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Impact of Elevated Levels of Atmospheric CO_2 and Herbivory on Flavonoids of Soybean (*Glycine max* Linnaeus)

Bridget F. O'Neill · Arthur R. Zangerl · Orla Dermody · Damla D. Bilgin · Clare L. Casteel · Jorge A. Zavala · Evan H. DeLucia · May R. Berenbaum

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Abstract Atmospheric levels of carbon dioxide (CO_2) have been increasing steadily over the last century. Plants grown under elevated CO_2 conditions experience physiological changes, particularly in phytochemical content, that can influence their suitability as food for insects. Flavonoids are important plant defense compounds and antioxidants that can have a large effect on leaf palatability and herbivore longevity. In this study, flavonoid content was examined in foliage of soybean (*Glycine max* Linnaeus) grown under ambient and elevated levels of CO_2 and subjected to damage by herbivores in three feeding guilds: leaf skeletonizer (*Popillia japonica* Newman), leaf chewer (*Vanessa cardui* Linnaeus), and phloem feeder (*Aphis glycines* Matsumura). Flavonoid content also was examined in foliage of soybean grown under ambient and elevated levels of O_3 and subjected to damage by the leaf skeletonizer *P. japonica*. The presence of the isoflavones genistein and daidzein and the flavonols quercetin and kaempferol was confirmed in all plants examined, as were their glycosides. All compounds significantly increased in concentration as the growing season progressed. Concentrations of quercetin glycosides were higher in plants grown under elevated levels of CO_2. The majority of compounds in foliage were induced in response to leaf skeletonization damage but remained unchanged in response to non-skeletonizing feeding or phloem-feeding. Most compounds increased in concentration in plants grown under elevated levels of O_3. Insects feeding on *G. max* foliage growing under elevated levels of CO_2 may derive additional antioxidant benefits from their host plants as a consequence of the change in ratios of flavonoid classes. This nutritional benefit could lead to increased herbivore longevity and increased damage to soybean (and perhaps other crop plants) in the future.

Keywords Flavonol · Isoflavone · *Popillia japonica* · *Aphis glycines* · *Vanessa cardui* · Global climate change · Plant-insect interactions

B. F. O'Neill · A. R. Zangerl · M. R. Berenbaum (✉)
Urbana-Champaign, Department of Entomology,
University of Illinois,
320 Morrill Hall, 505 S. Goodwin Avenue,
Urbana, IL 61801, USA
e-mail: maybe@uiuc.edu

O. Dermody
Pioneer Hi-Bred Switzerland S.A.,
DuPont Agriculture and Nutrition,
Via Cantonale/Galleria 3B,
CH-6928 Manno, Switzerland

D. D. Bilgin · E. H. DeLucia · M. R. Berenbaum
Urbana-Champaign, Institute for Genomic Biology,
University of Illinois,
1206 West Gregory Drive,
Urbana, IL 61801, USA

C. L. Casteel · E. H. DeLucia
Urbana-Champaign, Department of Plant Biology,
University of Illinois,
265 Morrill Hall, 505 S. Goodwin Avenue,
Urbana, IL 61801, USA

J. A. Zavala
Consejo Nacional de Investigaciones Cientificas y Technicas,
Catedra de Bioquimica, Facultad de Agronomia,
University of Buenos Aires,
Avenida San Martin 4453,
Buenos Aires, Argentina

Introduction

Atmospheric carbon dioxide (CO_2) levels have risen steadily since the start of the Industrial Revolution, from

280 to 387 ppm today (IPCC 2007). Current levels of atmospheric CO_2 are expected to double within the next 100 years (Ehhalt et al. 2001). Levels of ozone (O_3) also have been increasing in the lower atmosphere over this time period (Ehhalt et al. 2001). Numerous studies have been conducted on how various ecosystems might respond to changing atmospheric levels of these two gases (Kimball et al. 2002; Karnosky 2003; Booker et al. 2009; Lindroth 2010, this issue; Pinto et al. 2010, this issue). SoyFACE (Soybean Free Air gas Concentration Enrichment) is a research site at the University of Illinois, Urbana-Champaign, USA, for the study of the effects of elevated CO_2 and O_3 on a common midwestern agricultural ecosystem, that of *Glycine max* Linnaeus (soybeans). The United States produces 32% of the world's *G. max*, currently a $13 billion industry, and Illinois is one of the largest producers in the country (ASA 2008). Previous research conducted at this site revealed that damage by insect herbivores increased in elevated CO_2 treatments. This increase was associated with higher numbers of Japanese beetles (*Popillia japonica* Newman) found on those plants (Hamilton et al. 2005, Dermody et al. 2008). A subsequent study demonstrated that *P. japonica* fed a diet of *G. max* leaves grown in elevated CO_2 live longer and are more fecund than beetles that consume foliage grown under ambient conditions (O'Neill et al. 2008).

