves (per 100 g).

Sample code	Extraction yield (g)				
	Fermentation period (day)				
	0[a]	**1[b]**	**2[c]**	**3[d]**	**4[e]**
S_0V_1	0.565	1.925	2.382	2.751	3.264
S_0V_2	0.535	1.274	2.029	2.126	3.086
S_0V_3	0.593	1.963	2.351	3.376	5.741
S_0V_4	0.565	1.925	4.782	3.351	5.264
S_0V_5	0.635	1.632	3.325	3.476	4.621
S_0V_6	0.703	1.831	3.362	3.721	4.645
Mean (S_0)	0.599	1.758	3.039	3.134	4.437
Std. Dev.	0.061	0.266	1.015	0.588	1.064
S_1V_1	0.421	1.658	3.271	4.265	4.772
S_1V_2	0.973	2.532	4.623	3.260	5.720
S_1V_3	0.623	2.768	3.824	4.362	4.962
S_1V_4	0.421	2.025	2.421	3.726	4.532
S_1V_5	0.624	2.021	2.432	3.260	5.020
S_1V_6	0.433	1.982	3.726	4.803	5.726
Mean (S_1)	0.583	2.164	3.383	3.946	5.122
Std. Dev.	0.215	0.407	0.859	0.632	0.496
S_2V_1	0.542	2.426	3.561	4.063	4.823
S_2V_2	0.733	2.603	4.358	4.426	5.033
S_2V_3	0.603	2.086	3.263	3.088	4.729
S_2V_4	0.620	2.763	3.861	4.021	5.326
S_2V_5	0.803	2.326	4.335	4.235	4.230
S_2V_6	0.923	2.175	3.365	3.780	5.623
Mean (S_2)	0.704	2.397	3.791	3.936	4.961
Std. Dev.	0.143	0.256	0.477	0.468	0.487

Note: Superscript a-e represents color observed in the samples for each fermentation period: a = light yellow, b = brownish yellow, c = brownish yellow, d = brown, and e = dark brown.

Table 1 also reveals that the longer the fermentation period, the greater the extract mass produced. The extraction results of isoflavones from fermented soybeans range from light yellow to dark brown, indicating that the cyanide compounds have disappeared during soaking, making them safe for consumption. It can also be inferred from Table 1 that the extraction yield of isoflavones varies among soybean varieties. It is believed that this is due to the varying hardness or softness and density of the components in the seeds of each legume.

Isoflavones identification results: The HPLC analysis aimed to identify the presence of the isoflavone compounds daidzein, glycitein, and genistein in various fermented soybean leaf samples. Like other chromatographic techniques, HPLC analysis involved comparing the standard isoflavone compounds' retention times with each sample's retention times. Peaks with similar relative retention times to daidzein, glycitein, and genistein indicate the presence of these isoflavone compounds in the sample. To mitigate differences in conditions, the retention times for the standard daidzein, glycitein, and genistein compounds were determined on the same day as the retention times for each sample.

The quantitative analysis of isoflavone compounds was done by calculating the area under the chromatogram. Then, the concentration of daidzein, glycitein, and genistein isoflavone

compounds can be calculated by multiplying the percentage area of each isoflavone compound in the chromatogram by the extracted mass. The results of the identification of isoflavones from the foliage of several soybean varieties are presented in Table 2.

Table 1. Results of isoflavone identification from leaves of various soybean varieties (per 100 g).

