Anthropogenic Changes in Tropospheric Composition Increase Susceptibility of Soybean to Insect Herbivory

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ABSTRACT Increased concentrations of CO_2 and ozone are predicted to lower nutritional quality of leaves for insect herbivores, which may increase herbivory as insects eat more to meet their nutritional demands. To test this prediction, we measured levels of herbivory in soybean grown in ambient air and air enriched with CO_2 or O_3 using free air gas concentration enrichment (FACE). Under open-air conditions and exposure to the full insect community, elevated [CO_2] increased the susceptibility of soybeans to herbivory early in the season, whereas exposure to elevated [O_3] seemed to have no effect. In the region of the canopy exposed to high levels of herbivory, the percentage of leaf area removed increased from 5 to >11% at elevated [CO_2]. We found no evidence for compensatory feeding at elevated [CO_2] where leaf nitrogen content and C:N ratio were unaltered in plants experiencing increased herbivory. However, levels of leaf sugars were increased by 31% at elevated [CO_2] and coincided with a significant increase in the density of the invasive species *Popillia japonica* Newman (Japanese beetle). In two-choice feeding trials, Japanese beetles and Mexican bean beetles (*Epilachna varivestis* Mulsant.) preferred foliage grown at elevated [CO_2] to foliage grown at ambient [CO_2]. These data support the hypothesis that the increased level of sugar in leaves grown at elevated [CO_2] may act as a phagostimulant for the Japanese beetle. If these results apply more widely to soybean production, the expectation of agricultural yield increases as a result of increasing elevated [CO_2] may need to be reevaluated.

KEY WORDS elevated CO_2, elevated ozone, Japanese beetle, free air gas concentration enrichment, *Popillia japonica*

HUMAN ACTIVITY IS RAPIDLY altering the chemical composition of the air in ways that may profoundly affect the interactions between insects and plants. The concentrations of CO_2 ([CO_2]) and of tropospheric ozone ([O_3]) have increased by 31 and 36%, respectively, since the beginning of the Industrial Revolution (1750). In the northern hemisphere, ozone concentrations are rising at a rate of between 0.5 and 2.5%/y, and atmospheric [CO_2] is expected to double this century (Prather and Ehhalt 2001, Prentice 2001).

Elevated [CO_2] stimulates photosynthesis, growth, and productivity of terrestrial ecosystems (Koch and Mooney 1996, Curtis and Wang 1998, DeLucia et al. 1999, Ainsworth et al. 2002, Hamilton et al. 2002), whereas elevated [O_3] typically has a negative impact on these processes (Sandermann 1996, Long and Naidu 2002, Morgan et al. 2003). A common feature of growth at elevated [CO_2] and elevated [O_3] is an alteration of leaf chemical composition that often affects the palatability and nutritional quality of foliage for leaf-feeding arthropods (Allen et al. 1988, Lincoln 1993, Reid and Fiscus 1998, Norby et al. 1999). For example, plants grown at elevated [CO_2] and elevated [O_3] often produce leaves with a lower nitrogen and soluble protein content (Mulchi et al. 1992, Cotrufo et al. 1998), thereby reducing the nutritional value to herbivores. In addition, plants grown at elevated [CO_2] commonly accumulate sugars and starch in their foliage, also affecting palatability by altering C:N (Cotrufo et al. 1998, Long et al. 2004) To meet their nutritional requirements, some herbivores exhibit "compensatory feeding" by increasing their consumption of foliage with a lower N content (Bezemer and Jones 1998, Whittaker 1999). The changes in foliar composition observed in plants grown at elevated [CO_2] and elevated [O_3] may increase the susceptibility to herbivory.