Changes in the antioxidant makeup of foliage under elevated CO_2 may be a factor that contributes to enhanced *P. japonica* longevity. Oxidative stress has been linked to aging, with the long-term build-up of oxidation products in the body leading to diminished cellular function (Harman 1956). This damage is countered by antioxidants, molecules that prevent oxidative damage in cells by quenching oxygen free radicals (Tang et al. 2006). Dietary constituents, including some phytochemicals, with antioxidant properties are associated with enhanced longevity in several insect species (Orr and Sohal 1994). Previous studies on plants grown under elevated CO_2 have determined that antioxidant content increases in fruits (e. g., strawberries, *Fragaria x ananassa* Dutch, Wang et al. 2003) and in foliage (e. g., European silver birch, *Betula pendula* Roth, Saleem et al. 2001). *Glycine max* contains several compounds with antioxidant properties, including flavonoids and isoflavonoids (Lee et al. 2006), but if and how foliar content of these compounds changes when plants are grown under elevated CO_2 has yet to be determined.

Flavonoids and isoflavonoids are biosynthesized via the phenylpropanoid pathway (key enzyme: phenylalanine ammonia-lyase = PAL) and contribute to plant defense against oxidative stressors (Dakora and Phillips 1996), such as pathogens, herbivores, or abiotic factors. Plant wounding induces these compounds (Hagerman and Butler 1991). Individual flavonoids in artificial diets can be detrimental to insect growth by virtue of their prooxidant properties (Ahmad and Pardini 1990). A study by Johnson and Felton (2001), however, indicated that the relative proportions of individual flavonoids in the food source are important in determining biological activity. The total complement of flavonoids can override the prooxidant trend and instead benefit herbivores by providing them with increased antioxidant protection. Quercetin in particular can stimulate feeding and promote herbivore growth (Ruuhola et al. 2001; Saleem et al. 2001). Isoflavonoids can have negative effects on herbivores (Simmonds and Stevenson 2001; Yu et al. 2003) irrespective of composition.

We measured flavonoid content of *G. max* foliage grown under elevated and ambient levels of CO_2 and O_3, singly and in combination. We hypothesized that elevated levels of CO_2, O_3, and herbivore damage will increase the products of the PAL pathway. The PAL pathway requires high levels of carbon to generate its products, including flavonoids (Tsai et al. 2006). Plants grown under elevated CO_2 have a greater C:N ratio than plants grown under ambient levels of CO_2 (Ainsworth et al. 2002), thus increasing the amount of carbon available to be transformed into phenolics. Increased exposure to O_3 is hypothesized to increase flavonoid production in *G. max* tissue and counteract potential oxidative damage. Ground level O_3 is an atmospheric pollutant that cycles seasonally and can have large effects on agricultural systems (Mauzerall and Wang 2001). Ozone is a powerful oxidative stressor, reducing *G. max* yield and increasing the incidence of early senescence (Morgan et al. 2006). Because production of flavonoids is known to vary with types of herbivory (Izaguirre et al. 2007), we measured changes in flavonoid content in response to damage from multiple herbivore feeding guilds (Mewis et al. 2006). Damage to plants grown under elevated levels of CO_2 was inflicted by phloem-feeders (*Aphis glycines* Matsumura, soybean aphid), leaf-chewers (*Vanessa cardui* Linnaeus, painted lady butterfly), and leaf-skeletonizers (*P. japonica*, Japanese beetle). Damage to plants grown under elevated levels of O_3 was inflicted by the Japanese beetle only.

Methods and Materials

Description of Field Site All herbivore feeding and tissue collection took place at the SoyFACE site (South Farms, University of Illinois, Savoy, IL, USA). SoyFACE is an open Free Air gas Concentration Enrichment system that exposes large field plots of *G. max* to elevated CO_2 and elevated O_3, individually or in combination in a full factorial design (Long et al. 2004). Crops at SoyFACE are rotated between corn (*Zea mays*) and *G. max* cv. Pioneer 93B15. Treatment plots (ambient air with 387 μmol mol^{-1}

CO_2, elevated CO_2 with a target of 550 µmol $mol^{-1}CO_2$, addition of 1.2 x ambient levels O_3, and combination of the elevated CO_2 and O_3 treatments) have a diameter of 20 m, cover 350 m^2, and are at least 100 m from any other plots. Treatments were replicated in a randomized block design. There were four plots for each treatment for a total of 16 plots present in the field. Average O_3 concentration during the 2005 season was 63.3 nmol/mol. Average CO_2 concentration during the 2005 season was 552 µmol/mol, during the 2006 season, 550.3 µmol/mol, and during the 2007 season, 553 µmol/mol. Ozone levels in the experimental plots were measured with an O_3 analyzer (model 49C; Thermo Scientific Instruments, Franklin, MA, USA) and the concentration of CO_2 in the plots was measured with an infrared gas analyzer (model SBA-1; PP Systems, Hitchin, UK). As there were no observed effects of feeding on *G. max* foliage grown under elevated O_3 on *P. japonica* longevity at this site, we discontinued measuring changes in flavonoid concentrations in foliage grown under elevated levels of O_3 with *V. cardui* and *A. glycines* feeding damage.

