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ORIGINAL PAPER

Effects of elevated CO_2 and soil water content on phytohormone transcript induction in *Glycine max* after *Popillia japonica* feeding

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Abstract Plants will experience increased atmospheric CO_2 and drought in the future, possibly altering plant–insect dynamics. To investigate the combined effects of these components of global change on plant–insect interactions, three major hormone signaling pathways [jasmonic acid (JA), salicylic acid (SA), and ethylene (ET)] and related defenses were examined in undamaged soybean (*Glycine max*) leaves and after Japanese beetle (*Popillia japonica*) feeding; plants were grown under elevated CO_2 and reduced soil water both independently and simultaneously. Nutritional quality and Japanese beetle preference for leaf tissue grown under these different conditions also were determined. Elevated CO_2 increased the concentration of leaf sugars and dampened JA signaling transcripts but increased the abundance of SA compared with plants grown in ambient CO_2. A mild reduction in soil water content had no effect on leaf sugars but stimulated the induction of transcripts related to JA and ET biosynthesis after herbivory. When applied in combination, elevated CO_2 and reduced soil water content suppressed the expression of transcripts related to JA and ET gene transcription. Exposure to elevated CO_2 alone increased susceptibility of soybean to beetle damage. However, exposure to elevated CO_2 in combination with reduced soil water content negated the impact of elevated CO_2, leaving susceptibility unchanged. Predicting future crop resistance to pests must take into account interactions among individual components of global climate change.

Keywords Global change · Carbon dioxide · Soybean · Japanese beetle · Drought · Induced defenses · Plant–insect interactions

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Introduction

If current trends in global atmospheric change continue unabated, by 2050 plants will be growing in an atmosphere with 50 % more CO_2 than occurs today (Forster et al. 2007). Simultaneously, summer precipitation in mid-continental areas is projected to decrease in volume and frequency (Giorgi et al. 2001; Kling et al. 2003; Weltzin et al. 2003), and greater temperatures will increase crop water use and deplete soil moisture, resulting in a greater risk of droughts this century (Meehl et al. 2007). Typically, drought reduces yield and agricultural productivity (Goldblum 2009), whereas elevated CO_2 has the opposite effect (Ainsworth and Long 2005; Long et al. 2006). In addition, elevated CO_2 reduces plant stomatal conductance, which often reduces water use, conserves soil moisture, and may ameliorate drought stress (Leakey et al. 2009).

Plants grown under elevated CO_2 and drought experience chemical changes that can influence their suitability as food for insects. While exposure to elevated CO_2 decreases the nutritional quality of leaves by increasing C/N ratios

(Ainsworth et al. 2002), drought often increases available nitrogen in plant tissues (White 1984; Mattson and Haack 1987; Huberty and Denno 2004). Because nitrogen is the principal component in plant foliage limiting the growth of insect herbivores (Mattson 1980; Awmack and Leather 2002), increased CO_2 concentrations and drought may alter plant interactions with herbivores. However, changes in nitrogen alone do not explain the substantial variation in herbivore performance on plants grown under elevated CO_2 and drought individually or in combination (Thompson et al. 1993; Roth et al. 1997; Penuelas and Estiarte 1998; Huberty and Denno 2004; Joern and Mole 2005; Zvereva and Kozlov 2006; Stiling and Cornelissen 2007; Prichard et al. 2007; Dermody et al. 2008).

Defensive secondary metabolites produced by plants also influence suitability for insect herbivores. Plant defenses are regulated by a complex network of phytohormone-based signaling pathways (Bostock 2005). Three phytohormones in particular, jasmonic acid (JA), ethylene (ET), and salicylic acid (SA), have been studied extensively in connection with defense responses to biotic stressors, such as pathogens and herbivores (Glazebrook 2005; Jones and Dangl 2006; Howe and Jander 2008). Recently, we demonstrated that elevated CO_2 reconfigures these phytohormone signaling networks in soybean plants, resulting in decreased levels of defense chemicals and increased susceptibility to Japanese beetles (Casteel et al. 2008; Zavala et al. 2008, 2009). However, the interactive effect of elevated CO_2 and drought is not well understood, particularly with regard to the hormonal and metabolic responses that collectively mediate changes in plant–insect interactions.

