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Photosynthesis and Kaempferol Yields of Soy Leaves Under ABA Application and Mechanical Wounding

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Abstract

Environmentally sound plant treatments that can impose mild physiological stress and elicit bioaccumulation of useful phytochemicals such as kaempferols are limited. We tested ABA foliar application, 100 or 200 μM, and two types of leaf wounding, piercing or hole punching in young greenhouse-grown soy plants. Leaf gas exchange and A/C_i response, ΦPSII, pigments and antiradical activity were measured using the same leaf and kaempferols were measured in the leaf above. ABA 200 μM-treated plants had ≥ 20% less gas exchange and 17% less ETR, but greater V_{cmax} and J_{max} compared to control. They had 55% and 100% more stomatal limitation to P_{net} and ΦPSII, respectively, than control. Leaf-wounded plants showed the lowest stomatal limitation to either P_{net} or ΦPSII. Leaf piercing increased chlorophylls 39% and carotenoids 38% compared to control. Six kaempferols quantified were found to be mono-, di- and triglycosides. Each leaf treatment increased total kaempferol yield ranging from 42% in ABA 100 μM to 68% in ABA 200 μM treatment compared to control. In general, kaempferol yields were positively correlated to Pnet in ABA 100 μM-treated plants and to g_s in ABA 200 μM-treated plants but negatively correlated to P_{net} in leaf-pierced plants. ABA application and wounding affected the association between photosynthetic primary metabolism and kaempferol accumulation differently. Both ABA application and wounding are promising leaf treatments for eliciting kaempferol accumulation in young soy leaves.

Keywords: Photosynthesis; Kaempferols; Soybean; Stress; ABA; Leaf wounding

Abbreviations: A: Assimilation; ABA: Abscisic Acid; C_i: Intercellular [CO_2]; E: Transpiration Rate; ETR: Electron Transport Rate between PSII and PSI; F'_m: Maximum Fluorescence in light; g_s: Stomatal Conductance; J_{max}: Electron Transport driving RuBP Regeneration; P_{net}: Net Photosynthesis; R_d: Respiration during day; ROS: Reactive Oxygen Species; RuBP: Ribulose Bisphosphate; V_{cmax}: Maximum Rate of Carboxylation by rubisco; WUE: Instantaneous Water Use Efficiency; ΦPSII: Quantum Yield of Photosystem II; Γ: CO_2 Compensation Point.

Introduction

Soybean leaves are consumed in Asian countries including China as a seasonal vegetable or as preserved leaves [1,2]. Consumption of soy leaves or their extracts has been implicated in preventing type 2 diabetes, obesity, heart disease and cancer through a multitude of mechanisms such as enhancing pancreatic β-Cell function and suppressing hepatic lipid accumulation [1], downregulating adipogeneic transcription [3], inhibiting α-glucosidase [4], decreasing non-HDL to HDL cholesterol ratio [5], relaxing carotid arteries [6], inhibiting fatty acid synthase [7], cancer cell specific cytotoxicity [8], and reducing DNA damage [9]. Much of the bioactivity of soy leaves is thought to be linked to isoflavonoids, kaempferol glycosides and pterocarpans [10], which are absent or found in extremely low levels in soy seed [10,11]. Kaempferols' antioxidative, free radical scavenging and anti-inflammatory properties [12,13] also play a synergistic role in the aforementioned health benefits.

In plants, while ~350 known kaempferol glycosides are distributed ubiquitously from *Bryophytes* to *Anthophytes*, their specific functions are still poorly understood [14]. Their suggested or observed functions include UV protection [15], antioxidant activity [16], phytoalexins against pathogens [17,18], role in infestation of N fixing bacteria [19], attraction of feeding animals [20,21], flower color [22,23], seed production [24] and plant development [25].

In soybean, the kaempferol aglycone has shown inhibitory effects on photosynthesis [26,27] but its glycosides were less influential [26]. However, mesophyll cells of mature soy leaves had no kaempferols or their glycosides indicating that they may not directly affect photosynthesis [28]. Epidermis appears to be the primary leaf tissue of kaempferol accumulation in soy also as is the case in pea leaves [29] consistent with their UV protection and anti-pathogen functions. Soybean leaf tissue undergoes a programmed shift from isoflavone to flavonoid metabolism 3 days after germination, and mature soybean leaves are composed mainly of glycosides of kaempferol [30]. Kaempferol glycosides have been shown to vary in the leaves of different soybean cultivars [31], and certain combinations of kaempferol glycosides were associated with photosynthetic rate [32].

How plants balance the allocation of resources derived from carbon assimilation between the growth-related primary metabolism vs the defense-related secondary metabolism is an intriguing and complex phenomenon. Among the explanations on how plants achieve this balance, the "growth-differentiation balance model" [33-35], considered to be the most integrative [36], stipulates that the defense-related secondary metabolism is increased under the conditions of lower than maximum gross productivity or at least moderate stress. How kaempferol levels in soy leaves change under the stress treatments that affect carbon assimilation, and the correlation between kaempferol levels and specific gas exchange variables under such treatments is not well-understood. Given the variety of useful bioactivities of soy

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kaempferols, finding stress treatments that increase kaempferol yield and are environmentally sound is important. Thus, the main objectives of this study were to:

a) determine the physiological responses of leaf carbon assimilation and photosystem function to two concentrations of exogenously applied ABA and two types of mechanical wounding in young soy leaves and,

b) determine the concentrations of major kaempferol glycosides in the leaf closest to the leaf used for physiological measurements.