In this study, we examined the effect of growth at elevated [CO_2] and elevated [O_3] on the susceptibility of soybean to herbivory. Free air gas concentration enrichment (FACE) technology was used to elevate the [CO_2] and [O_3] to the levels predicted for the middle of this century. The advantage of using

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FACE experiments to investigate herbivory is that insects are allowed unfettered access to the experimental plots. Importantly, open-air experiments do not artificially restrict insect population size or diversity and include multi-trophic interactions. Another advantage of FACE is that plants are grown in the field where restrictions of rooting volume and nutrient supply common in controlled environments are absent (Long et al. 2004). Limited rooting volume may prevent induction of secondary metabolites (Baldwin 1988) or interact with elevated [CO_2] to alter leaf tissue composition and hence palatability (Long et al. 2004). The genetically uniform soybean (*Glycine max*) planted at the University of Illinois SoyFACE facility provided us with an attractive model system to test predictions of altered atmospheric composition on insect herbivory in the field. Results from greenhouse and open-top chamber studies suggest that elevated atmospheric [CO_2] and [O_3] will increase herbivory on soybean. Lincoln et al. (1984) found that the rate of feeding by soybean loopers (*Pseudoplusia includens*Walker) on soybean was positively correlated with [CO_2]. Experiments with soybean in open-top chambers have shown that increased [O_3] resulted in a significant increase in defoliation by adult Mexican bean beetle (*Epilachna varivestis*) (Chappelka et al. 1988). Moreover, beetle larvae tended to weigh more and develop faster on plants exposed to O_3, and in feeding tests, adult beetles preferentially fed on foliage that had been exposed to O_3 (Endress and Post 1985).

This study examined the following questions. (1) Does elevated [CO_2] or [O_3] alter susceptibility of soybeans to herbivory in the field? (2) Does elevated [CO_2] or [O_3] alter leaf chemical composition and lead to compensatory feeding? (3) Does elevated [CO_2] or [O_3] alter insect populations? (4) Given a choice, do common soybean herbivores exhibit a preference for foliage from soybeans grown at elevated [CO_2]?

Materials and Methods

Experimental Site. The Soybean Free Air gas Concentration Enrichment (SoyFACE) facility (University of Illinois, 40°03′21.3″ N 88°12′3.4″ W) was established to examine the responses of an agroecosystem to elevated tropospheric CO_2 and O_3. This site has been in continuous cultivation to arable crops for >100 yr, and cultural practices are typical for this region of Illinois. Twelve 20-m-diameter experimental plots were nested within the 16 ha planted with soybean. Each plot was surrounded by a segmented ring of pipe that released gas at high velocity at the surface of the crop canopy (Miglietta et al. 2001). Four plots were fully instrumented controls with ambient CO_2 and O_3; four plots maintained CO_2 concentrations at 550 μl/CO_2 liter air; and four plots maintained O_3 concentrations at 1.2× ambient levels. The elevated CO_2 treatment reflects the level anticipated by 2050 (Houghton et al. 2001). At the time of this research, an elevated CO_2 plus elevated O_3 treatment was not available. Fumigation was operated from planting until harvest during daylight hours. Plots were separated by 100 m to minimize cross-contamination. As part of the management of the SoyFACE site, the entire field was sprayed with 4 F carbaryl (Sevin; Aventis Crop Science, Research Triangle Park, NC) just after the first insect collection period to prevent an infestation of Japanese beetle (*Popillia japonica*) from killing the plants. Carbaryl is a nonsystemic contact insecticide with a short residence time of 3–7 d. No other pest management was applied for the rest of the field season.

Susceptibility to Herbivory. Because herbivore damage is heterogeneous throughout plant canopies in space and time, total damage is a function of the position and number of leaves present when a particular herbivore starts feeding. For each of the two sampling periods (17–18 July and 12 August 2002), we first determined the "zone of herbivory" and then sampled within this zone. Thus, our estimates provide a description of susceptibility to herbivory rather than an estimate of the total leaf area removed. To determine the zone of herbivory, we examined leaves on 10 haphazardly selected plants from each plot and scored them for damage. For every leaf, we visually estimated the percentage tissue removed. Most herbivory was localized on leaves at nodes 3–5 from a total of 9 nodes in July and from nodes 8–10 from a total of 16 nodes in August. Most damage occurred when leaves were at the top of the canopy and exposed to full sunlight with leaves subsequently added above as the plant grew. To estimate susceptibility to herbivory in the entire field, we haphazardly selected 100 plants from each plot and randomly chose one leaf from each plant from the subset of leaves in the zone of herbivory. Leaves were harvested, placed on a light box to provide high contrast, and photographed with a digital camera (Sony Cyber-Shot S85, Sony, New York, NY).