An essential component of all defense responses in plants is the capacity to rapidly modify the transcriptome. In this study, the interacting effects of reduced soil water content and elevated CO_2 on transcriptional and chemical components of phytohormone signaling and related defense responses were examined in soybeans after Japanese beetle damage. Plants were grown in the field at the soybean free air gas concentration enrichment (SoyFACE) research site in a full factorial experiment with elevated CO_2 and reduced soil water content. Leaf area removed by Japanese beetles was quantified in a 4-way choice experiment among field-grown tissues from the four treatments in the laboratory. To determine the interactive effect of reduced soil water content and CO_2 exposure on phytohormone signaling, we analyzed the transcripts previously demonstrated to be regulated by CO_2 exposure. We examined the abundance of 1-aminocyclopropane-1-carboxylate synthase (*acc1*), which is involved in the regulation and biosynthesis of ET, and two genes in the octadecanoid signaling pathway, which control the accumulation of JA (allene oxide synthase (*aos*) and hydrogen peroxide lyase (*hpl*)). In addition, the abundance of the phytohormones JA and SA and related direct defense metabolites were examined. To dissect the influence of defense responses versus nutritional changes, important components of insect nutrition were also measured in leaf tissue.

Materials and methods

Site description and field feeding experiment

Research was performed at the SoyFACE experiment at the University of Illinois, Urbana–Champaign (40°02′N, 88° 14′W, 228-m above sea level; http://soyface.illinois.edu; Long et al. 2004), where large plots of soybean (Pioneer 93B15) are exposed to elevated concentrations of CO_2. The experiment consists of eight 20-m-diameter octagonal plots (282.8-m^2) distributed within four randomized blocks. Soybean was rotated annually with corn, and no N fertilizer was added during years of soybean production, according to standard agronomic practice in this region. Soybeans had been growing for 45 days at the beginning of the experiment and were in the vegetative developmental stage.

Soybean plots either were at current ambient CO_2 concentration ($\sim$386 μmol mol^{-1}) or fumigated to a target CO_2 concentration of 550 μmol mol^{-1} (average elevated CO_2 concentration for the 2008 growing season: $\sim$553 μmol mol^{-1}), the concentration predicted to occur in 2050 (Forster et al. 2007). Within each plot, one 8×4 m^2 subplot was exposed to ambient levels of rainfall, and one subplot of equal size was exposed to reduced levels of rainfall, resulting in four treatments: (1) ambient CO_2, ambient soil water content; (2) ambient CO_2, reduced soil water content; (3) elevated CO_2, ambient soil water content; and (4) elevated CO_2, reduced soil water content.

The reduction in rainfall was achieved with retractable awnings that were deployed manually at dusk when nighttime rainfall was predicted. The awnings were retracted at dawn the following day. In the second 2 weeks of July 2008 when this experiment was conducted, nighttime rain interception reduced soil volumetric water content in the top 25 cm by up to 30 % in ambient CO_2 plots and by up to 24 % in elevated CO_2 plots (no significant difference in soil moisture could be detected between plots at ambient and elevated CO_2; data not shown). Over 2 years of experimentation, decreases in soil moisture of this magnitude caused reductions in stomatal conductance of up to 16 % in ambient CO_2 and up to 19 % in elevated CO_2 (S.B. Gray, unpublished data).

Using commercially available traps (Bag-a-Bug®, A.G. Organics, Prosper, TX), Japanese beetles (*Popillia japonica* Newman) were collected from soybeans adjacent to the SoyFACE site and starved for 24 h prior to infestation. In

each of the treatment subplots, the youngest fully expanded trifoliate of 12 undamaged plants was enclosed in a mesh bag (1 mm × 4 mm mesh) to prevent movement of insects. Five adult Japanese beetles were added to nine of the enclosed trifoliates, while three undamaged controls received no beetles. Starved beetles were allowed to feed until ~10 % of the leaf area was consumed (~1 h) and then removed. Leaves from three undamaged (0 h) and damaged trifoliates were collected 2, 6, and 72 h after beetle removal from separate plants from each treatment subplot. The three leaves collected at each time point were pooled for each block, resulting in 4 samples for each of the 4 subplot treatments. Tissue was frozen in liquid nitrogen, ground to a fine powder, and stored at −80 °C. The complete experiment was replicated in four blocks, resulting in 64 tissue samples ($n = 4$).

RNA preparation

Total RNA was extracted from the frozen tissue samples (0 and 2 h) using Trizol reagent (Invitrogen, Carlsbad, CA). The early time points only (0 and 2 h) were examined for transcriptional changes based on previous observations of induction patterns in soybean (Casteel 2010). RNA integrity was determined using a 1.2 % formaldehyde agarose gel (Sambrook et al. 1989) and a microfluidic visualization tool (Bioanalyzer, Agilent Technologies, USA, http://www.agilent.com).