Materials and Methods

Plant material, growth conditions and treatments

Soybean (*Glycine max* L. Merr., cultivar IA 2032) seeds were obtained from USDA, New Orleans. Presoaked seeds were overplanted in Sungro Metromix 380 in 5 gal (30 cm diam) pots and thinned to one plant per pot at V2 stage. Plants were grown in greenhouse without supplemental light (~1200 μmol m^{-2} s^{-1} of photon flux during day, and 22-30°C min-max temperature) during Fall. All plants were watered daily to field capacity, and fertilized weekly with Scotts Peters Professional 20-20-20 (10 g/8 L, 250 mL per pot first four weeks and 500 mL thereafter - Scotts Sierra Horticultural Products Co., Marysville, OH). Leaf wounding and ABA (abscisic acid, Sigma Cat. #A1049) spray treatments were done three times at V6, V9 and R1 stages before measurements. For leaf wounding, each fully expanded leaflet of each trifoliate was either pierced at 15 places with a dissecting pin or holed with a paper whole puncher at five places on either side of midrib avoiding major veins at each treatment time. Plants receiving ABA treatments were separated from other plants and sprayed either 100 or 200 μM aqueous ABA solution with a drop of Tween 20 using hand spray bottles to complete wetness at each treatment time. Plants receiving other treatments were sprayed with DI water. In plants with leaf wounding, the third fully expanded leaf and leaves above were left unwounded for measurements taken during R1 stage.

Leaf gas exchange and fluorescence measurements

Middle leaflet of the third fully expanded leaf from top on the main stem was used for measurements with LICOR 6400-40 photosynthesis system with leaf fluorescence chamber attached (LICOR Inc. Lincoln, NE). Measurement conditions were 1300 μmol m^{-1} s^{-1} photon flux (based on light saturation measured), 200 μmol s^{-1} flow, 400 μmol mol^{-1} CO_2, 23°C block temperature and ~40% sample RH. Fluorometer settings were, 5 intensity, 20 kHz modulation, 10 gain, 0.8 duration, 8 intensity and 20 kHz modulation for flash. Readings of F'_m, ΦPSII and ETR were adjusted to compensate for the Rectangular Single Flash used during measurements as per Loriaux et al. [37]. Gas exchange and leaf fluorescence measurements of each leaflet were taken when g_s and P_{net} stabilized on the digital display.

A/C_i curves and stomatal limitations

Data were collected using A/C_i fluorescence autoprogram feature with same measurement conditions as above. Sample CO_2 concentrations of 400, 300, 200, 100, 0, 400, 400, 600, 800, 1000, 1500 and 2000 μmol mol^{-1} were used. Resulting C_i (converted to Pa units) and A values were analyzed using Photosyn Assistant ver. 1.2 (Dundee Scientific, Dundee, UK) for rubisco carboxylation rate V_{cmax}, electron transport driving RuBP regeneration rate J_{max}, triose phosphate utilization rate TPU, day respiration R_d and CO_2 compensation point Γ. Stomatal limitation in each plant was computed as l=(A"–A')/A" for assimilation [38] and as l=(ΦPSII" – ΦPSII')/ΦPSII" for quantum yield, where l=stomatal limitation, A" or ΦPSII"=reading at 390 μmol m^{-2} s^{-1} of C_i, and A' or ΦPSII'=reading at 390 μmol m^{-2} s^{-1} of sample CO_2 concentration. Second order polynomial equations of Microsoft Excel trend line function ($R^2 \geq 0.85$) were used to derive the respective A and ΦPSII values.

Pigment assays

Two leaf discs (0.6 cm diam) taken from the same leaflet used for above measurements were left in 2 mL methanol at 4°C in dark for 24 h. Extract was centrifuged for 5 min at 10,000 rpm and absorbance was read at λ =665.2, 652.4 and 470 nm using a BioTek Synergy HT microplate reader (Winooski, VT). Concentrations of chl a, b and carotenoids were measured according to Lichtenthaler [39].

Total antiradical activity assay

Antiradical activity was assayed according to modified Schwarz et al. and Yu et al. [40,41]. Briefly, five leaf discs from the same leaflet used for above measurements were ground with liquid N_2 and stirred in 800 μL 95% ethanol for 30 min. Homogenate was centrifuged for 10 min at 10,000 rpm. To assay antiradical activity of samples (S), 50 μL of supernatant and 150 μL of 100 μM DPPH (2,2-diphenyl-1-picrylhydrazyl, DPPH•, Sigma Cat. #D9132) in 95% ethanol were combined in triplicate wells on a 96 well plate, with 50 μL of supernatant and 150 μL of 95% ethanol in triplicate wells as blank. For control (C), 200 μL of DPPH was blanked with 200 μL of 95% ethanol. Ascorbic acid (100 μM) in 95% ethanol was used as standards. Plate was incubated at 37°C while shaking for 45 min before absorbance was read at 515 nm at 37°C in the same plate reader as above in pigment assays. Antiradical activity was measured as ((C-S)/C) × 100, where C and S are absorbance of control and sample, respectively.