Herbivore Enclosures for P. japonica Adults and V. cardui Larvae Mesh cages for containing study species and excluding non-study species were constructed with 1× 4 mm plastic mesh material covering a PVC-pipe framework. Cages were raised over the course of the season with the height of the plants, with a maximum height of 1 m, 1 m wide, and 1 m deep covering ~30 plants and a total volume of 1 m^3. For *P. japonica* and *V. cardui*, two cages were erected in all elevated CO_2 and ambient plots, at opposite ends of the plot. For *P. japonica*, two cages were also erected in each elevated O_3 plot. *P. japonica* and *V. cardui* experiments were done separately, as natural populations of each species were present in the field at different points during the season.

Collection of P. japonica Emerging *P. japonica* adults were collected from the east side of Meadowbrook Park (Urbana, IL, USA) in the last week of June 2005. Sixty beetles were added to one of the two cages in each plot, 30 individuals on June 29, and 30 individuals on June 30. The second cage was left sealed to prevent any damage to the plants until sampled. Seventy additional beetles were added to cages on July 8, to replace those that had escaped.

Acquisition of V. cardui V. cardui larvae were obtained from Carolina Biological Supply (Burlington, NC, USA). Natural populations at the SoyFACE site were not large enough to use for this experiment, but we observed natural population cycles for the timing of placement of larvae onto the experimental plants. Ten larvae were bagged (1.4 mm mesh bags) over two neighboring trifoliate leaves chosen from the top four trifoliate leaves on each plant on July 14, 2006, with 10 plants bagged in one cage in each ambient and elevated CO_2 plot. The larvae were bagged on the plants in addition to being in the cage, as they were small enough to fit through the mesh of the cage. The second cage was left sealed to prevent any damage from occurring to the plants until they were sampled.

Collection of A. glycines A. glycines adults were obtained from a campus laboratory colony. Fifty aphids were bagged over one trifoliate chosen from the top four trifoliates on each of 10 plants on August 6, 2007, in each ambient plot and on another 10 plants in each elevated CO_2 plot. Plants without visible insect damage were selected. Ten plants were bagged without aphids for control tissue.

Collecting Leaf Tissue from Herbivore-Damaged and Control Plants Damaged and undamaged leaf tissues were sampled 2.5 d and 2 wk after beetles started feeding, 1 and 3 d after *V. cardui* larvae started feeding, and 1, 3, and 7 d after aphids started feeding. Tissue was sampled at multiple time points after herbivore feeding began because flavonoid and isoflavonoid levels are induced over time, and we

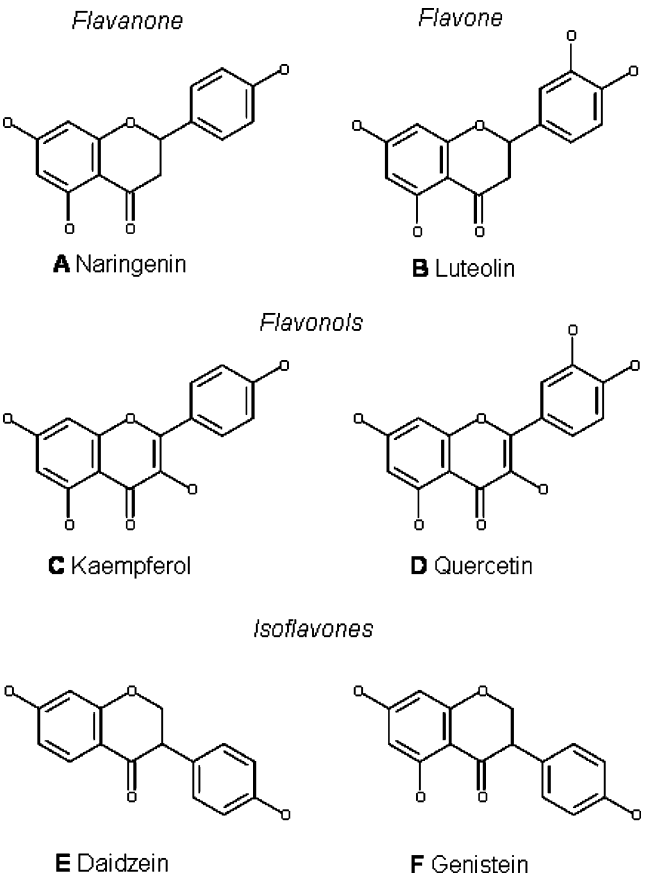

Fig. 1 Structures of the six flavonoid compounds identified in *Glycine max* foliage and phloem. **a**. Naringenin, **b**. Luteolin, **c**. Kaempferol, **d**. Quercetin, **e**. Daidzein, and **f**. Genistein. Figures drawn with SymyxDraw 3.2 (Symyx Solutions, Inc., San Ramon, CA)

wanted to capture their increase and decrease. Tissue always was selected from the top three leaf trifoliates of the sampled plants. On plants with herbivores, leaves exhibiting between 20–40% herbivore damage (as estimated by eye) but that did not have the herbivore currently feeding on them were selected. This allowed us to sample damaged tissue without disrupting feeding. For flavonoid analysis, four leaf disks were punched from one lateral leaflet from 5 plants with a metal cork borer (bore diam ~ 1.5 cm.). Leaf veins and spots of damage (either created mechanically or by herbivores) were avoided when leaves were punched so that equal leaf area was obtained in all disks. Disks from each cage were collected in paper envelopes that were immediately sealed and placed in liquid nitrogen.

