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Role of cysteine proteinase inhibitors in preference of Japanese beetles (*Popillia japonica*) for soybean (*Glycine max*) leaves of different ages and grown under elevated CO_2

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Abstract Elevated levels of CO_2, equivalent to those projected to occur under global climate change scenarios, increase the susceptibility of soybean foliage to herbivores by down-regulating the expression of genes related to the defense hormones jasmonic acid and ethylene; these in turn decrease the gene expression and activity of cysteine proteinase inhibitors (CystPIs), the principal antiherbivore defenses in foliage. To examine the effects of elevated CO_2 on the preference of Japanese beetle (JB; *Popillia japonica*) for leaves of different ages within the plant, soybeans were grown at the SoyFACE facility at the University of Illinois at Urbana-Champaign. When given a choice, JB consistently inflicted greater levels of damage on older leaves than on younger leaves, and there was a trend for a greater preference for young leaves grown under elevated CO_2 compared to those grown under ambient CO_2. More heavily damaged older leaves and those grown under elevated CO_2 had reduced CystPI activity, and JB that consumed leaves with lower CystPI activity had correspondingly greater gut proteinase activity. Younger leaves with higher CystPI activity and photosynthetic rates may contribute disproportionately to plant fitness and are more protected against herbivore attack than older foliage. Cysteine proteinase inhibitors are potent defenses against JB, and the effectiveness of this defense is modulated by growth under elevated CO_2 as well as leaf position.

Keywords Within-plant movement · Plant–insect interactions · Global change · Plant defenses · Optimal defense theory · Free-air CO_2 enrichment

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Introduction

Levels of atmospheric CO_2 are rising and are expected to double from the preindustrial level of 280 μmol mol^{-1} before the end of this century (Prather et al. 2001). Although high atmospheric CO_2 levels increase photosynthesis and crop yield, insect attacks can offset some of this fertilization effect (Long et al. 2006; DeLucia et al. 2008; Zavala et al. 2008a). This phenomenon can most readily be assessed when experiments are conducted in the open air in agricultural fields. Free-air concentration enrichment (FACE) experiments currently provide the most realistic measure of the future impact of elevated CO_2 on crop yields (Ainsworth et al. 2008).

There are some general patterns of plant responses to increased atmospheric CO_2, including decreases in leaf nitrogen and increases in total nonstructural carbohydrates (Ainsworth et al. 2002; Zvereva and Kozlov 2006). However, the effect of elevated CO_2 on plant interactions with insect herbivores is variable and depends on species. Soybeans (*Glycine max*), the most widely grown seed legume in the United States, are preferred by the invasive herbivore Japanese beetle (JB; *Popillia japonica* Newman: Coleoptera) when grown under elevated CO_2 over those grown under ambient CO_2 (Hamilton et al. 2005; DeLucia et al. 2008; Dermody et al. 2008).

In soybeans, elevated CO_2 down-regulates the expression of genes related to defense signaling. The expression of genes coding for the defense hormone jasmonic acid (JA; lipoxygenase 7, *lox7*, lipoxygenase 8, *lox8*) and ethylene (ET; 1-aminocyclopropane-1-carboxylate synthase, *acc-s*) decrease under elevated CO_2 (Chen et al. 2005; Saravitz and Siedow 1996; Casteel et al. 2008; Zavala et al. 2008a). Because the expression of cysteine proteinase inhibitors (CystPIs) is regulated directly by JA (Bolter and Jongsma 1995), the downregulation of these genes, in turn, reduces the expression of the two inducible CystPI genes (*N2* and *R1*) and reduces the corresponding enzyme activity (Botella et al. 1996; Zhao et al. 1996; Zavala et al. 2008a). CystPIs of soybeans are specific deterrents to JBs because they attach to the primary proteinases in the digestive tracts of beetles (Kim and Mullin 2003; Zavala et al. 2008a). Furthermore, gut cysteine proteinase activity was higher in beetles consuming foliage of soybeans grown under elevated CO_2 than in beetles consuming soybeans grown in ambient CO_2 (Zavala et al. 2008a), consistent with the enhanced growth and fecundity of these beetles on plants grown in elevated CO_2 (O'Neill et al. 2008).