Quantitative real-time RT-PCR (qRT-PCR)

The expression levels of genes coding for *aos* (allene oxide *synthase 2*; *NCBI* ACCESSION: NM_001249516), *hpl* (hydrogen peroxide lyase; NCBI ACCESSION: DQ340251), and *acc1* synthase (1-aminocyclopropane-1-carboxylate; NCBI ACCESSION: NM_001249929) were analyzed with quantitative real-time RT-PCR (qRT-PCR), with *con6* (contig 6) as an internal standard according to Casteel (2010). Contig 6 is constitutively expressed (Libault et al. 2008), and stable expression was verified across samples using qRT-PCR. Total RNA (1 µg) was DNAse-treated (RQ1 DNase, Promega, Madison, WI) and reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) using oligo-$dT_{12–18}$ as a primer. Primers used for qRT-PCR were designed using Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=NcbiHomeAd) according to Casteel (2010). Reactions were carried out using 5 µl of the "SYBR green PCR master mix" (Applied Biosystems, Foster City, CA), with 800 nM of primer, in the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA). The PCR conditions were run according to Casteel (2010). The technical replicates on the plate for each sample were averaged, and relative expression was calculated for these data with the relative standard curve method (Applied Biosystems, Foster City, CA). Fold change was calculated for each sample by comparing them to the average control value.

Phytohormones

JA and SA were measured according to Wu et al. (2007), 2 and 6 h after the beetles were removed from damaged plants as well as from the undamaged controls. Approximately 150 mg from each frozen tissue sample was transferred to FastPrep tubes (Qbiogene, Carlsbad, CA) containing 900 mg of Zirmil beads (1.1 mm; Saint-Gobain ZirPro, Mountainside, NJ, USA). Using a bead beater, samples with 1 ml of ethyl acetate and internal standards were then homogenized for 2 min (100 ng of D_2-JA and D_4-SA per 1 ml of ethyl acetate). After centrifugation at 12,000*g* for 20 min at 4 °C, 500 µl was removed and transferred to a new tube. An additional 1 ml of ethyl acetate without internal standards was added to the remaining tissue, which was then re-extracted and centrifuged as described. The supernatants were combined (500 µl from the first extraction and 250 µl from the re-extraction) and then dried by evaporation on a vacuum concentrator. Samples were then dissolved in 500 µl 70 % (v/v) methanol, vortexed for 10 min, and subsequently centrifuged at 12,000*g* for 10 min.

Measurements were taken on a liquid chromatograph-mass spectrometry system (Shimadzu LCMS-2010 EV, Shimadzu, Columbia, MD, US) according to Wu et al. (2007). Analytes were separated on a C18 reversed phase HPLC column (250 × 2 mm 5 µm i.d.; Phenomenex, Torrance, CA, USA) using a gradient of 0.05 % formic acid in water (solvent A) and 0.05 % formic acid in methanol (solvent B) at a flow rate of 300 µl min^{-1}. The initial condition of 10 % B was kept for 5 min and increased to 100 % solvent B over 25 min. The mass spectrometer was operated in a negative electro-spray ionization mode. The hormones (JA and SA) were quantified by comparing their peak areas with those from internal standards.

Plant defenses

Measurements of two important plant defenses, cysteine proteinase inhibitor (CystPI) and polyphenol oxidase (PPO), were made 72 h after the beetles were removed. Both CystPI and PPO are inducible by JA (Mayer 2006), and CystPIs of soybeans are specific deterrents to Japanese beetles because they attach to and inhibit the primary proteinases in the digestive tract (Kim and Mullin 2003; Zavala et al. 2008). CystPI activity was measured as in Zavala et al. (2008); 200 mg of frozen leaf powder was

extracted in 1 ml of a 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA. Samples were vortexed for 10 s and centrifuged at 12,000*g* for 15 min. CystPI activity was measured in samples on a plate reader at 37 °C for up to 20 min at 410 nm against a papain standard by following the release of p-nitroaniline after the addition of a synthetic substrate, p-Glu-Phe-Leu-pNA.

Polyphenol oxidase activity was measured as in Anderson and Morris (2001). Briefly, 50 mg of frozen leaf powder was extracted in a 50 mM MOPS buffer (pH 6.5, Sigma Aldrich) with 10 mM L-DOPA as a phenolic substrate (L-DOPA solution was made fresh daily). Tubes were constantly rotated for 0.5 h at room temperature and then were centrifuged for 5 min at 12,000*g*. The change in absorbance at 475 nm was determined and compared with a substrate-only control. One unit of PPO activity was defined as a change of 0.001 absorbance unit/min/ml. All reactions were conducted at 20 °C.