Collecting Phloem from Aphid-Damaged and Control Plants A second leaf was selected from the top three leaf trifoliate leaves (again avoiding the trifoliate on which aphids were feeding) for phloem sap collection 1 d, 3 d, and 1 wk after aphids started feeding. Leaves were selected from aphid-damaged and control plants at each time point. Leaves were cut at the petiole, and the petiole was placed immediately in a 5-ml solution of 10 mM EDTA, pH 7 (Walter and DiFonzo 2007). The open tube tops and leaf petioles were Parafilm-wrapped to seal them, and the tubes were left in the dark at 5°C for 24 h. Phloem samples were kept at −80°C until analyzed.

Foliar Flavonoid Analysis for Herbivore-Damaged and Control Samples Six leaf disks (~1.77 cm^2 each) were taken from each sample, ground with a 6-mm glass bead (Fisher Scientific) in a Bead Beater (Wig L Bug, Crescent Dental Mfg. Co., Chicago, IL, USA), and combined with 500 µl methanol. Samples were vortexed and left at room temperature for 1 h. Samples then were sonicated for 1 min and centrifuged at 15,000 rpm for 5 min. Flavonoid compounds were separated on a reverse-phase high-pressure liquid chromatograph (HPLC) (712 WISP, Waters Corporation, USA) using a 250×4.6 mm ID 5 µm Capcell Pak C18 column (AG120, Shiseido Fine Chemicals, Japan) and a diode array detection system that has the capacity to

Table 1 Effects of elevated CO_2 and O_3 level (between-subject effects) on flavonoid amounts in undamaged and beetle-damaged soybean foliage[a]

Flavonoid	CO_2 *P* value	CO_2 *F* value	Ambient/elevated CO_2 mean µg/cm^2 leaf area	Ambient/elevated CO_2 s.e.	O_3 *P* value	O_3 *F* value	Ambient/elevated O_3 mean µg/cm^2 leaf area	Ambient/elevated O_3 s.e.	CO_2 x O_3 *P* value	CO_2 x O_3 *F* value
Genistein	0.088	3.440	0.257 0.118	0.053 0.053	**0.027**	**6.340**	**0.093** **0.282**	**0.053** **0.053**	**0.010**	**9.330**
Kaempferol	0.157	2.283	0.419 0.494	0.035 0.035	**0.025**	**6.529**	**0.393** **0.520**	**0.035** **0.035**	0.221	1.670
Kaempferol diglycoside	0.053	4.620	0.223 0.141	0.027 0.027	0.069	3.990	0.144 0.220	0.027 0.027	**0.001**	**17.364**
Kaempferol triglycoside 1	0.469	0.559	0.374 0.308	0.062 0.062	0.556	0.367	0.314 0.367	0.062 0.062	0.060	4.328
Kaempferol triglycoside 2	0.070	3.952	0.405 0.100	0.108 0.108	**0.018**	**7.474**	**0.043** **0.462**	**0.108** **0.108**	**0.030**	**6.079**
Luteolin diglycoside	0.224	1.644	0.130 0.168	0.021 0.021	**0.009**	**9.827**	**0.103** **0.196**	**0.021** **0.021**	0.242	1.513
Naringenin methyl hexose	0.766	0.093	0.140 0.131	0.020 0.020	**0.022**	**6.962**	**0.099** **0.173**	**0.020** **0.020**	**0.019**	**7.336**
Quercetin	0.797	0.069	0.317 0.334	0.045 0.045	**0.046**	**4.941**	**0.254** **0.397**	**0.045** **0.045**	0.191	1.925
Quercetin diglycoside	0.913	0.012	0.871 0.857	0.093 0.093	0.064	4.159	0.730 0.998	0.093 0.093	**0.019**	**7.312**
Quercetin hexose	0.959	0.003	0.223 0.221	0.028 0.028	0.103	3.119	0.187 0.257	0.028 0.028	0.177	2.057
Quercetin triglycoside 1	0.322	1.067	0.460 0.545	0.058 0.058	0.053	4.591	0.415 0.590	0.058 0.058	0.160	2.242
Quercetin triglycoside 2	**0.013**	**8.422**	**0.308** **0.448**	**0.034** **0.034**	**0.013**	**8.442**	**0.308** **0.448**	**0.034** **0.034**	0.943	0.005

[a] Changes in flavonoid amounts across the control and beetle-damage treatments over time were compared in foliage grown under ambient and elevated levels of CO_2 and O_3 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. The repeated measures effects from this analysis are separately reported in Tables 2 and 3.

measure wavelengths in the 210–400 nm range. The mobile phase consisted of 0.05% formic acid in H_20 (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 1 ml/min. The sample injection volume was 10µl, and components were eluted with the following solvent gradient: from 0–1 min solvent A was 85%; from 1 to 10 min solvent A was decreased to 70%; from 10 to 20 min solvent A was decreased to 62%; from 20 to 25 min solvent A was decreased to 20%; from 25 to 30 min solvent A was maintained at 20%; finally, solvent A was increased to 85% for 5 min. Between each injection, a mixture of 85% solvent A and 15% solvent B was run for 15 min.