Plant defenses in general and PI activity in particular vary among different tissues on an individual plant (Denno and McClure 1983; Zavala and Baldwin 2004; Zavala et al. 2004), and this variation in defense and other aspects of tissue quality cause herbivores to adjust their feeding positions (Zavala et al. 2008b). In addition to variation in defense, the effect of herbivory on plant fitness also depends on which tissues are damaged. For example, when *Manduca sexta* caterpillars feed on leaves at higher stalk positions on *Nicotiana attenuata* plants, they decrease plant fitness and increase their rate of mass gain and level of gut proteinase activity compared to larvae that remain on basal leaves (Zavala and Baldwin 2006; Zavala et al. 2008b). Elucidating the effects of elevated CO_2 on JB preference and CystPI activity heterogeneity within the soybean plant will increase our understanding of the consequences of down-regulation of defense on leaves of different ages.

To examine the effects of elevated CO_2 and leaf position within individual plants on JB feeding preference, we conducted an experiment at the SoyFACE facility at the University of Illinois at Urbana-Champaign. The SoyFACE facility, which elevates CO_2 under fully open-air field conditions, allowed us to investigate the consequences of increasing the susceptibility of soybean to naturally occurring JBs. Specifically, we examined whether growth under elevated CO_2 downregulated CystPI activity differentially within the plant, whether elevated CO_2 affected leaf damage from JBs within the plant, and whether JBs displayed different levels of digestive cysteine proteinase activity in their guts when consuming leaves of different ages grown under elevated CO_2. A reduction in the CystPI activity in younger leaves, which increases foliage quality, may increase feeding by insects and may thereby offset some of the predicted increases in agricultural productivity associated with greater levels of CO_2 in the atmosphere (Long et al. 2006).

Materials and methods

Experiments were conducted in the Soybean Free Air Concentration Enrichment (SoyFACE) facility at the University of Illinois, Urbana-Champaign (40°02′N, 88°14′W, 228 m above sea level; http://www.soyface.uiuc.edu). SoyFACE consists of soybeans (*Glycine max* cultivar 93B15, Pioneer Hi-Bred, Des Moines, IA, USA) grown in octagonal plots 20 m in diameter distributed within four randomized blocks (for an extended site description see Ainsworth et al. 2004). Within each block, one control plot was at the current ambient CO_2 of 380 μmol mol^{-1} and one plot was fumigated to a target CO_2 concentration of 550 μmol mol^{-1}. The experimental plots were separated by at least 100 m to prevent cross contamination of CO_2. The desired enrichment in each plot was maintained by varying the rate and position of gas release depending on the wind speed and direction. One-minute averages of CO_2 concentration were ±20% of the target for >95% of the time. At current rates of anthropogenic emissions, the targets for CO_2 represent predicted atmospheric levels in 2050 (Prather et al. 2001).

To determine if beetle leaf preferences were age dependent and if elevated CO_2 affected this preference, we surveyed naturally attacked leaves at different positions on plants grown under either elevated or ambient CO_2. Ten plants were randomly selected in each FACE plot, and for each plant the total number of leaves and the number of leaves with visible damage were recorded in each of four foliage age classes; the values for individual plants were averaged within a plot, as the unit of replication for statistical analysis was the individual FACE plot ($n = 4$). Leaves were classified into three age categories: mature (MA, older than first fully expanded leaf), medium (M, fully expanded leaf), and young (Y, less than 100% leaf expansion).