Leaf kaempferol assay

The leaf immediately above the leaf that was used for above measurements was used for the kaempferol assay. Approximately 200 mg of the lyophilized leaf was homogenized in 1 mL methanol followed by sonication for 1 h. Samples were filtered (0.4 μm) before injecting 10 μL on to a Waters HPLC system using a 4. 6 × 150 mm C18 column (10 μm particle size). The system included a Waters 600E System Controller combined with a Waters UV-VIS 996 detector. Elution was carried out at a flow rate of 1.0 mL/min with the following solvent system: A=acetonitrile, B=water 0.1% TFA; 5% A for 5 min, then 5% A to 90% A in 24 min followed by holding at 90% A for 6 min. The solvent acetonitrile (HPLC grade Sigma Cat. #271004) was purchased from Sigma Chemical Company. Water was obtained using a Millipore system and used during sample preparation procedures and HPLC analyses. Samples were injected in triplicate and measured using peak areas at 260 nm. Kaempferol glycosides were quantified by comparing peak area responses with those of kaempferol (Cat.#60010 Sigma Chemical Company).

Mass spectral analysis of kaempferol glycosides

The mass spectrometer used was a Finnigan MAT LCQ ion trap (San Jose, CA, USA) equipped with a heated nebulizer atmospheric pressure chemical ionization (APCI) interface. HPLC effluent at 1 mL/min was introduced directly into the interface without splitting using a source temperature of 500°C. Positive ion mode was used with a sprayer needle voltage of 4 kV. The capillary temperature was 210°C. The full scan mass spectra of the flavones from m/z 100-1000 were measured using 500 ms for collection time and three micro scans were summed.

The instrument was set to measure total ion chromatograms (TICs) in full scan MS mode to measure protonated $(M+H)^+$ ions.

Statistical analysis

Statistical significance of the treatments for each response variable was tested using general linear model on SPSS version 19.0.0.1 (International Business Machines, [42]). Pairwise comparisons after significant ANOVA were performed with Tukey's HSD.

Results and Discussion

Long standing efforts to increase yields of crops including soybean have often involved genetic improvement and development of cultural practices that promote photosynthetic primary metabolism. However, these methods often targeted no or little improvement in the crop's nutritional or medicinal value resulting from secondary phytochemicals generally elicited under suboptimal abiotic or biotic environmental conditions. Thus, finding agronomic methods that can impart health-promoting phytochemical quality and understanding how these methods impact the balance between carbon assimilatory primary metabolism and the accumulation of the secondary phytochemicals of interest remain important research goals. We focused on ABA and mechanical wounding treatments for several reasons. First, both abiotic and biotic stresses involve ABA as a natural plant signaling molecule and leaf wounding as a result of pest, wind or hail damage. Secondly, these stress treatments do not severely reduce the total leaf area so that the plant's contribution of photosynthetic primary metabolism toward crop yield, either leaf or seed, is only mildly affected. Thirdly, both treatments are eco-friendly. However, plants may respond differently to exogenously applied vs endogenous ABA [43] and mechanical wounding vs pest damage using distinct pathways for defense [44,45]. Analyses of physiological and phytochemical responses to ABA or wounding treatments in our study, therefore, were attempts to find cultural practices that can influence the balance between the photosynthetic primary metabolism and kaempferol accumulation rather than to directly understand the defense orchestrated by endogenously synthesized ABA through kaempferol biosynthesis under stress or how leaf damage by natural causes would affect kaempferol yield.

Effects of ABA treatments on physiological variables

Both ABA 100 and 200 µM applications reduced the gas exchange variables such as P_{net}, g_s, E and C_i. However, only the reductions by ABA 200 µM, >20% in each of these variables compared to control, were statistically significant (Table 1). Instantaneous water use efficiency increased 32% in ABA 200 µM-treated plants due to their larger reduction in E than in P_{net} compared to control. Furthermore, ABA 200 µM application reduced the variables of energy harvest and transport namely, F'_m, ΦPSII and ETR by 16%, 10% and 17%, respectively, compared to control (Table 2). Stomatal limitations of net photosynthesis and quantum yield were 55% and 100% greater, respectively, in ABA 200 µM-treated plants than control (Figure 1). ABA's role in stomatal closure through a multitude of molecular and physiological mechanisms within the guard cells including ABA receptor-involved activation of genes that encode enzymes [46,47] and other proteins involved in cellular dehydration tolerance [48], collaborative signaling using reactive oxygen species [49], Ca^{2+} channel activation [50], anion efflux causing membrane depolarization-dependent inhibition of inward K^+ channels [51], effects on aquaporins [52], etc. is well-known. Li et al. [1,3] found that exogenously applied ABA slowed photosynthetic dark reactions generating H_2O_2 and a glut of C_i which then promoted further stomatal closure whereby mainly regulating plant's water balance. In soybean, Ward and Bunce [53] also found combined and proportional reductions in leaf gas exchange variables and ΦPSII in response to exogenously applied ABA. However, RuBP content increased but RuBP carboxylation efficiency decreased in ABA-treated plants in their study. They also concluded that this decrease in carboxylation efficiency was not due to a change in activation status or total activity of rubisco as ABA treatment had no effect on them [53]. In our study, both V_{cmax} and J_{max} were increased by ABA 200 µM treatment with the quickest response of carbon assimilation to early increases in C_i compared to other treatments (Table 3, Figure 2). However, effects of ABA on V_{cmax} and J_{max} were fairly equal as seen by the same V_{cmax}:J_{max} ratio as in control. Also, R_d was relatively high under ABA 200 µM treatment (Table 3) which likely caused higher Γ. Increased biochemical variables of assimilation and R_d of ABA-treated plants in our study are consistent with the observations that exogenously applied ABA increased shoot and root growth, size of young leaves and pod yield in field grown soybean [54].