A sample containing all flavonoid peaks present in this soybean variety was analyzed by LC/MS (model 2010ev Shimadzu, Kyoto) and diode array detection by employing the same separation and column for quantitative analyses. Sample peak areas were measured at 254 nm. Mass spectra, UV spectra and retention times were used to identify compound peaks in each sample, and commercially available standards were employed to convert peak areas to µg/cm^2 leaf area. The amount of standard injected in g divided by the area of the peak produced was used as the calibration factor. The area of the sample peak multiplied by the calibration factor, then multiplied by the dilution factor (total extract volume divided by the volume injected), and all divided by the total leaf area extracted (number of disks multiplied by the area per disk) was the flavonoid amount in the plant, µg/cm^2 leaf area. Peaks were matched across samples, and concentrations were compared by double repeated measures analysis of variance (*ANOVA*) with a between-subject effect of CO_2 treatment, and a between-subject effect of O_3 treatment in the beetle experiments. Damage and date were considered as repeated measures because matched insect-damaged and control samples were taken from the same CO_2 and O_3 treatment plots for each time point (SPSS 9.0, Chicago, IL, USA).

Phloem Flavonoid Analysis of Aphid-Damaged and Control Samples Phloem samples were dried in a centrifugal evaporator (RC 10.22, Jouan Inc., USA). Five hundred µl of methanol were added to each sample, and samples were analyzed by HPLC using the same protocol as for foliage. Statistical analysis was conducted with the same protocol as used for foliage (SPSS 9.0, Chicago, IL, USA).

Table 2 Effects of beetle-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean µg/cm^2 leaf area	Standard error
Genistein	0.065	4.108	Undamaged	0.162	0.043
			Damaged	0.213	0.036
Kaempferol	0.377	0.842	Undamaged	0.438	0.032
			Damaged	0.475	0.032
Kaempferol diglycoside	**0.005**	**11.625**	Undamaged	**0.148**	**0.024**
			Damaged	**0.217**	**0.019**
Kaempferol triglycoside 1	**0.005**	**11.949**	Undamaged	**0.273**	**0.039**
			Damaged	**0.409**	**0.055**
Kaempferol triglycoside 2	0.995	0.000	Undamaged	0.253	0.103
			Damaged	0.252	0.058
Luteolin diglycoside	0.929	0.008	Undamaged	0.150	0.024
			Damaged	0.148	0.016
Naringenin methyl hexose	**0.025**	**6.540**	Undamaged	**0.100**	**0.019**
			Damaged	**0.171**	**0.020**
Quercetin	0.193	1.905	Undamaged	0.294	0.038
			Damaged	0.357	0.040
Quercetin diglycoside	0.064	4.172	Undamaged	0.776	0.096
			Damaged	0.952	0.056
Quercetin hexose	0.337	1.002	Undamaged	0.242	0.030
			Damaged	0.201	0.026
Quercetin triglycoside 1	0.632	0.241	Undamaged	0.519	0.056
			Damaged	0.486	0.050
Quercetin triglycoside 2	**0.044**	**5.066**	Undamaged	**0.312**	**0.030**
			Damaged	**0.444**	**0.044**

[a] Flavonoid amounts across the two atmospheric treatments over time were compared in control and beetle-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

Results

The isoflavone genistein (Fig. 1f), the flavone luteolin (Fig. 1b), the flavanone naringenin (Fig. 1a), and the flavonols quercetin (Fig. 1d) and kaempferol (Fig. 1c) were identified in all foliage irrespective of damage, as were glycosides of these compounds.

Flavonoid Results for Beetle-Damaged and Control Tissues The only main effect of CO_2 involved a single flavonoid; one of the two quercetin diglycosides increased significantly under elevated CO_2 (Table 1). In contrast, seven (genistein, kaempferol, kaempferol triglycoside, luteolin diglycoside, naringenin methyl hexose, quercetin, and quercetin triglycoside) of the 12 flavonoids increased in plants exposed to elevated ozone (Table 1). Five compounds not belonging to any particular flavonoid class were significantly affected by interactions between CO_2 and O_3. These interactions were of one type; large increases in flavonoids associated with elevated ozone were wholly or largely suppressed in elevated CO_2 treatments (data not shown). There also was a significant interaction between CO_2 and time with kaempferol triglycoside doubling in concentration at ambient CO_2 (from 0.267±s.e. 0.052 to 0.480±s.e. 0.092, μg/cm^2 leaf area) but remaining unchanged at elevated CO_2 (from 0.337±s.e. 0.052 to 0.279±s.e. 0.092, μg/cm^2 leaf area) (d.f.=63, F=5.322, P=0.040).