A separate experiment was conducted to examine the potential interactions between elevated CO_2, leaf position, and defense. Forty-four days after emergence, 30 undamaged vegetative-stage soybeans were randomly selected in each FACE plot, and the uppermost, fully expanded trifoliate leaf (M) on each of eight plants was infested with five adult Japanese beetles (*Popillia japonica* Newman; JB); an additional eight plants were infested with five adult JB apiece on trifoliate leaves expanded from 25% to less than 100% (Y); and four plants served as controls. Beetles were collected as they emerged from a nearby grass field and were the same age. To measure constitutive and induced CystPI activity in leaves, half of the treatment and control leaves were harvested for analysis one day after infestation, whereas the other half were harvested three days after infestation. To ensure that the control leaves remained undamaged and that the insects remained where they were placed, leaves were enclosed in plastic mesh (mesh size: 1 × 4 mm) secured around the petiole. Adult JBs were collected from the SoyFACE site from plants outside the plots 24 h prior to infestation.

A greenhouse experiment was conducted to examine the preference of JBs for leaves of different ages under ambient CO_2 only. Potted plants grown from the same seed as in SoyFACE were 35 days old at the beginning of the experiment. Ten plants were infested with ten JB adults each, and beetles were allowed to wander freely over the entire plant. Plants were bagged (plastic mesh; 1 × 4 mm) individually to keep the beetles on the plants and assessed for damage three days after infestation, as described below.

A second "preference" experiment was conducted in the field to examine preferences under ambient and elevated CO_2. Beetles were allowed to choose freely among leaves of different ages on individual plants. In each FACE plot, ten undamaged, vegetative-stage soybean plants were enclosed in a plastic mesh bag to include all leaves up to one trifoliate beyond the first fully expanded trifoliate (MA). Five bags were infested with ten adult JB, which were allowed to feed freely on the plant for three days. Five control plants were also enclosed to prevent natural damage from occurring. We estimated JB feeding damage on each trifoliate for four leaf positions (MA, M, Y and YY). Expanding leaves were divided into two categories in this experiment (young, Y, 25–100% leaf expansion; very young, YY, less than 25% leaf expansion). The ranking scale for damaged leaves was: 1 = no damage; 2 = damage present; 3 ≤ 1/3 leaf defoliated; 4 = 1/3–1/2 leaf defoliated; 5 > 1/2 leaf defoliated. Leaves were harvested, flash-frozen in liquid nitrogen, and stored at −80°C for a subsequent analysis of CystPI activity.

Cysteine proteinase inhibitor activity

We determined constitutive and induced CystPI activity in leaves using flash-frozen tissue ground to a fine powder. Control leaves (four at each time point) and those from each of the treatments (four at each time point) were combined to form one sample from each FACE plot; the unit of replication for statistical analyses was the individual FACE plot ($n = 4$). To determine cysteine proteinase inhibitor (CystPI) activity, leaf powder was extracted with 50 mM phosphate buffer (pH 7.2) containing 150 mM NaCl and 2.0 mM EDTA (4 mL extraction buffer g^{-1} fresh weight of tissue). Extracts were collected after vortexing for 10 s and centrifuging at 12,000×*g* for 15 min. CystPI activity levels in the leaves were measured against papain by following the release of *p*-nitroaniline (*p*NA; 37°C for up to 20 min at 410 nm) after adding the synthetic substrate *p*-Glu-Phe-Leu-pNA (Filipova et al. 1984). Briefly, papain (30 μl of 28 μg ml^{-1}) was incubated in a 96-well microplate with 0–10 μl supernatant of plant extracts at 37°C for 10 min before addition of the substrate. Protein concentrations were measured (Bradford 1976) using BSA (bovine serum albumin) as a standard. The molar concentration of active papain present in the commercial preparation (Sigma, St. Louis, MO, USA) was determined by titrating a known concentration of the inhibitor *trans*-epoxysucciny-L-leucyl-amido (4-guanidino) butane (E-64; 1–100 μl of 2 μM) against papain until all activity had been inhibited (Zucker et al. 1985).