Leaf nutrients

To measure carbohydrates and protein content, ground leaf tissue was extracted according to Jones et al. (1977). Tissue was extracted five times in 80 % (v/v), buffered (2 mM HEPES, pH 7.8) ethanol at 80 °C for 20 min. Glucose, fructose, and sucrose concentrations were determined using a continuous enzymatic substrate assay as in Jones et al. (1977) and modified by Ainsworth et al. (2007). For protein determination, pellets of the ethanol extraction were dissolved at 95 °C in 0.1 M NaOH. The pH of the NaOH solution was then adjusted to 4.9, and protein concentration was determined using a commercial kit (BCA protein assay kit; Thermo Scientific; Rockford, IL, USA) with BSA (bovine serum albumin) as a standard. Total CystPI and PPO calculations were adjusted to control for differences in protein loading.

Choice experiment

The youngest fully expanded soybean trifoliate was collected from 4 plants that had been growing for 45 days in the following four treatments: (1) ambient CO_2, ambient soil water content; (2) ambient CO_2, reduced soil water content; (3) elevated CO_2, ambient soil water content; and (4) elevated CO_2, reduced soil water content. Leaves were harvested from SoyFACE plots, and leaf disks (2.5 cm diameter) were cut from each treatment and placed equidistant from the center in Petri dishes (15 cm diameter) on filter paper moistened with distilled water. The act of cutting leaf disks may influence preference by inducing defenses; however, selecting a larger-diameter leaf disk would minimize the effect of localized induction on the suitability of the remaining leaf tissue (Jones and Coleman 1988). Japanese beetles were collected and starved as described previously. Beetles were placed in the center of the Petri dish and allowed to feed for 24 h, after which they were removed. Photographs were taken of the leaf disks to analyze leaf area removed with image analysis software (Image J, http://rsbweb.nih.gov/ij/).

Statistical analyses

The transcript and metabolite values were analyzed with a $2 \times 2 \times 2$ (CO_2 exposure × reduced soil moisture × herbivory) factorial mixed model analysis of variance (ANOVA) followed by Fisher's protected least significant difference (LSD) post hoc comparisons in all experiments. For the comparisons, CO_2 exposure, reduced soil moisture, and herbivory were considered to be fixed effects and block as a random effect. Hormone and defense data were transformed to achieve normality. Leaf area removed in the choice experiment was analyzed using a 2×2 ANOVA (CO_2 exposure and reduced soil moisture), followed by Fisher's protected LSD post hoc comparison. Data were analyzed using SAS, version 9.0 (SAS Institute).

Results

To determine the direct and interacting effects of elevated CO_2 and reduced soil water content on herbivore feeding behavior, Japanese beetles were allowed to feed for 24 h with equal access to leaves grown under ambient and elevated CO_2, and under ambient and reduced soil water content, in a four-way choice experiment. Soybean foliage grown under elevated CO_2 was preferred by beetles compared with foliage grown under ambient CO_2 (main effect of CO_2 exposure; $P < 0.05$; Fig. 1). Beetles removed 13.2 % of leaves grown under elevated CO_2, compared with only 3.5 % of leaves grown under ambient CO_2 (Fig. 1). There was no significant effect of reduced soil water content on leaf area removed by Japanese beetles ($P > 0.05$; Fig. 1).

The two genes in the octadecanoid pathway leading to the production of JA and green leaf volatiles, *aos* and *hpl*, were up-regulated by Japanese beetle herbivory (Fig. 2 a, b; main effect of herbivory: $P < 0.01$), and for *hpl*, this up-regulation was strongly modulated by simultaneous exposure to reduced water availability (CO_2 × herbivory and reduced soil moisture × herbivory interaction: $P < 0.01$; Fig. 2a, b). The overall fold change relative to undamaged controls following herbivory averaged across the other treatments was 1.23 and 1.17 for *aos* and *hpl*, respectively. Reduced water availability up-regulated *aos* and *hpl* (main effect of reduced soil moisture: $P < 0.01$; Fig. 2a, b), reducing the impact of CO_2 when applied in combination