Thus, in our study, the main nonstomatal limitation to photosynthesis observed in ABA-treated plants was the reduced ΦPSII. Reduced ΦPSII under ABA treatments has been attributed to impaired trans-thylakoid proton motive force [55]. Consistent with the reduced ΦPSII, F'_m and ETR were also lower in ABA 200 µM-treated plants (Table 2). Most of the chlorophyll fluorescence in a healthy leaf originates from the light harvesting antennae complex of photosystem II [56]. However, leaf chlorophyll and carotenoid concentrations were unaffected by ABA treatment under the conditions of our study (Figure 3). Thus, reduced F'_m likely indicates a transient, rather than a chronic, weakening or underutilization of the antennae strength of photosystem II in ABA-treated plants. This lower energy capture likely caused the reduced ETR as well. However, ETR/P_{net} ratio was the same (data not presented) in ABA-treated plants and control indicating that thylakoid redox status adjusted in ABA-treated plants to match the carbon assimilation. Thus, alternative electron sinks such as photorespiration may not have been a major reason for the reduced carbon assimilation in ABA-treated plants as also shown by Li et al. [57].

Treatment	P_{net} (µmol m^{-2} s^{-1})	g_s (mmol m^{-2} s^{-1})	C_i (µmol mol^{-1})	WUE (µmol mol^{-1})	E (mol m^{-2} s^{-1})	Leaf Temperature (°C)
Control	13.6b	380b	253b	4.4a	3.2b	23.2a
ABA 100 µM	12.5b	322b	246b	4.6a	2.8b	23.5a
ABA 200 µM	10.7a	204a	202a	5.8b	1.9a	24.2a
Leaf piercing	14.3b	400b	246b	4.6a	3.3b	23.2a
Leaf hole punching	12.5b	382b	268b	4.0a	3.1b	23.3a

P_{net}, net photosynthesis; g_s, stomatal conductance; C_i, intercellualar CO_2 concentration; WUE, instantaneous water use efficiency (P_{net}/E); E, transpiration rate. N=10 – means from two different days' measurements of five replications; Differences between means followed by different letters in a column are statistically significant (P<0.05).

Table 1: Gas exchange characteristics and leaf temperature under treatments.

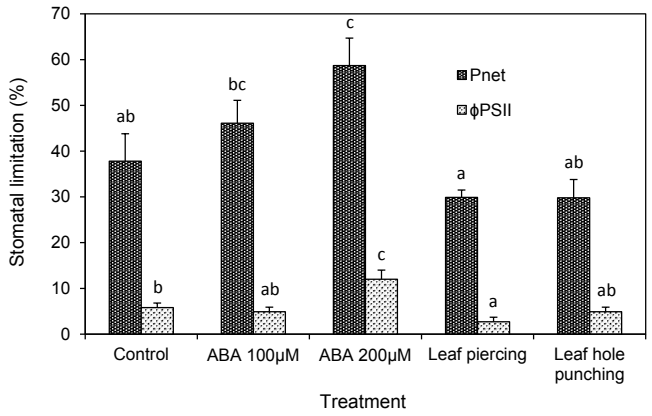

Figure 1: Stomatal limitations of net photosynthesis (P_{net}) and quantum yield of photosystem II (ΦPSII) under the treatments. Error bar=SE, N=5. Different letters above the error bar indicate statistically significant difference across treatments for the given variable (P<0.05).

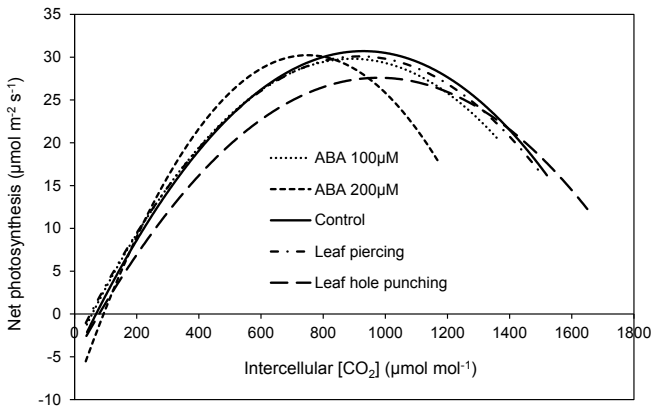

Figure 2: Second degree polynomial A/C_i curves for the plants under different leaf treatments (N=5). Control, y=-4E-05x^2+0.0772x-5.1435, R²=0.8274; ABA 100 μM, y=-4E-05x^2+0.0761x-4.1612, R²=0.7441; ABA 200 μM, y=-7E-05x^2+0.1052x-9.2175, R²=0.7898; Leaf piercing, y=-4E-05x^2+0.0736x-3.6489, R²=0.7016; Leaf hole punching, y=-3E-05x^2+0.0667x-5.0474, R²=0.8169.