Beetle damage induced significantly increased concentrations of naringenin methyl hexose, kaempferol diglycoside, kaempferol triglycoside, and quercetin triglycoside (Table 2). Over the course of the sampling period, concentrations of six compounds (genistein, kaempferol, kaempferol diglycoside, naringenin methyl hexose, quercetin, and quercetin triglycoside) increased from the first to the second collection date (Table 3).

Flavonoid Results for Caterpillar-Damaged and Control Tissues There were no significant effects of elevated CO_2 (Table 4) on flavonoid concentration, and no significant interactions between effects of elevated CO_2 and damage by caterpillars. Levels of genistein were significantly higher in foliage damaged by *V. cardui* (Table 5). Quercetin triglycoside decreased in abundance from the first to the second collection date (Table 6).

Table 3 Effects of time (repeated measure) on flavonoid levels in undamaged and beetle-damaged soybean foliage[a]

Flavonoid	P value	F value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	**0.021**	**7.015**	First collection	**0.097**	**0.026**
			Second collection	**0.278**	**0.067**
Kaempferol	**< 0.001**	**23.472**	First collection	**0.338**	**0.031**
			Second collection	**0.575**	**0.038**
Kaempferol diglycoside	**0.027**	**6.384**	First collection	**0.114**	**0.034**
			Second collection	**0.250**	**0.032**
Kaempferol triglycoside 1	0.215	1.718	First collection	0.302	0.037
			Second collection	0.379	0.065
Kaempferol triglycoside 2	0.054	4.552	First collection	0.099	0.034
			Second collection	0.407	0.145
Luteolin diglycoside	0.840	0.042	First collection	0.153	0.027
			Second collection	0.145	0.020
Naringenin methyl hexose	**0.029**	**6.189**	First collection	**0.089**	**0.024**
			Second collection	**0.183**	**0.023**
Quercetin	**0.034**	**5.757**	First collection	**0.272**	**0.032**
			Second collection	**0.379**	**0.045**
Quercetin diglycoside	0.719	0.136	First collection	0.840	0.079
			Second collection	0.888	0.105
Quercetin hexose	0.232	1.581	First collection	0.192	0.025
			Second collection	0.252	0.036
Quercetin triglycoside 1	**0.006**	**10.932**	First collection	**0.368**	**0.037**
			Second collection	**0.637**	**0.072**
	0.241	1,524	First collection	0.428	0.040
			Second collection	0.328	0.053

[a] Flavonoid amounts in the control and beetle-damage treatments and across the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant P values at the 0.05 level are in bold text.

Table 4 Effects of CO_2 (between-subjects effect) on flavonoid levels in undamaged and caterpillar-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.904	0.016	Ambient	0.083	0.024
			Elevated	0.122	0.024
Kaempferol diglycoside	0.605	0.297	Ambient	0.157	0.045
			Elevated	0.192	0.045
Kaempferol triglycoside 1	0.467	0.604	Ambient	0.569	0.052
			Elevated	0.627	0.052
Quercetin diglycoside	0.925	0.010	Ambient	1.195	0.091
			Elevated	1.208	0.091
Quercetin hexose	0.582	0.339	Ambient	0.771	0.060
			Elevated	0.721	0.060
Quercetin triglycoside 1	0.910	0.014	Ambient	0.663	0.048
			Elevated	0.671	0.048
Quercetin triglycoside 2	0.690	0.175	Ambient	0.961	0.100
			Elevated	1.021	0.100

[a] Flavonoid amounts over time and across the control and caterpillar-damage treatments were compared in foliage grown under ambient and elevated levels of CO_2 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. The repeated measures effects from this analysis are separately reported in tables 5 and 6.

Flavonoid Results for Aphid-Damaged and Control Foliar Tissues Concentration of only one flavonoid, quercetin, increased under elevated CO_2 conditions (Table 7). Two of the quercetin triglycosides, kaempferol triglycoside, quercetin hexose, and genistein increased from the first to the third collection date, whereas luteolin diglycoside declined in abundance from the first to the third collection date (Table 8). There were no significant effects of aphid damage (Table 9), and no significant interactions between effects of elevated CO_2 and aphid feeding.

Flavonoid Results for Aphid-Damaged and Control Phloem The only confirmed flavonoid in phloem was the isoflavone daidzein (Fig. 1e). This isoflavone increased in concentration from the first to the third collection date (Table 8) and showed a marginally significant increase in concentration in response to aphid damage (Table 9). There were no significant interactions.