Sample code	Fermentation period (day)	Isoflavone content (g)			Total (g)
		Daidzein	Glycitein	Genistein	
S_0V_1	0	0.055	0.012	0.102	0.164
	1	0.442	0.075	0.527	1.044
	2	0.486	0.306	0.726	1.518
	3	0.529	0.081	0.428	1.038
	4	0.625	0.221	0.632	1.478
S_0V_2	0	0.053	0.018	0.098	0.169
	1	0.472	0.065	0.568	1.105
	2	0.508	0.276	0.823	1.607
	3	0.432	0.101	0.503	1.036
	4	0.673	0.218	0.672	1.563
S_0V_3	0	0.075	0.021	0.032	0.128
	1	0.363	0.072	0.542	0.977
	2	0.535	0.125	0.726	1.386
	3	0.482	0.186	0.563	1.231
	4	0.672	0.221	0.677	1.570
S_0V_4	0	0.027	0.035	0.068	0.130
	1	0.373	0.086	0.576	1.035
	2	0.672	0.135	0.803	1.610
	3	0.385	0.176	0.432	0.993
	4	0.702	0.252	0.723	1.677
S_0V_5	0	0.036	0.015	0.089	0.140
	1	0.403	0.073	0.438	0.914
	2	0.628	0.165	0.765	1.558
	3	0.532	0.132	0.482	1.146
	4	0.650	0.307	0.679	1.636
S_0V_6	0	0.053	0.021	0.092	0.166
	1	0.274	0.085	0.482	0.841
	2	0.681	0.062	0.733	1.476
	3	0.408	0.183	0.416	1.007
	4	0.732	0.323	0.725	1.780
S_1V_1	0	0.081	0.032	0.087	0.200
	1	0.265	0.092	0.464	0.821
	2	0.702	0.112	0.701	1.515
	3	0.432	0.178	0.424	1.034
	4	0.725	0.223	0.618	1.566
S_1V_2	0	0.011	0.082	0.076	0.169
	1	0.278	0.012	0.487	0.777
	2	0.735	0.085	0.734	1.554
	3	0.487	0.168	0.520	1.175
	4	0.786	0.250	0.602	1.638
S_1V_3	0	0.086	0.023	0.082	0.191
	1	0.280	0.078	0.425	0.783
	2	0.792	0.082	0.720	1.594
	3	0.402	0.155	0.415	0.972
	4	0.775	0.220	0.698	1.693
S_1V_4	0	0.072	0.027	0.076	0.175
	1	0.226	0.085	0.432	0.743
	2	0.682	0.088	0.765	1.535
	3	0.532	0.111	0.328	0.971
	4	0.745	0.253	0.688	1.686
S_1V_5	0	0.016	0.032	0.083	0.131
	1	0.278	0.075	0.428	0.781
	2	0.725	0.065	0.752	1.542
	3	0.512	0.131	0.318	0.961
	4	0.787	0.278	0.723	1.788
S_1V_6	0	0.072	0.018	0.063	0.153
	1	0.365	0.085	0.456	0.906
	2	0.626	0.016	0.678	1.320
	3	0.418	0.160	0.322	0.900
	4	0.654	0.225	0.765	1.644
S_2V_1	0	0.062	0.017	0.072	0.151
	1	0.461	0.072	0.576	1.109
	2	0.529	0.205	0.822	1.556
	3	0.460	0.123	0.512	1.095
	4	0.703	0.273	0.680	1.656
S_2V_2	0	0.025	0.027	0.068	0.120
	1	0.364	0.073	0.583	1.020
	2	0.662	0.142	0.813	1.617
	3	0.373	0.182	0.443	0.998
	4	0.725	0.268	0.733	1.726
S_2V_3	0	0.043	0.032	0.072	0.147
	1	0.264	0.072	0.501	0.837
	2	0.671	0.068	0.713	1.452
	3	0.418	0.192	0.423	1.033
	4	0.742	0.314	0.783	1.839
S_2V_4	0	0.071	0.013	0.078	0.162
	1	0.235	0.063	0.435	0.733
	2	0.712	0.133	0.721	1.566
	3	0.443	0.165	0.435	1.043
	4	0.733	0.217	0.627	1.577
S_2V_5	0	0.080	0.032	0.082	0.194
	1	0.276	0.067	0.432	0.775
	2	0.762	0.082	0.745	1.589
	3	0.432	0.163	0.405	1.000
	4	0.725	0.212	0.623	1.560
S_2V_6	0	0.023	0.012	0.070	0.105
	1	0.328	0.065	0.418	0.811
	2	0.735	0.053	0.720	1.508
	3	0.423	0.121	0.328	0.872
	4	0.795	0.263	0.762	1.820

Based on Table 2, daidzein was found to be highest in the Dena 2 variety on the fourth day with shading treatment on producing plants (S_2V_6; 0.795 g). Meanwhile, glycitein was found to be highest at 0.314 g in the Denasa 1 variety (V_3) with shading treatment on producing plants also (S_2V_3). On the other hand, genistein was most successfully extracted from sample S_0V_2, which had no shading treatment in the Mutiara 1 variety, at 0.823 g. Overall, among those three types of isoflavones, the highest content was found in S_2V_6, Dena 2, on the fourth day with shading treatment on producing plants.