Leaf antiradical activity increased 25% under ABA 100 μM treatment compared with the control (Figure 4). However, the 9% increase in antiradical activity by ABA 200 μM treatment was insignificant (P=0.12). Part of this increased antiradical activity may be attributed to the increased leaf kaempferol levels (see below) which are known free radical scavengers [12,13]. However, given that ABA 200 μM treatment had generally greater leaf kaempferol levels than ABA 100 μM treatment, although statistically insignificant, ABA 200 μM treatment is expected to produce higher antiradical activity. One probable reason for lower antiradical activity in ABA 200 μM application is that this ABA concentration may have decreased other radical scavenging metabolites such as glutathione (GSH) as reported by Okuma et al. [58]. Furthermore, Mittler and Blumwald [49] found that ABA itself can induce reactive oxygen species as part of its signaling network. Therefore, leaf extracts from ABA 200 μM-treated plants may have had more free radicals than the extracts from ABA 100 μM-treated plants.

Effects of leaf mechanical wounding on physiological variables

Leaf-pierced plants had the highest P_{net} and g_s although statistically significant only compared to ABA 200 μM-treated plants (Table 1). Similarly, they had the highest ΦPSII, F'_m and ETR, significant differences compared to ABA 200 μM-treated plants (Table 2). Leaf-pierced plants also showed statistically insignificant increases in V_{cmax} and J_{max} with an A/C_i response similar to control (Table 3, Figure 2). This strong photosynthetic capacity in leaf-pierced plants was also supported by their 39% higher total chlorophyll and 38% higher total carotenoids compared to control plants (Figure 3). Besides conferring light harvesting complexes and photoprotection, this pigment enrichment may add to the nutritional quality of the young leaves in leaf-pierced plants. Furthermore, leaf-pierced plants had the lowest stomatal limitation to either P_{net} or ΦPSII (Figure 1) among all treatments. For instance, leaf-pierced plants had 49% and 77% less stomatal limitations to P_{net} and ΦPSII, respectively, compared to ABA 200 μM-treated plants although only the 53% less stomatal limitation to ΦPSII was significantly different from the control. Both leaf-pierced and -holed plants had similar levels of antiradical capacity (Figure 4) compared to control suggesting that an ROS boost may not have occurred and a redox status conducive to carbon assimilation was still maintained following wounding. Leaf-holed plants, however, didn't show above indicators of an elevated photosynthetic capacity but had gas exchange and quantum use variables that were more comparable to control. Furthermore, leaf-holed plants had a reduced slope and lower A_{max} in A/C_i curves than the other treatment groups resulting in non-significantly lower V_{cmax} and J_{max} compared to all other treatments

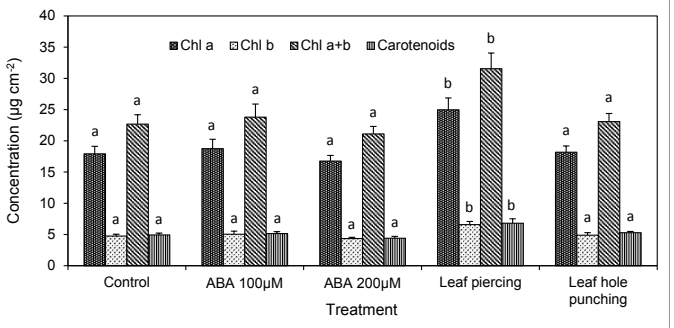

Figure 3: Leaf chlorophyll and total carotenoid contents affected by treatments. Error bar=SE, N=5. Different letters above the error bar indicate statistically significant difference across treatments for the given variable (P<0.05).

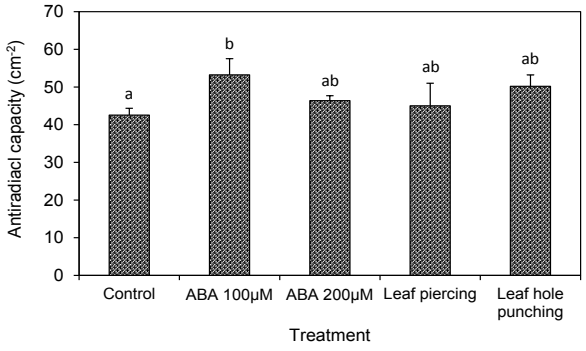

Figure 4: Leaf antiradical capacity affected by treatments. Error bar=SE, N=5. Different letters above the error bar indicate statistically significant difference across treatments (P<0.05).

or control. In addition to the greater loss of tissue, total kaempferol levels were slightly lower in leaf-holed plants (Table 4) than leaf-pierced plants. Liu et al. [59] found that treatment of apple leaves with quercetin or kaempferol inhibited the ABA-induced stomatal closure by decreasing levels of ROS in the guard cells. In our study, leaf-pierced plants had slightly higher leaf kaempferol levels (next section) which along with less tissue loss likely contributed to higher stomatal activity as seen in their lower stomatal limitations to both gas exchange and ΦPSII compared to leaf-holed plants. Antiradical activities (Figure 4) did not parallel the leaf kaempferol levels likely reflecting the different degrees of contribution by non-kaempferol antioxidants to free radical scavenging under the two types of leaf wounding.