Discussion

Elevated CO_2 affected only concentrations of a single flavonoid in *G. max*. Plants grown under elevated amounts of CO_2 have higher quercetin:kaempferol ratios, and quercetin may be acting as an antioxidant for these plants,

Table 5 Effects of caterpillar-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	**0.001**	**36.102**	Undamaged	**0.074**	**0.022**
			Damaged	**0.131**	**0.020**
Kaempferol diglycoside	0.863	0.032	Undamaged	0.179	0.049
			Damaged	0.170	0.027
Kaempferol triglycoside 1	0.937	0.007	Undamaged	0.600	0.051
			Damaged	0.596	0.034
Quercetin diglycoside	0.873	0.028	Undamaged	1.208	0.097
			Damaged	1.294	0.049
Quercetin hexose	0.769	0.094	Undamaged	0.732	0.078
			Damaged	0.760	0.042
Quercetin triglycoside 1	0.825	0.053	Undamaged	0.658	0.057
			Damaged	0.677	0.048
Quercetin triglycoside 2	0.605	0.297	Undamaged	0.945	0.122
			Damaged	1.037	0.097

[a] Flavonoid amounts were compared across atmospheric treatments over time in control and caterpillar-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

Table 6 Effects of collection date (repeated measure) on flavonoid levels in undamaged and caterpillar-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.976	0.001	First collection	0.095	0.022
			Second collection	0.109	0.013
Kaempferol diglycoside	0.453	0.643	First collection	0.204	0.042
			Second collection	0.145	0.054
Kaempferol triglycoside 1	0.560	0.380	First collection	0.612	0.031
			Second collection	0.584	0.053
Quercetin diglycoside	0.244	1.669	First collection	1.263	0.044
			Second collection	1.140	0.104
Quercetin hexose	0.192	2.159	First collection	0.773	0.030
			Second collection	0.719	0.059
Quercetin triglycoside 1	**0.019**	**10.232**	First collection	**0.712**	**0.025**
			Second collection	**0.623**	**0.046**
Quercetin triglycoside 2	0.541	0.420	First collection	1.016	0.091
			Second collection	0.966	0.069

[a] Flavonoid amounts across control and caterpillar-damaged tissues in the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

quenching reactive oxygen species (ROS) (Qiu et al. 2008). Elevated levels of atmospheric CO_2 have been suggested to cause oxidative damage to plants, so additional amounts of antioxidants may well be produced to counter this increased damage (Cheeseman 2006). Ozone, a strong oxidizing agent, was a far more effective inducer of flavonoid production in this study. This inducing effect, however, was almost entirely absent when CO_2 also was elevated. One possible explanation for this interaction is that soybean leaf stomatal conductance is decreased in elevated CO_2 atmospheres, thus reducing ozone fluxes into the leaf and buffering its effects (Bernacchi et al. 2007; Mishra et al. 2008).

The significant increase in quercetin and the non-significant decrease of kaempferol changes the flavonoid ratios found in plants grown under elevated CO_2 and may change the total balance for herbivores also from prooxidant to antioxidant (Galati et al. 2002). Similar shifts in the quercetin:kaempferol ratio have been observed in plants grown under temperature stress (Albert et al. 2009). Quercetin has a higher quenching capability than kaempferol (Rice-Evans et al. 1996), and it was theorized that under temperature stress more quercetin was produced because of this quenching ability (Albert et al. 2009). Similar effects may occur in soybeans growing under

Table 7 Effects of CO_2 (between-subject effect) on flavonoid lvels in undamaged and aphid-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.473	0.587	Ambient	0.163	0.034
			Elevated	0.126	0.034
Quercetin	**0.015**	**11.510**	Ambient	**0.452**	**0.099**
			Elevated	**0.927**	**0.099**
Luteolin diglycoside	0.607	0.295	Ambient	0.290	0.036
			Elevated	0.318	0.036
Kaempferol triglycoside 1	0.260	1.547	Ambient	0.893	0.219
			Elevated	1.278	0.219
Quercetin hexose	0.937	0.007	Ambient	1.220	0.199
			Elevated	1.243	0.199
Quercetin triglycoside 1	0.215	1.923	Ambient	0.831	0.295
			Elevated	1.411	0.295
Quercetin triglycoside 2	0.158	2.593	Ambient	1.335	0.251
			Elevated	1.907	0.251

[a] Flavonoid amounts over time and across the control and aphid-damaged treatments were compared in foliage grown under ambient and elevated levels of CO_2 by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text. Repeated measures effects from this analysis are reported in Tables 8 and 9.

Table 8 Effects of collection date (repeated measure) on flavonoid levels in undamaged and aphid-damaged soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	**0.028**	**7.880**	First collection	**0.061**	**0.015**
			Third collection	**0.273**	**0.058**
Quercetin	0.800	0.234	First collection	0.762	0.204
			Third collection	0.698	0.118
Luteolin diglycoside	**< 0.001**	**54.017**	First collection	**0.379**	**0.061**
			Third collection	**0.095**	**0.023**
Kaempferol triglycoside 1	**0.001**	**33.137**	First collection	**0.626**	**0.141**
			Third collection	**1.598**	**0.285**
Quercetin hexose	**0.015**	**10.919**	First collection	**0.639**	**0.120**
			Third collection	**2.183**	**0.442**
Quercetin triglycoside 1	**0.019**	**9.742**	First collection	**0.627**	**0.118**
			Third collection	**1.490**	**0.238**
Quercetin triglycoside 2	**0.004**	**20.481**	First collection	**1.020**	**0.199**
			Third collection	**2.403**	**0.397**