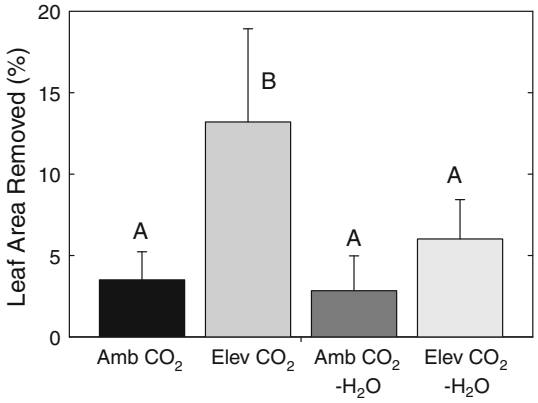

Fig. 1 Leaf area removed after 24 h by Japanese beetles given a choice of soybean foliage grown under ambient or elevated CO_2 concentrations and at ambient or reduced soil water content (designated "drought"). Values represent the mean ± SE; *letters* indicate significant differences between ambient and elevated CO_2 treatments ($P < 0.05$)

with reduced soil water content (reduced soil moisture × CO_2 interaction: $P < 0.05$; Fig. 2a, b). While no main effect of CO_2 was detected for *hpl*, CO_2 down-regulated *aos* abundance (Fig. 2a, b; main effect of CO_2: $P < 0.05$) and interacted strongly with herbivory to influence the expression of *aos* and *hpl* (CO_2 × herbivory interaction: $P < 0.01$; Fig. 2a, b). The constitutive levels of both genes were down-regulated by exposure to elevated CO_2 prior to herbivory. However, after herbivory under elevated CO_2, both genes were up-regulated to similar levels as plants grown under ambient conditions. Because the initial levels (undamaged) were lower for plants grown under elevated CO_2 but the induced levels after herbivory were the same compared with ambient grown plants, the induction capacity was amplified under elevated CO_2.

In contrast to *aos* and *hpl*, there was no main effect of herbivory on the expression of *acc*, an important gene regulating the production of ET (Fig. 2c; main effect of herbivory, $P > 0.05$). Reduced soil water content, however, increased expression of *acc* when averaged across the other treatments (1.17-fold; main effect of reduced soil moisture: $P < 0.01$), and exposure to elevated CO_2 down-regulated the expression of *acc* when averaged across the other treatments (−0.64-fold; main effect of CO_2, $P < 0.01$). The increase in expression of *acc* by reduced soil water content was dampened when plants were exposed to herbivory and elevated compared with ambient CO_2 (reduced soil moisture × CO_2 × herbivory interaction, $P < 0.01$).

Neither herbivory nor reduced soil water content affected SA concentration in soybean, but SA levels were higher in plants grown under elevated CO_2 compared with those grown under ambient air (Fig. 3a; SA ambient CO_2: 6,530.7 ± 618.0 ng/g of tissue fresh weight, SA elevated