Peterson et al. [60] reported that leaf mechanical wounding by clipping a piece of each leaflet in a leaf with scissors in six legume crops had no significant effect on photosynthesis 24 hrs after injury. In soybean, previous works found the similar results when leaflets were wounded by either cork-borer or hole-puncher [61]. We were not able to find reports on the effect of soy leaf piercing on physiological variables. However, the differential physiological response that we observed between leaf piercing and holing is consistent with the concept that injury types can be classified into guilds (groups or types) based on the within-group-homogeneities of plant response to those guilds [62].

Enhanced or sustained P_{net} and related structural and physiological variables that were observed in response to mechanical wounding can also be attributed to reduced source/sink ratio as observed by others in soybean [63] and other species [64]. In our study, plants were treated through early vegetative stages to early flowering when all the measurements were taken. Thus, wound healing rather than wound-induced leaf senescence was observed during the experiment which probably caused a reduced source/sink ratio requiring more carbon assimilation to provide row materials for wound healing. Furthermore, soluble carbohydrates from current photosynthesis and wound-induced jasmonic acid (methyl jasmonate) were found to co-regulate the expression of genes encoding vegetative storage proteins (VSPs), a group of glycoproteins that accumulate in young shoot tissue as a temporary sink for carbon and nitrogen usable for wound healing in leaf-wounded soy plants [65,66]. When current photosynthesis was inhibited in leaf-wounded soy plants VSP accumulation was negated [66].

Effects of treatments on kaempferol glycoside yield

Six leaf kaempferol glycosides were identified based on UV and MS spectra as shown in the HPLC chromatogram in Figure 5. Using mass spectrometry, K1 was identified as a kaempferol diglycoside (m/z 611, 449, 287), K2 as a kaempferol triglycoside (m/z 757, 741, 595, 449, 287), K3 as a kaempferol triglycoside (m/z 741, 595, 449, 287), and K4, K5, and K6 as kaempferol monoglycosides (m/z 449, 287). Concentrations of all leaf kaempferol glycosides quantified increased under each ABA treatment causing the total kaempferol concentration to rise 42% in ABA 100 μM and 68% in ABA 200 μM-treated plants compared to control (Table 4). However, the difference between the kaempferol glycoside concentrations under the two ABA treatments was insignificant. Similarly, all six kaempferol glycoside concentrations were significantly higher in leaf-wounded plants compared to control except for the 21% increase of K1 in leaf-pierced plants. Total kaempferol glycoside concentration was 55% greater in leaf-pierced plants and 43% greater in leaf-holed plants compared to control. Two leaf wounding treatments also had statistically similar concentrations of kaempferol glycosides (Table 4).

According to the growth-differentiation balance model, soy kaempferol glycoside concentrations in our study were expected be negatively correlated with photosynthesis and related variables. However, since photosynthesis and related physiological responses to ABA treatments were different from those of wounding treatments and, as far as known to us, correlations between soy leaf kaempferol glycosides and photosynthesis-related physiological responses have not been reported we studied the correlations of kaempferol glycosides to P_{net} and g_s (Table 5) under the leaf treatments. We found that these correlations differed depending on the leaf treatment and also on the specific physiological variable, P_{net} vs g_s. For instance, kaempferol glycoside concentrations and P_{net} showed a more positive correlation

Treatment	F'_m	ΦPSII	ETR
Control	1604ab	0.56b	105.3bc
ABA100 μM	1527ab	0.55ab	94.1ab
ABA200 μM	1339a	0.50a	86.5a
Leaf piercing	1866b	0.61b	110.1c
Leaf hole punching	1632ab	0.57b	96.9abc

F'_m, maximum light-adapted fluorescence; ΦPSII, quantum yield of PSII; ETR, electron transport rate between PSII and PSI. N=10 – means from two different days' measurements of five replications; Differences between means followed by different letters in a column are statistically significant (P<0.05).

Table 2: Leaf photochemical properties under the treatments.

Treatment	V_{cmax} (μmol m^{-2} s^{-1})	J_{max} (mmol m^{-2} s^{-1})	J_{max}:V_{cmax} (μmol mol^{-1})	R_d (μmol mol^{-1})	Γ (Pa)
Control	67.6ab	192.7a	2.8a	10.2ab	9.0a
ABA 100 μM	79.8b	213.2a	2.7a	10.6ab	9.9ab
ABA 200 μM	115.7c	328.5b	2.9a	16.3b	12.4b
Leaf piercing	77.9b	213.0a	2.7a	9.8a	8.1a
Leaf hole punching	52.5a	181.0a	2.9a	8.7a	9.7ab

V_{cmax}, rubisco carboxylation rate; J_{max}, electron transport driving RuBP regeneration; J_{max}:V_{cmax} ratio; R_d, daytime respiration; Γ, CO_2 compensation point, N=5; Differences between means followed by different letters in a column are statistically significant (P<0.05).