Figure 1 illustrates the trend in isoflavone content based on the type of isoflavone from sample with no shading treatment (S_0). In the case of genistein, the trend shows an increase in all types of samples but a sharp decrease on the third day of fermentation. For daidzein, it is almost similar to the genistein trend, except for sample S_0V_1, which shows an increase trend until the fourth day. Meanwhile, the trend in glycitein content varies among samples.

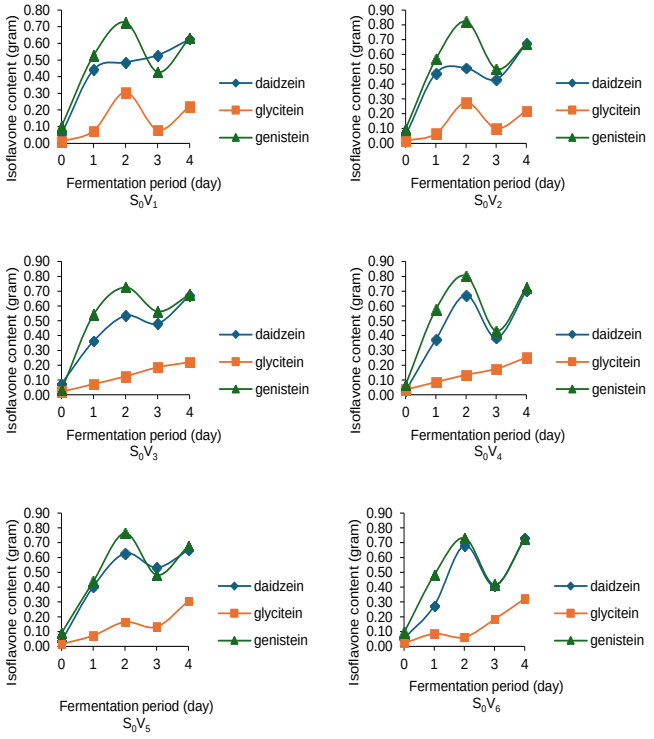

Figure 1. Isoflavone content in the treatment without shade (S_0) with various soybean varieties.

Figures 2 and 3 illustrate the trends in the content of isoflavones for the three types of isoflavones with treatment on non-producing and producing plants, respectively. When comparing the data for shaded treatments with data for treatments without shading, in the case of genistein, both for non-producing and producing plants, the results show a similar pattern, with an upward trend but a sharp decrease on the third day. As for daidzein, the trend is almost the same as the genistein trend, except for sample S_2V_1, which is the Anjasmoro variety with shade treatment on producing plants, where the decrease is not as sharp on the third day. However, the trend in glycitein content varies among samples and does not exhibit a consistent pattern.

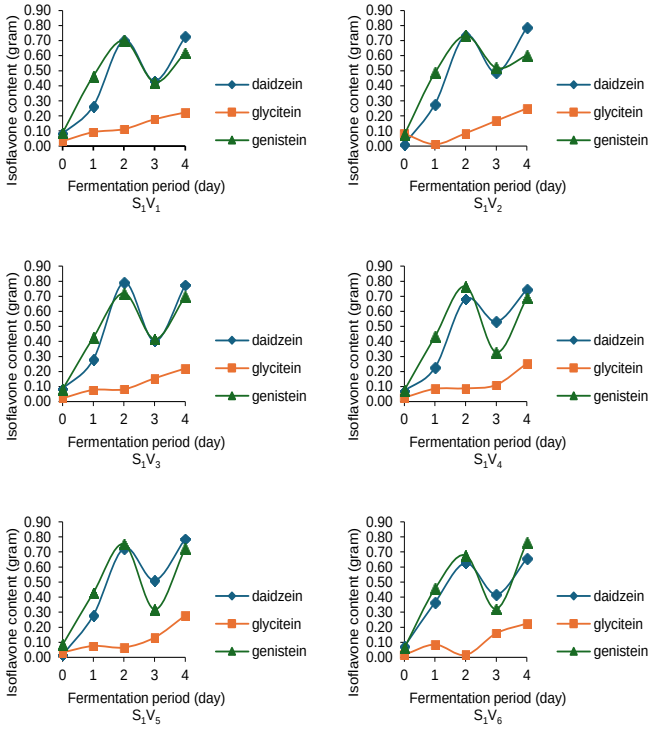

Figure 2. Isoflavone content in treatment shading on immature plants (S_1) with various soybean varieties.