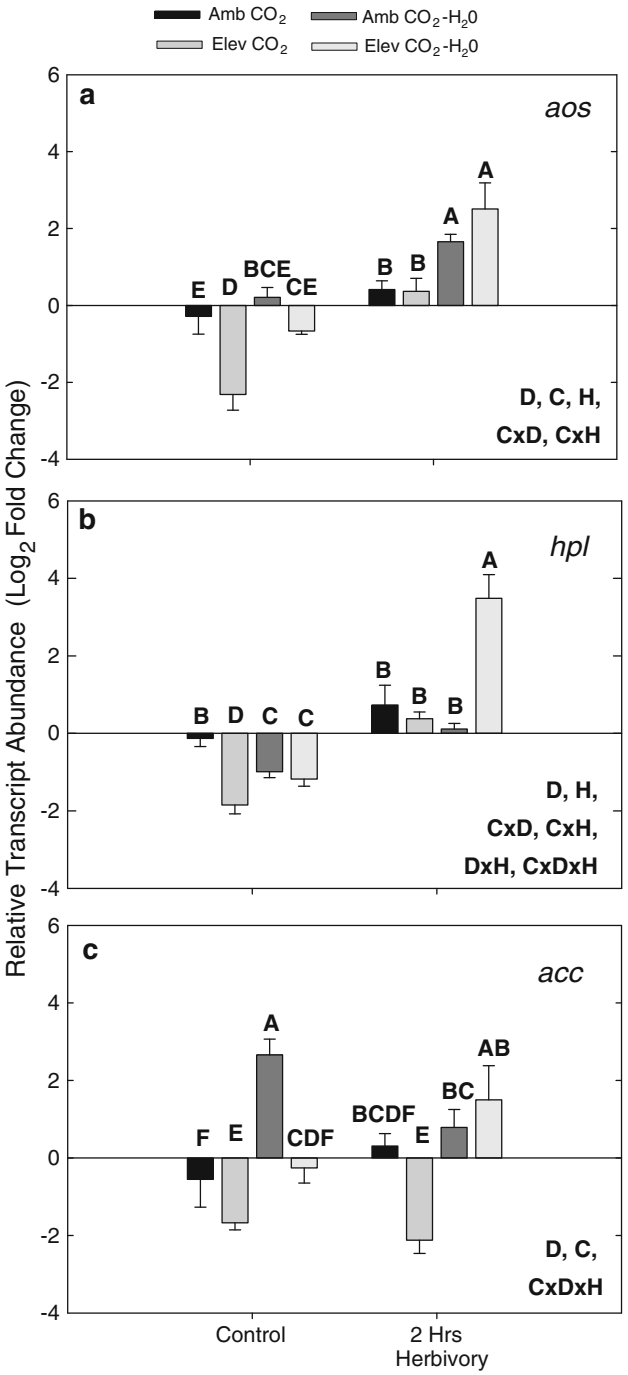

Fig. 2 Transcript abundance of three genes from fully expanded leaves of soybean grown under elevated or ambient CO_2 concentration, and at ambient or reduced soil water content (designated "drought"; *D*), before and after a burst of Japanese beetle feeding: **a** allene oxide synthase (*aos*), **b** 1-aminocyclopropane-1-carboxylate synthase (*acc1*), **c** hydrogen peroxide lyase (*hpl*). Data for all treatments are relative to transcript abundance in soybean grown at ambient CO_2 and at ambient soil water content prior to beetle feeding. A statistically significant ($P < 0.05$) main effect of CO_2 exposure, drought, or herbivory is indicated by *C*, *D*, or *H*, respectively. Interaction terms are included where significant. *Letters* indicate significant differences between treatments. Values represent the mean ± SE

CO_2: 8,481.4 ± 649.7 ng/g of tissue fresh weight; main effect of CO_2; $P = 0.005$). Growing soybeans under reduced water availability had no significant impact on SA levels (Fig. 3a). Simultaneous exposure to reduced soil moisture and CO_2 modified the induction pattern of JA after herbivory in soybeans (reduced soil moisture × herbivory and CO_2 exposure × reduced soil moisture × herbivory interaction; $P < 0.1$; Fig. 3b). ET was not examined in this study as it is highly volatile and difficult to measure under field conditions.

The activity of the defensive compounds CystPI and PPO in soybean leaves averaged across all treatments was 4.7 and 3.1 nmol/g of tissue fresh weight, respectively, and their activities were not affected by elevated CO_2, reduced soil water content, or herbivory ($P > 0.05$; data not shown).

Growth under elevated CO_2 increased leaf fructose and glucose content (main effect of CO_2; $P < 0.01$; Table 1), and there was a trend for increased sucrose (main effect of CO_2; $P = 0.065$; Table 1). Herbivory by Japanese beetles decreased leaf carbohydrates (main effect of herbivory; $P < 0.05$), and herbivory modulated the effect of elevated CO_2 on carbohydrates. After herbivory, fructose, glucose, and sucrose content decreased (main effect of herbivory; $P < 0.05$; Table 1); the reduction of fructose and glucose in leaves was more pronounced under elevated CO_2 compared with ambient conditions (CO_2 × herbivory interaction; $P < 0.001$; Table 1). No main effect of reduced soil water content on sugars or protein could be detected. And total protein content was not affected by any of the treatments (Table 1; $P > 0.05$).