[a] Flavonoid amounts across control and aphid-damaged tissue in the two atmospheric treatments were compared over time by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

elevated CO_2 stress. Genistein levels in *G. max* were decreased under elevated CO_2 treatment at the $P=0.1$ level. This decrease in genistein content in plants grown under elevated CO_2 is fortuitous for most herbivores, as genistein reduces most insects' longevity and overall fitness (Piubelli et al. 2005). The multiple changes in *G. max* flavonoid content under elevated CO_2 conditions may contribute to the increased longevity experienced by *P. japonica* on *G. max* foliage grown under elevated carbon dioxide (O'Neill et al. 2008). This hypothesis could be tested by supplementing soybean foliage grown under ambient levels of CO_2 with enough quercetin to equal the quercetin:kaempferol ratio found in foliage grown under elevated levels of CO_2, even with the higher levels of kaempferol found in the foliage grown under ambient levels of CO_2.

The differential flavonoid response of *G. max* to leaf-skeletonization, non-skeletonizing leaf chewing, and phloem sucking feeding damage suggests that the induced defense responses of plants depend on the type of damage inflicted (Felton et al. 1994; Kaplan et al. 2008). Whereas leaf damage in the form of skeletonization induces changes in several flavonoid and isoflavonoid levels, feeding damage by the

Table 9 Effects of aphid-damage (repeated measure) on flavonoid levels in soybean foliage[a]

Flavonoid	*P* value	*F* value	Treatment	Mean μg/cm^2 leaf area	Standard error
Genistein	0.237	1.726	Undamaged	0.102	0.028
			Damaged	0.187	0.050
Quercetin	0.333	1.107	Undamaged	0.622	0.105
			Damaged	0.757	0.084
Luteolin diglycoside	0.071	4.786	Undamaged	0.246	0.028
			Damaged	0.362	0.043
Kaempferol triglycoside 1	0.731	0.129	Undamaged	1.048	0.219
			Damaged	1.124	0.149
Quercetin hexose	0.720	0.141	Undamaged	1.184	0.197
			Damaged	1.279	0.180
Quercetin triglycoside 1	0.735	0.126	Undamaged	1.181	0.352
			Damaged	1.061	0.144
Quercetin triglycoside 2	0.550	0.400	Undamaged	1.517	0.257
			Damaged	1.724	0.225

[a] Flavonoid amounts across the two atmospheric treatments over time were compared in undamaged and aphid-damaged foliage by double repeated measures *ANOVA*. Significant *P* values at the 0.05 level are in bold text.

caterpillar *V. cardui* increased levels of one genistein glycoside, and feeding by the aphid *A. glycines* did not affect production of any compound. The increase in flavonoid content over time is to be expected as these compounds are induced by herbivore damage (Hagerman and Butler 1991), and by increased photooxidative stress, particularly in August when the aphid experiments were conducted (Stark et al. 2008).

The increase in flavonoid content in *G. max* tissue grown under elevated O_3 conditions is also expected as O_3 is a powerful oxidative agent (Sager et al. 2005). This increase is consistent with the results of other studies that have looked at changes in genes involved in the flavonoid biosynthesis pathway in *G. max* foliage in response to elevated O_3 conditions (Bilgin et al. 2008). The increase in content of all flavonoid compounds regardless of class could be utilized by the plant as a general response against adverse environmental conditions (Bilgin et al. 2008). This increase in content of all flavonoids in *G. max* foliage grown under elevated O_3 leaves the quercetin:kaempferol balance unchanged, helping to explain the lack of an effect on longevity of *P. japonica* feeding on this foliage (O'Neill et al. 2008) and contributing to the lack of an effect on herbivory on plants grown under elevated levels of O_3 at this research site (Dermody et al. 2008).

In a separate study, tissue from plants in the beetle treatments from this experiment was examined *via* microarray to investigate transcriptional responses to elevated CO_2 and leaf skeletonization (Casteel et al. 2008). Transcriptional changes involved in flavonoid and isoflavonoid biosynthesis supported our findings that abundance increased after beetle damage (e.g., putative flavonol synthase, 2′-hydroxydihydrodaidzein reductase, isoflavone reductase-like protein), with or without elevated CO_2, and increased flavonol biosynthesis (e.g., flavonol synthase-like oxidoreductase) under elevated CO_2 conditions (Casteel et al. 2008).

These changes in defense compound/antioxidant concentrations were examined in the context of damage inflicted by only a single herbivore; impacts of different types of damage inflicted simultaneously have yet to be determined. Given the multiplicity of responses that depend on the nature of feeding damage, the prediction of future impacts of global atmospheric changes on the soybean agroecosystem will be difficult. The changes in the quercetin: kaempferol ratio of *G. max* foliage under elevated CO_2 though, suggests that the benefits of *G. max* foliage to select herbivores may be great.

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