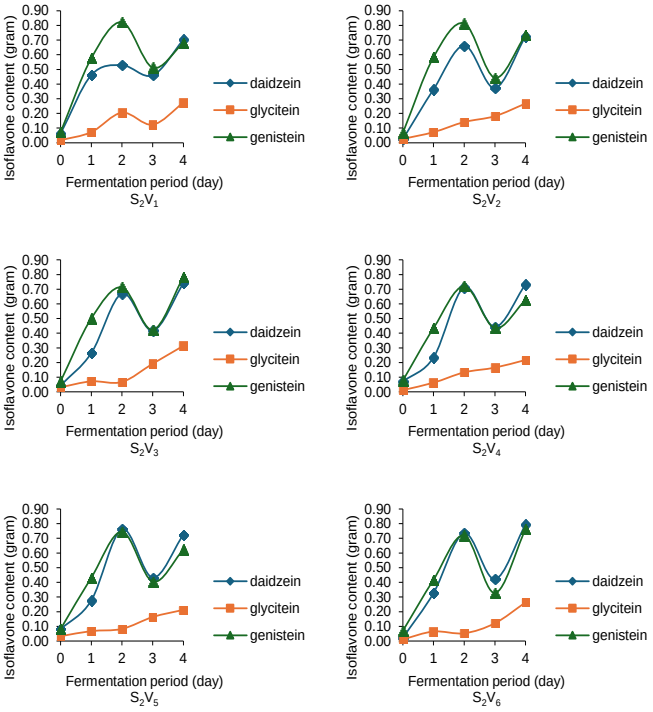

Figure 3. Isoflavone content in treatment shading on producing plants (S_2) with various soybean varieties.

Statistical analysis: This analysis was conducted to determine whether there were significant differences between treatments using data from extraction and isoflavone mass yield. Before conducting the analysis to compare means between groups, a prerequisite analysis was performed, including normality and homogeneity tests, to determine whether the analysis should follow a parametric or non-parametric method. Table 3 shows the results of normality testing on the extraction mass data and isoflavone mass data. The normality test for the extract mass dataset indicates that the data from days 1, 2, and 3 are normally distributed according to both the Kolmogorov-Smirnov and Shapiro-Wilk tests (p-value > 0.05). However, for days 0 and 4, there are conflicting results between the two tests, so normality cannot be assumed for these days. Additionally, for all isoflavone components (daidzein, glycitein, and genistein) and the total isoflavone content, both the Kolmogorov-Smirnov and Shapiro-Wilk tests show that the data are not normally distributed, as all p-values are less than 0.05.

Table 3. Normality test results.

Dataset	Variable	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Extract Mass	Day 0	0.206	18	0.043	0.918	18	0.118
	Day 1	0.136	18	0.200*	0.964	18	0.671
	Day 2	0.157	18	0.200*	0.944	18	0.337
	Day 3	0.100	18	0.200*	0.974	18	0.873
	Day 4	0.174	18	0.153	0.89	18	0.039
Isoflavone Mass	Daidzein	0.124	90	0.002	0.917	90	0.000
	Glycitein	0.162	90	0.000	0.926	90	0.000
	Genistein	0.142	90	0.000	0.891	90	0.000
	Total Isoflavone	0.148	90	0.000	0.892	90	0.000

Note: *This is a lower bound of the true significance; [a]Lilliefors significance correction.

Based on the normality results, only the datasets from extract mass on days 1, 2, and 3 were further analyzed for homogeneity. Table 4 shows the results of the homogeneity testing. Based on the results, all p-values from Levene's test are greater than 0.05, indicating that the assumption of homogeneity of variances is met for all variables tested.