Cysteine proteinase activity in the guts of insects

After three days of feeding on either young or medium-age leaves of plants grown under ambient or elevated CO_2, JBs were removed for analysis of gut cysteine proteinase activity. Midguts were removed from the five beetles on each leaf and combined with the midguts from beetles on the four replicates for either Y or M leaves to create one composite sample for each FACE plot. Midguts were stored at −20°C.

The composite samples of midguts of beetles from each field plot were pulverized in liquid nitrogen with a mortar and pestle. Proteinases from midguts were extracted by homogenizing tissue with Tri-K citrate (30 mM, pH 6.0) 1:1 and incubated on ice for 30 min. The suspension was centrifuged at 12,000 rpm for 15 min at 4°C, and the resulting supernatant used as a source of JB gut proteinase activity.

Gut cysteine proteinase activity was estimated using the chromogenic substrate *p*-Glu-Phe-Leu-pNA (Filipova et al. 1984); 10 μl of the 18× diluted enzyme were added to 20 μl of 0.38 mM *p*-Glu-Phe-Leu-pNA (in 0.1 M $NaPO_3$, 0.3 M KCl, 0.1 mM EDTA, 3 mM dithioerythreitol, pH 6.0) and incubated at 37°C. Absorbance at 410 nm from wells on the microtiter plate was measured at 20 s intervals for 20 min with JB enzymes. Initial rates of hydrolysis were estimated from the slopes of the resulting absorbance versus time graphs. Assays were linear over the assay period. One cysteine proteinase activity unit (CAU) was defined as the amount of enzyme required to produce 1 mM of 4-nitroaniline per minute at 37°C using *p*-Glu-Phe-Leu-pNA as a substrate under given assay conditions.

Statistical analyses

Data were analyzed using multifactor analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc comparisons ($P < 0.05$) for all experiments (SAS Institute 9.1, Cary, NC, USA). Percentage of leaves damaged by herbivores, cysteine proteinase activity and CystPI activity in "no choice" experiments were analyzed by two-way ANOVA. Cysteine proteinase activity in choice experiments was analyzed using multifactorial ANOVA. Leaf damage ratings in the greenhouse "preference" experiment were analyzed using ANOVA. Leaf damage ratings in the field were analyzed by ANOVA and correlated with CystPI concentrations using graphical analysis software (GraphPad Software Inc., San Diego, CA, USA).

Results

To determine the effects of elevated CO_2 on insect preference within soybean plants, we performed a survey of the damage on the upper zone of the canopy, where herbivores are more active. Damage was greater in old than in young leaves (age effect: $P < 0.01$); the average proportions of damaged young, medium and old leaves were 12.5 ± 1.9, 35.1 ± 2.1 and 52.1 ± 2.1, respectively (Fig. 1). There was a trend ($P = 0.052$) for greater damage under elevated CO_2 (35.6%) than under ambient CO_2 (30.9%; Fig. 1). The CO_2 × age interaction term was not statistically significant ($P = 0.56$).

CystPI activity was consistently lower in leaves grown under elevated CO_2 (1.8 ± 0.1 nmol mg protein^{-1}) than under ambient CO_2 (2.6 ± 01 nmol mg protein^{-1}; $P < 0.01$), and lower in medium-age (1.9 ± 0.1 nmol mg protein^{-1}) than in young leaves (2.6 ± 0.2 nmol mg protein^{-1}; $P < 0.01$). Insect damage significantly increased CystPI activity (undamaged: 1.7 ± 0.1 nmol mg protein^{-1}; damaged: 2.6 ± 0.2 nmol mg protein^{-1}; Fig. 2), and the magnitude of induction increased with time (time × damage interaction: $P < 0.01$). An effect of elevated CO_2 on the induction of CystPI could not be resolved statistically (CO_2 × time × damage interactions, $P = 0.78$).