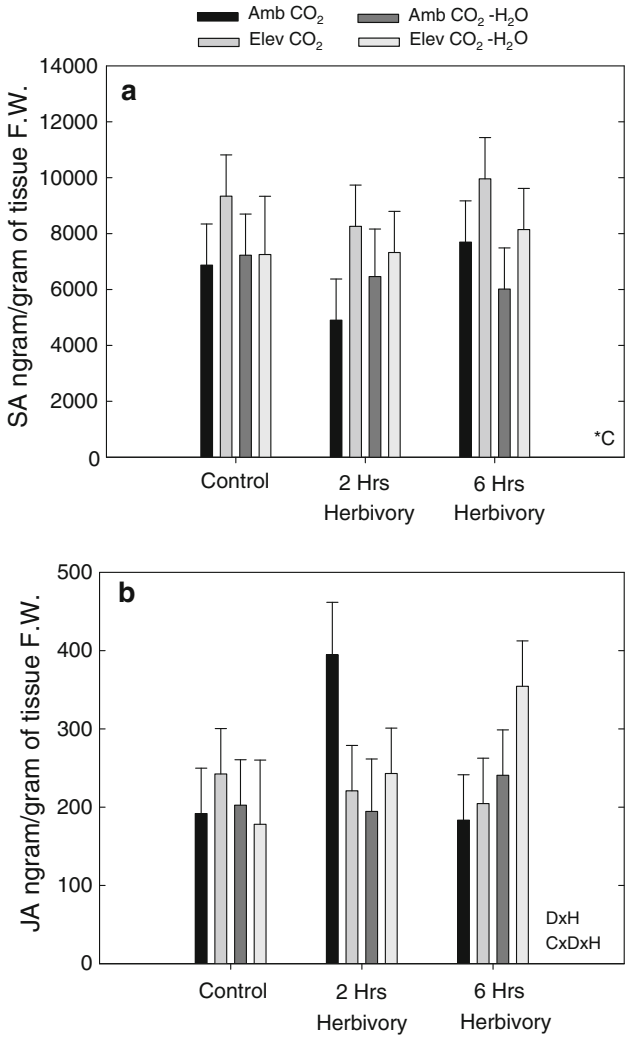

Fig. 3 Accumulation of (**a**) salicylic acid (SA) and (**b**) jasmonic acid (JA) in soybean grown under elevated or ambient CO_2 concentration, and at ambient or reduced soil water content (designated "drought"; *D*), before and after a burst of Japanese beetle feeding. A statistically significant main effect of CO_2 exposure, drought, or herbivory is indicated by *C*, *D,* or *H*, respectively ($P < 0.05$, $^*P < 0.1$). Interaction terms are included where significant. Values represent the mean ± SE

Discussion

The interactive effects of elevated CO_2 and reduced water availability on defense signaling and susceptibility of soybean to herbivory are complex. Consistent with previous results (Casteel et al. 2008, Zavala et al. 2008, 2009), growth under elevated CO_2 down-regulated constitutive expression levels of transcripts, *aos* and *hpl*, related to the production of direct and indirect defenses (Fig. 2a, b; significant CO_2 × herbivory interaction), and *acc*, a key gene in the production of ET (Fig. 2c; significant CO_2 × herbivory interaction). Strikingly, even a mild reduction in soil water content had the opposite effect on phytohormone signaling transcripts. Acting with elevated CO_2, reduced soil moisture increased the expression of *aos* and *hpl* (Fig. 2b; significant CO_2 × reduced soil moisture interaction), and reduced soil moisture acting independently increased expression of *acc* (Fig. 2b; significant main effect of reduced soil moisture). Despite the lack of significant difference in CystPI or PPO among treatments, the action of these genes and subsequent defenses were evident in Japanese beetle feeding (Fig. 1). As observed previously (Hamilton et al. 2005; Zavala et al. 2008, 2009), there was a substantial increase in tissue removed by beetles on plants grown under elevated CO_2 (Fig. 1). However, when plants were exposed simultaneously to reduced soil water content, this CO_2-induced increase in susceptibility to herbivory was eliminated (Fig. 1). Results presented here illustrate the capacity of other components of global change, in this case reduced soil moisture, to modulate the effect of elevated CO_2 on plant–insect interactions.

Sugars are feeding stimulants for Japanese beetles (Ladd 1986), and increased sugar content in leaves grown under elevated CO_2 (Table 1) may have contributed to the

Table 1 Changes in protein and sugar concentration in soybean grown under ambient and elevated CO_2 concentration with or without reduced soil moisture at SoyFACE, in undamaged plants and after herbivory by Japanese beetles (mean ± SEM)

Nutritional component	Undamaged				+Japanese beetle herbivory				
	Control	$+CO_2$	$-H_2O$	$+CO_2/-H_2O$	Control	$+CO_2$	$-H_2O$	$+CO_2/-H_2O$	Sig*
Protein	34.0 (2.9)a	29.0 (3.7)a	33.6 (3.3)a	31.8 (1.4)a	37.6 (3.5)a	33.6 (2.4)a	32.9 (2.3)a	30.7 (4.9)a	ns
Fructose	0.50 (0.08)a	1.06 (0.08)b	0.51 (0.06)a	0.90 (0.08)b	0.59 (0.10)a	0.53 (0.07)a	0.63 (0.01)a	0.60 (0.04)a	C, H, C × H
Glucose	2.0 (0.2)a	3.4 (0.2)b	1.3 (0.09)a	3.0 (0.09)a	1.2 (0.1)a	1.4 (0.3)a	2.0 (0.3)a	1.5 (0.3)a	C, H, C × H
Sucrose	1.9 (0.6)a	3.0 (0.8)b	2.7 (0.3)ab	4.8 (1.1)b	1.6 (0.8)a	2.0 (0.2)a	2.0 (0.3)a	1.8 (0.2)a	H