Based on the prerequisite analysis, the datasets decided to use Kruskal-Wallis are from the extract mass dataset on days 0 and 4, as well as the dataset from isoflavone mass on all variables. Meanwhile, the dataset from extract mass on days 1, 2, and 3 were analyzed via ANOVA since the prerequisites are satisfied.

Table 5 presents the results of the Kruskal-Wallis test. The p-values for the assessed variables are greater than 0.05, indicating that there is no statistically significant difference in the total extract mass among the shading treatment groups, whether based on fermentation on day 0 and 4. Additionally, the shading treatment also indicates no significant difference in isoflavone mass generated, including in daidzein, glycitein, genistein, and total isoflavone.

According to Table 6, there are significant differences in shading treatments of extract mass during fermentation on days 1 and 3, with p-values (Sig.) of 0.011 and 0.039, respectively, both of which are less than the significance level of 0.05. However, no significant difference was observed in the data from day 2 fermentation.

In summary, these findings imply that while shading treatments may not significantly affect the total extract mass or isoflavone content overall, they could have varying effects at different stages of fermentation.

DISCUSSION

Table 4. Homogeneity test results.

Variable*	Factors	Levene Statistic	df1	df2	Sig.
Day 1	Based on Mean	1.134	2	15	0.348
	Based on Median	0.290	2	15	0.752
	Based on Median and with adjusted df	0.290	2	11.048	0.754
	Based on trimmed mean	1.090	2	15	0.361
Day 2	Based on Mean	1.392	2	15	0.279
	Based on Median	1.239	2	15	0.318
	Based on Median and with adjusted df	1.239	2	11.1	0.327
	Based on trimmed mean	1.386	2	15	0.280
Day 3	Based on Mean	0.761	2	15	0.484
	Based on Median	0.564	2	15	0.581
	Based on Median and with adjusted df	0.564	2	12.117	0.583
	Based on trimmed mean	0.757	2	15	0.486

Note: *Extract mass dataset.

Table 5. Kruskal-Wallis test.

Statistics	Variable					
	Day 0[a]	Day 4[a]	Daidzein[b]	Glycitein[b]	Genistein[b]	Total Isoflavone[b]
Kruskal-Wallis H	0.926	0.231	0.060	0.000	0.184	0.053
Df	1	1	1	1	1	1
Asymp. Sig.	0.336	0.631	0.807	0.982	0.668	0.819

Note: [a]Data from extract mass; [b]Data from isoflavone mass; The grouping variable is shading treatment (S).

Table 6. ANOVA test.

Variable	Factors	Sum of Squares	df	Mean Square	F	Sig.
Day 1	Between Groups	1.252	2	0.626	6.216	0.011
	Within Groups	1.511	15	0.101		
	Total	2.763	17			
Day 2	Between Groups	1.701	2	0.85	1.278	0.307
	Within Groups	9.977	15	0.665		
	Total	11.677	17			
Day 3	Between Groups	2.607	2	1.303	4.052	0.039
	Within Groups	4.825	15	0.322		
	Total	7.432	17			

Note: All data are from the extract mass.

Soybean cultivation using shading techniques plays an important role in the soybean industry. The effect of shading on soybean plant growth and physiological metabolite variables can directly influence robust growth, soybean survival rates, and the quality of the harvested soybeans. This study was conducted to determine the most effective shading treatment for soybean growth, which can ultimately assist cultivators in developing the soybean industry on a large scale.

The research results indicate a decrease in the levels of daidzein and genistein on day 3 for all treatments. The study by Hashim *et al.* (2018) showed that the optimal conditions for these two metabolite compounds occurred on the second day of tempeh fermentation, with respective contents of 1.34 and 0.94 mg/g for daidzein and genistein. According to Xiao *et al.* (2016) and Liu *et al.* (2023), during the fermentation process on the second day, the activity of *R. oligosporus* causes the conversion of isoflavone glucosides into aglycones, which in turn enhances β-glucosidase activity. Therefore, daidzein and genistein are expected to be optimal on the second day (McCue and Shetty, 2003).