Because cysteine proteinases are the principal protein-digesting enzymes in Coleoptera, we quantified the impact of changes in soybean CystPI on cysteine proteinase activity in the guts of JBs. We estimated proteinase activity in the guts of JBs that fed on either young or medium leaves of plants grown under ambient or elevated CO_2. No difference in gut cysteine proteinase activity could be resolved for beetles that fed on old versus young leaves ($P = 0.11$; Fig. 3). However, beetles that consumed foliage grown under elevated CO_2 had substantially higher gut proteinase activities (young leaves: 55.5% greater, $P = 0.002$; medium leaves: 35.2% greater, $P = 0.04$) than those that fed on leaves of plants grown under ambient CO_2 (Fig. 3). Our results suggest that reductions in CystPI activity in plants grown under

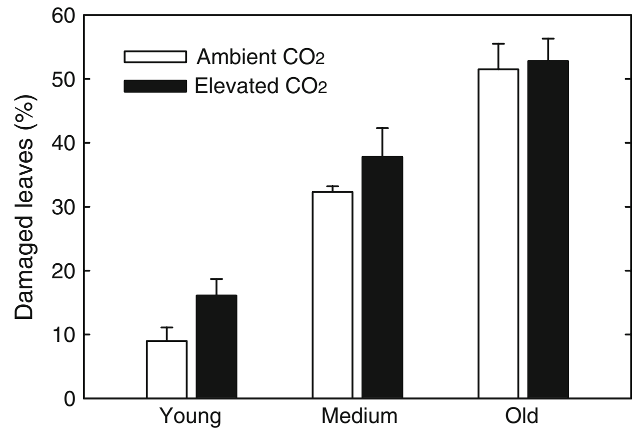

Fig. 1 The proportion of total soybean leaves in each age class, grown under either ambient or elevated CO_2, that exhibited visible damage by insect herbivores (mean ± SEM). We performed a survey of the damage on the upper third of the canopy, where herbivores are more active

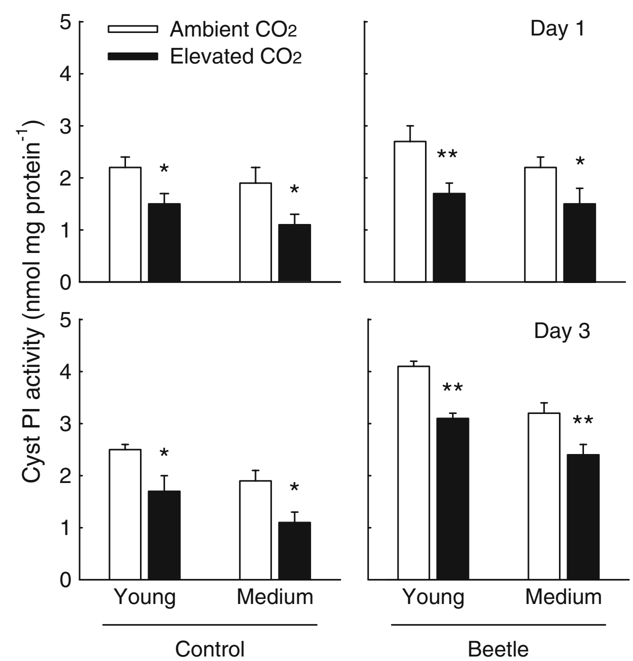

Fig. 2 Cysteine proteinase inhibitor (CystPI) activities (mean ± SEM) of fully expanded leaves (*medium*) and leaves expanded by less than 50% (*young*) of soybeans grown under either elevated or ambient CO_2. Activities one day and three days after JB infestation are shown. *Asterisks* indicate the level of significant difference between the ambient and elevated CO_2 treatments (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$)