All values are expressed in μmol/g of tissue FW

A statistically significant ($P < 0.05$) main effect of CO_2, reduced soil moisture (designated "drought"), or herbivory is indicated by C, D, or H, respectively. Interaction terms are included where significant. "ns" indicates that no significant differences were observed. Values sharing the same letter in rows were not significantly different (LSD post hoc test, $P < 0.05$)

increased preference for foliage from well-watered plants (Fig. 1). Exposure to elevated CO_2 increased constitutive levels of leaf sugars, but reduced soil water content had no impact on carbohydrates (fructose, sucrose, and glucose; Table 1). However, the increased sugar content observed in the "droughted" plants grown under elevated CO_2 did not result in higher leaf area removed (Fig. 1). This loss of susceptibility is likely a result of increased production of defenses from modulated signaling pathways (Fig. 1a, b, c).

While this study examined only PPO and one proteinase inhibitor (CystPI) and that a statistical difference was not observed, changes in transcript level suggest that other JA- or ET-regulated defenses may have been affected by the treatments. In addition to JA and ET, SA plays a critical role in defense signaling to biotic stress. Consistent with previous results (Casteel 2010), soybean grown under elevated CO_2 had elevated levels of SA across treatments, regardless of soil water content. Generally, the SA defense pathway is activated after biotrophic pathogen attack initiating pathogen-specific defenses (Glazebrook 2005; Jones and Dangl 2006; Pieterse and Dicke 2007). Indeed, recent studies examining pathogen infections in soybeans grown under elevated CO_2 found reduced occurrence of downy mildew (*Peronospora manshurica*) a biotrophic pathogen. Insofar as increased SA is associated with increased production of defenses against pathogens, soybean plants may experience reduced pathogen infection under elevated CO_2 concentrations (Eastburn et al. 2010). Furthermore, increased SA may affect the final balance of phytohormone defense signaling networks. For example, SA generally interacts antagonistically with the JA/ET pathways controlling herbivore resistance (Doares et al. 1995; Maleck and Dietrich 1999; Felton and Korth 2000; Howe and Jander 2008; Leon-Reyes et al. 2010), and this relationship may influence a plant's ability to establish a targeted response to herbivore threats. Deciphering the influence of elevated CO_2 on hormonal networks will be essential to understanding how plants will orchestrate targeted defense responses in future environments.

Variation in phytohormones and plant defenses observed in this study was high and may be an experimental artifact related to the low number of repetitions for specific assays ($n = 3 - 4$) and the fact that these plants were grown in the field without protection from damage. Individual leaves used in these experiments were free of visible damage, but this was not the case for all trifoliates on an individual plant, as all field plants had some degree of damage by beetles or other folivores. In addition, pathogens may not have been visible in undamaged leaves chosen in field environments where infection is common (Eastburn et al. 2010). Although differences in plant defenses were not resolved following exposure to elevated CO_2 or reduced soil moisture, singly or in combination, effects on insect behavior were detected (Fig. 1). This outcome highlights the importance of examining plants under field conditions, which more accurately reflect the conditions plants experience in agricultural fields compared with growth chambers.

The ability of plants to quickly modify their transcriptome is critical in an ever-changing environment. Elevated CO_2 increases susceptibility of soybean to herbivory (Zavala et al. 2008, 2009), but the consequences of interactions with other components of global environmental change are largely unknown. In this study, we demonstrated that reduced soil moisture attenuated CO_2-induced transcriptional changes and allowed soybean plants to defend themselves more effectively against Japanese beetle herbivory. The amelioration of CO_2-induced chemical changes in the transcription of signaling and defense genes by reduced soil moisture is similar to the amelioration of CO_2-induced chemical changes by ozone (Casteel et al. 2008), suggesting that studying interactive effects of global

change will be essential in predicting plant–insect dynamics in the future. Although plant defenses vary greatly across species and even across cultivars of soybean, the jasmonate pathway is highly conserved in plants (Meyer et al. 1984; Herrmann et al. 1989). Examining the impact of drought and elevated CO_2 on this signaling pathway in other plant species may contribute to a greater understanding of the implications of global climate change on future crop productivity.

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