On the third day, the total daidzein and genistein decreased because these compounds were consumed by *R. oligosporus* (Liu *et al.*, 2023). Then, on the fourth day, the study results showed an increase in daidzein and genistein, with some minor fluctuations, as supported by Hashim *et al.* (2018). It can be concluded that the optimal conditions for these two compounds occur on days 2 and 4. This finding is supported by Kuligowski *et al.* (2016), who stated that the optimal fermentation period lasts until the fourth day. Furthermore, Ban *et al.* (2020), explained that the decrease in isoflavon levels may occur due to metabolic oxidation events, as evidenced by the aging of soybean leaves (turning yellow). Therefore, if the process is extended to the fifth day, isoflavon levels are likely to continue to decrease.

The fluctuation in total isoflavones may be caused by several factors. One of the main factors is the reactive nature of isoflavone compounds in bonding with other compounds. Other factors include harvest time, planting location, planting time, and weather conditions (Mustonen *et al.*, 2018). This is evident in the research findings, which show that different soybean varieties with different shading treatments produce different total isoflavone levels. For example, the Dena 2 variety shows the highest total isoflavone content compared to other varieties with no shading treatment. In addition, Dena 2 with shading treatment produces the highest total isoflavone content, surpassing all other shading treatments. This is probably affected by the growth of the given shading treatment and the soaking to fermentation procedure.

The anticipated impact of shading treatments on isoflavone content in soybeans did not manifest as expected in this study. Instead, the significant effects were observed during the fermentation period regarding the mass extract. This unexpected outcome may be attributed to the intricate nature of metabolic processes in plants, wherein shading treatments could have indirectly influenced biochemical pathways, leading to alterations in extract mass instead. Furthermore, the effects of shading might have manifested through factors such as plant physiology, nutrient availability, or stress response, which affect overall metabolic activity and subsequently, extract mass (Eum *et al.*, 2020). It is also plausible that the observed discrepancy reflects a time lag in responses, where changes in extract mass occurred earlier or were more sensitive to the applied treatments compared to isoflavone

content (Kim and Kim, 2020). Additionally, interactions with other variables within the experimental setup and methodological considerations could have contributed to this unexpected outcome. Further investigation into these underlying mechanisms is warranted to gain a comprehensive understanding of the observed phenomenon.

In this study, the amounts of three isoflavones were reported in grams through HPLC analysis. Therefore, it is necessary to explain the relationship between their molecular weights. The analysis was conducted using HPLC, where chromatograms were identified by matching UV spectra and retention times of standard isoflavones and comparing them with literature data. The isoflavone contents were also converted into molar concentrations based on the molecular weight of each compound. The mobile phase used in the experiment was ethanol, which is a polar compound. Similarly, daidzein, genistein, and glycitein are also polar compounds. Therefore, all of them were well-extracted through the mobile phase. According to Jung *et al.* (2020), isoflavones exhibit nearly the same polar characteristics. However, based on the retention time in the HPLC chromatogram, daidzein had the fastest retention time, followed by glycitein, and genistein had the slowest retention time. This is because daidzein has the lightest molecular weight, 254 g/mol, while genistein and glycitein are 270 and 284 g/mol, respectively. The second in line is glycitein, even though it has a heavier molecular weight than genistein. Glycitein possesses unique characteristics due to the presence of a methoxy group at the C-6 position of the isoflavone nucleus. Research by Yuk *et al.* (2016) also indicated the same order of retention time for daidzein, glycitein, and genistein.

***Conclusion*:** The study explores the impact of different shading treatments on soybean growth and physiological metabolite indices to ensure robust growth, high survival rates, and superior quality. The study indicates that shading treatments might not have a substantial impact on the overall total extract mass or isoflavone content. However, they may exert diverse effects during different phases of fermentation. In addition, the study shows that the longer the fermentation period, the greater the isoflavone extract mass produced. The extraction yield of isoflavones varies among soybean varieties due to varying hardness and density of components. Factors such as variety, growing region, and harvest season significantly impact the physical and chemical properties of both shaded and sun-exposed soybean seeds. Meanwhile, the fluctuation in isoflavone compounds is likely due to factors such as reactivity and ease of oxidation, variety, harvest time, planting location, and climatic conditions. Different soybean varieties and shading treatments produce varying outcomes, with the highest isoflavone content of 1.820 g being Dena 2 with shading treatment on producing plants (S_2V_6).

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