Table 3: Biochemical variables of assimilation derived from A/C_i curves.

Treatment	K1 (20.69)	K2 (21.19)	K3 (21.33)	K4 (22.47)	K5 (22.78)	K6 (23.00)	Total
Control	311.98a	971.15a	501.64a	652.13a	970.14a	226.64a	3633.71a
ABA 100 μM	447.29b	1372.79b	713.66b	1009.39b	1344.87ab	275.63b	5163.66b
ABA 200 μM	431.92b	1681.79b	879.96b	1146.27b	1640.67b	310.93b	6091.57b
Leaf piercing	377.26ab	1693.44b	800.99b	1080.80b	1373.32b	306.48b	5632.31b
Leaf hole punching	433.86b	1713.76b	749.24b	943.07b	1097.15ab	285.64b	5222.74b

K#=Kaempferol glycoside with retention time (min) in parentheses, N=4; Differences between means followed by different letters in a column are statistically significant (P<0.05).

Table 4: Concentrations of leaf kaempferol glycosides (μg g^{-1} DW) detected by HPLC.

Treatment	Gas exchange Variable	K1 (20.69)	K2 (21.19)	K3 (21.33)	K4 (22.47)	K5 (22.78)	K6 (23.00)	Total
Control	P_{net}	0.97	0.3	0.32	0.38	-0.27	0.6	0.2
	g_s	0.78	-0.55	-0.31	-0.48	-0.6	-0.23	-0.53
ABA 100 μM	P_{net}	0.18	0.74	0.59	0.93	0.94	0.92	0.85
	g_s	0.72	-0.34	-0.08	-0.09	-0.15	-0.13	-0.13
ABA 200 μM	P_{net}	0.47	0.12	0.34	-0.23	0.05	-0.13	0.09
	g_s	0.61	0.99	0.75	0.72	0.75	0.66	0.84
Leaf piercing	P_{net}	-0.65	-0.78	-0.95	-0.93	-0.8	-0.86	-0.85
	g_s	-0.01	0.26	0.58	0.49	0.25	0.46	0.34
Leaf hole punching	P_{net}	-0.06	0.09	-0.05	0.02	-0.32	0.43	-0.06
	g_s	-0.3	0.54	0.31	0.37	0.1	0.76	0.35

K#=Kaempferol glycoside with retention time (min) in parentheses; N=4.

Table 5: Pearson correlation coefficients between each kaempferol glycoside concentration and two key gas exchange variables (P_{net}, net photosynthesis; g_s, stomatal conductance) under different treatments.

in ABA 100 μM-treated plants compared to control with the exception of K1. A similar positive correlation of kaempferol glycoside levels was observed with g_s rather than with P_{net} in ABA 200 μM-treated plants. In contrast, a negative correlation between kaempferol glycoside concentrations and P_{net} was seen in leaf-pierced plants while there were no clear correlations between kaempferol glycoside concentrations and photosynthesis-related variables in leaf-holed plants (Table 5).

Fairly positive correlation between kaempferol glycoside levels and gas exchange variables in ABA-treated plants suggests that carbon skeletons feeding kaempferol biosynthetic pathway were more directly dependent on current photosynthetic carbon assimilation compared to leaf wounded plants. This is also consistent with the growth-differentiation balance model [33-35] which stipulates that primary metabolism parallels secondary metabolism or synthesis of defense compounds under relatively low or restricted resource availability. Under ABA treatments this restriction was likely a physiological condition, the low g_s. The greater stomatal limitation to both ΦPSII and P_{net} in ABA 200 μM-treated plants may explain their more positive correlation between kaempferols and g_s compared to ABA 100 μM-treated plants. Reasons for the differences in correlation coefficients of kaempferol glycosides with P_{net} vs with g_s under the two ABA treatments, though not obvious, may be due to the different degrees of association between P_{net} and g_s and slight differences in the nonstomatal regulation of P_{net} between the two treatments.

As discussed earlier under the effects of leaf mechanical wounding on physiological variables, leaf-pierced plants had slightly elevated photosynthetic capacity boosted by a stronger pigment bed and an elevated g_s especially compared to ABA treatments. Leaf-pierced plants had 5% greater g_s than even the control plants though this increase was statistically insignificant. Thus, the negative correlations between the yields of kaempferol glycosides and P_{net} under leaf piercing likely corresponds to the more optimum resource availability or conducive conditions for photosynthetic primary metabolism of the growth-differentiation balance model during which secondary metabolism