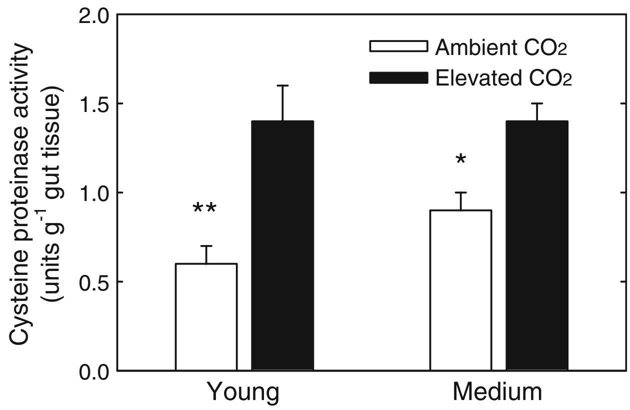

Fig. 3 Digestive cysteine proteinase activity units (CAU) in the guts of Japanese beetles that fed for three days on either young or fully expanded leaves of soybeans grown under either elevated or ambient CO_2 (mean ± SEM). *Asterisks* indicate the level of significant difference between the ambient and elevated [CO_2] treatments (*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$)

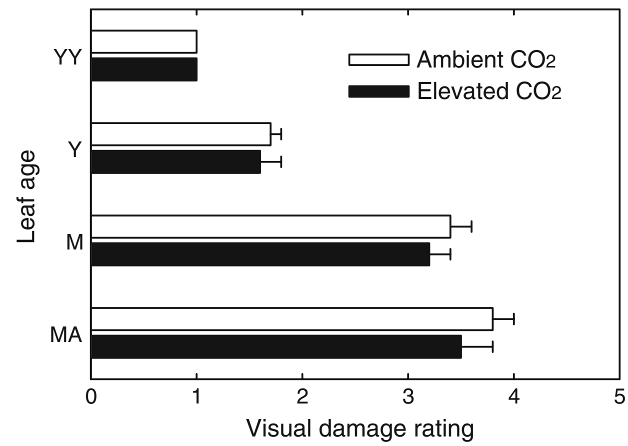

Fig. 4 Percentage damage (mean ± SEM) after three days of JB infestation on different trifoliate leaf ages of plants grown under either ambient or elevated CO_2: mature (*MA*), medium (*M*), young (*Y*) and very young (*YY*)

elevated CO_2 improved foliage digestibility and increased the digestive proteinase activity in JB guts.

When given the opportunity to feed freely among the top four soybean trifoliates, beetles inflicted more damage on old leaves than on young leaves (average visual damage rating: YY = 1.0 ± 0.0; Y = 1.3 ± 0.1; M = 2.1 ± 0.3, MA = 2.3 ± 0.4; $P < 0.01$; Fig. 4). No effect of elevated CO_2 on the amount of leaf damage was resolved in this experiment. CystPI activity decreased significantly as the leaves aged (CystPI activity, nmol mg protein^{-1}: YY = 2.8 ± 0.2; Y = 1.6 ± 0.2; M = 0.9 ± 0.1, MA = 1.7 ± 0.1; $P < 0.01$) and was significantly lower for leaves grown under elevated CO_2 (2.4 ± 0.2) than under ambient CO_2 (2.0 ± 0.1; $P < 0.01$). Insect damage caused an increase in CystPI activity (undamaged leaves: 1.7 ± 0.1; damaged leaves: 2.7 ± 0.2, $P < 0.01$), and, when all data were combined, CystPI activity at the end of the experiment was linearly correlated with the amount of damage (Y = CystPI × $x + b$; CystPI = 0.64 ± 0.16; $b = 0.73 \pm 0.44$; $R^2 = 0.35$; $P = 0.0004$). Our results suggest that CystPI activity modulates JB preferences and feeding damage among the top four soybean trifoliates.

The greenhouse experiment also showed that younger leaves experienced less beetle damage after three days of attack than older leaves ($P \leq 0.0001$; data not shown).