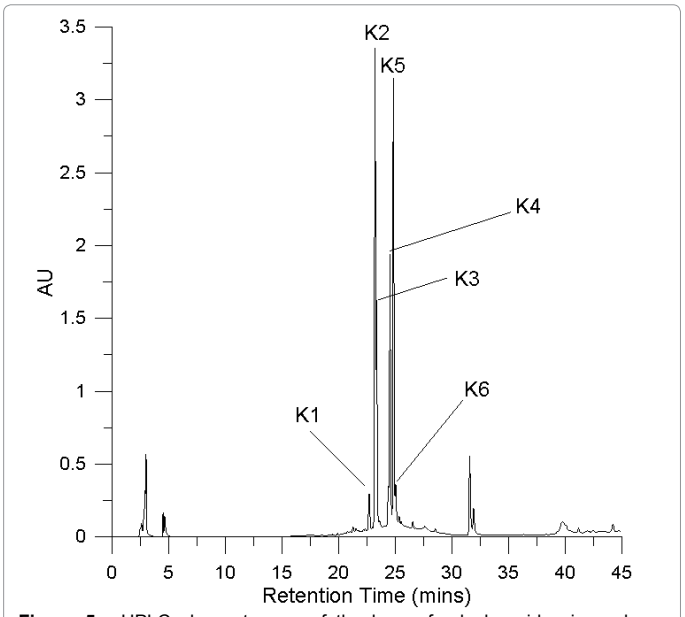

Figure 5: HPLC chromatogram of the kaempferol glycosides in soybean leaves. K1 through K6 were the identified kampferol glycosides.

toward kaempferol accumulation has probably passed its peak but is still higher relative to control. Furthermore, kaempferol glycosides may play a role in the systemic defense against wounding [24] or in wound healing itself. In leaf-holed plants, however, the generally negative correlation between kaempferol glycosides and P_{net} seen in leaf-pierced plants was absent, and no discernible pattern of correlation between kaempferol glycoside yields and gas exchange physiological variables was observed (Table 5). Based on the physiological variables discussed earlier and due to greater loss of leaf tissue, leaf-holed plants experienced a different type and severity of stress compared to the leaf-pierced plants. Thus, the different wound healing and antioxidative

demands in leaf-holed plants may attribute to the different patterns of the associations of kaempferol glycoside yields and gas exchange variables compared to leaf-pierced plants. These differential associations of primary vs secondary metabolic responses between the two leaf wounding treatments are also consistent with the within-group-homogeneities of plant response to different guilds [62].

Kaempferols are produced by the actions of five, namely, glycolytic, pentose phosphate, shikimate, phenylpropanoid and kaempferol flavonoid pathways. Glycolytic and pentose phosphate pathways produce phosphoenolpyruvate and erythrose-4-phosphate which feed shikimate pathway producing phenylalanine. Phenylpropanoid metabolism uses phenylalanine to produce *p*-Coumaroyl CoA which then is used to produce chalcone isomers that feed kaempferol biosynthesis by falavonol synthase and flavanone 3 hydrolase [67,68]. Buttery et al. [32] reported that certain combinations of the nonallelic flavonol glycoside genes in soybean, especially the genotype that produces kaempferol 2^{G}-glucosylgentiobioside, named K9, was associated with low chlorophyll levels, photosynthesis, stomatal density and specific leaf weight. They suggested that such genotypes are eliminated or reduced in soy breeding programs since photosynthesis is highly correlated with seed yield in soybean. Therefore, the cultivar used in this study, a high yielding IA 2032, is unlikely to have been a K9 line. While the inhibitory effects of kaempferol aglycone on photosynthesis *in vitro* has been well-known [26,27] glycosides were less effective [26]. However, Cosio and McClure [28] found that kaempferol has no direct inhibitory effect on photosynthesis in soybean since neither kaempferol, nor its glycoside including K9 was found in mesophyll cells but only in epidermis. Thus, inhibitory effects of kaempferol on photosynthesis were suggested to be indirect through its effects on formation of stomata and other features of leaf development [69,70]. Furthermore, aglycones of kaempferol and other related flavonoids such as quercetin which is an early product of flavonoid biosynthetic pathway are found to inhibit polar transport of auxin causing localized auxin accumulation. Auxin may play a role in controlling stomatal opening and resource allocation under stress [71-73]. Thus, the patterns of correlation between kaempferol glycoside levels and gas exchange variables in our study also likely resulted from differential effects of leaf treatments on the growth and resource allocation associated with source-sink balance rather than solely the direct effects of kaempferols themselves [74].

In conclusion, elicitation of kaempferols or their glycosides is an important step toward improving nutritional and pharmaceutical potential of soy leaves whether used as vegetable, ingredients in foodstuff and traditional medicine or raw material for extraction. However, increasing phytochemical quality without compromising the total plant productivity is a challenge as phytochemicals such as kaempferols are produced as part of plant's stress response. We found that kaempferol-rich young soy leaves can be produced by mild stress treatments by way of foliar ABA spray and wounding to already mature plants thus avoiding major growth effects. Although ABA and leaf wounding influenced photosynthetic primary productivity differently they both increased leaf kaempferol glycoside yields. While ABA-elicited kaempferol accumulation occurred with reduced P_{net} it still showed a positive correlation to a given gas exchange variable depending on the concentration of ABA applied. Kaempferol accumulation elicited by leaf wounding occurred with no reduction in P_{net} showing different patterns of correlation to gas exchange variables depending on the type of the wounding treatment. Results of this study show that appreciable increase in kaempferol yield and thus phytochemical or nutritional quality of soy leaves is achievable with environmentally friendly foliar treatments.

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