Discussion

Cysteine proteinase inhibitors are potent defenses in soybeans against herbivory by JBs, and the magnitude of this defense is modulated by the concentration of CO_2 in the atmosphere as well as leaf age. Elevated CO_2 increased JB feeding damage on soybean foliage by reducing CystPI activity levels (Hamilton et al. 2005; Dermody et al. 2008; Zavala et al. 2008a). Here we demonstrate that CystPI activity also modulates JB attack and preference within the plant; CystPI activity was higher in young than old leaves, and insect damage was inversely related to CystPI activity (Figs. 1, 4). Although we could not resolve a significant difference when JB consumed young versus old leaves, digestive cysteine proteinase activity was higher in the guts of beetles that consumed leaves grown under elevated CO_2 (Fig. 3), confirming the inverse relationship between defense (CystPI activity in leaves) and digestibility (gut PI activity). Younger leaves with higher photosynthetic rates, which presumably afforded greater contributions to the final yield (Larcher 2003; Morgan et al. 2004), were more protected against herbivore attack by having higher CystPI activities (Fig. 2). These results are consistent with the higher JA levels found in younger leaves of soybeans (Creelman and Mullet 1995).

Many herbivores perceive variations in the PI activities of plants and modify their feeding behavior to minimize their exposure to these potentially toxic chemicals (Bernays and Chapman 1994; Zavala and Baldwin 2004; Zavala et al. 2008b). Although trypsin PI of *N. attenuata* decreased *M. sexta* performance, larval movement within the plant alleviated the inhibitory effect of PI on gut proteinase activity and its effect on larval mass (Zavala and Baldwin 2004; Zavala et al. 2004, 2008b). Similarly, our results showed that CystPIs affected beetle preference, decreasing herbivory on foliage with higher CystPI levels (Fig. 4). Herbivores can respond to high PI levels in the diet by (a) overproducing or down-regulating PI-sensitive proteinase (Broadway and Duffey 1986; Zavala et al. 2008b), (b) overproducing PI-insensitive proteases (Bayes et al. 2005; Jongsma et al. 1995), (c) degrading the inhibitors (Giri et al. 1998), or (d) changing their feeding positions on the plant (Zavala and Baldwin 2004). Selective feeding on older, more poorly defended soybean leaves may allow JBs to optimize their growth under a variety of atmospheric conditions.

When allowed to feed freely on the top of the canopy, beetles damaged highly defended younger leaves less than older and less defended leaves (Fig. 4). Our results suggest that in spite of the high protein content of younger leaves (J. Zavala, unpublished data), JBs damage younger leaves initially and then move on to the older leaves to alleviate the inhibitor effect. Although elevated CO_2 decreased Cys-tPI activity in foliage, we could not resolve a preference between foliage grown under ambient or elevated CO_2 environments (Fig. 4). However, the field survey showed more insect damage in younger leaves of plants grown under elevated rather than ambient CO_2, suggesting either that the bags used in the control experiments affected JB behavior or that other invasive insects, such as grasshoppers, made use of the availability of foliage with high nutrition quality (Fig. 1).

Although many defense traits are elicited after herbivore attack and younger leaves are more protected against herbivores than older leaves, our survey in the soybean field showed that elevated CO_2 disrupts plant defenses, which increases herbivore attack on younger leaves (Fig. 1). Elevated CO_2 may affect the expression of other soybean traits regulated by JA and make plants more vulnerable to herbivores other than beetles, such as soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae) and western corn rootworm *Diabrotica virgifera virgifera* LeConte (Dermody et al. 2008). Herbivores respond in part by seeking more poorly defended plants and more poorly defended tissues within individual plants. SoyFACE experiments demonstrated that elevated CO_2 affects the expression of soybean inducible genes related to defense traits, including serine proteinase inhibitors, isoflavonoid content, and polyphenol oxidase activity (Casteel et al. 2008). The higher insect damage on younger leaves of plants grown under elevated CO_2 found in FACE experiments is a potential mechanism to account for the offset of the predicted increases in agricultural productivity associated with greater levels of CO_2 in the atmosphere (Ainsworth et al. 2008; Long et al. 2006).

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