The digital images of each leaf (n = 100 for each plot) were converted to binary black and white, and their areas were measured using ScionImage software (Scion, Frederick, MD). We measured the area of each damaged leaf and estimated the total leaf area before herbivory by reconstructing the missing area. The vast majority of herbivory was from chewing insects; other forms of leaf damage were not quantified.

Insect Censuses. We surveyed insect populations by sweep net sampling on 11 July and again on 14 August 2002. Weather conditions were sunny to partly cloudy, with wind velocity <6 m/s. Each plot was swept four times with a standardized 40 sweeps (net diameter of 40 cm) over a total length of 30.4 m/plot. Total sweeping time for all plots on each date was ≈1.5 h. Insects in nets were placed on dry ice and transferred to the laboratory for identification.

Tissue Analysis. Leaf samples for carbohydrate analysis were taken at mid-day on 17 July and 15 August. One leaf disc (3 cm^2) was removed from a vein-free area of a lateral leaflet from the uppermost, undamaged, fully expanded trifoliate leaf, wrapped in foil, and frozen immediately in liquid nitrogen. Leaves received most herbivore damage when they were at the top of the canopy, and we assumed that leaves sampled for chemical analyses reflected the quality of leaves when they were most exposed to herbivores. Samples were analyzed for glucose, fructose, sucrose, and starch content as described previously by Rogers et al. (2004). Briefly, samples were powdered in liquid nitrogen and sugars extracted in 90% (vol:vol) ethanol. The glucose, fructose, and sucrose contents were determined from the ethanol extract using a continuous enzymatic substrate assay adapted for microwell plates. Starch in the pellet resulting from the ethanol extraction was digested in 32% (vol:vol) perchloric acid and assayed using a phenol-sulfuric acid assay.

For analysis of leaf N, C, water content, and specific leaf area (SLA; leaf mass per area), an additional 24 undamaged leaves were collected from each plot. A vein-free area was sampled from each leaflet, weighed, and dried at 70°C to constant mass. Concentrations of leaf carbon and nitrogen were measured on dried tissue by micro-Dumas combustion (ECS 4010; COSTECH Analytical Instruments, Valencia, CA).

Feeding Trials. We performed two feeding trials to assess preferences of herbivores for control versus CO_2-fumigated leaves. In the first trial, a single (field collected) adult Japanese beetle was placed in each of 11 containers (1-l clear, polypropylene) containing one undamaged soybean leaf chosen from an ambient and one from an elevated-CO_2 plot. Beetles were placed on the floor of the container at an equal distance from both leaves and were allowed to feed for 24 h. Leaves were analyzed for missing tissue with digital photography, and the area eaten was converted to mass using treatment-specific SLA. In the second trial (50 replicate containers, one beetle per container), we used Mexican bean beetle (*E. varivestis*), an important herbivore in other soybean-growing regions (beetles were obtained from a colony maintained by Charles Helm, IL Natural History Survey). Leaves for these studies were collected in late July and early August.

Data Analysis. The treatments were blocked and analyzed with a mixed model analysis of variance (ANOVA), with treatment and date as main fixed effects and block as a random component with plot as the replicate unit ($n = 4$; SAS version 8; SAS Institute, Cary NC). Interaction terms were included in the ANOVA. Treatment differences were analyzed using the Bonferroni means separation procedure. The level of significance was taken to be $P \leq 0.05$. To fulfill the assumptions of ANOVA, leaf area data were square root transformed before analysis. Values presented are least squares means.

Results

In July, leaf damage (on an area basis) in the active zone of herbivory was 57% greater in elevated [CO_2] plots compared with control plots ($F = 12.07$; df = 2,6; $P < 0.05$; Fig. 1A). There was no effect of elevated [O_3] on the susceptibility of leaf tissue to herbivory. In August, the overall levels of herbivory were lower,

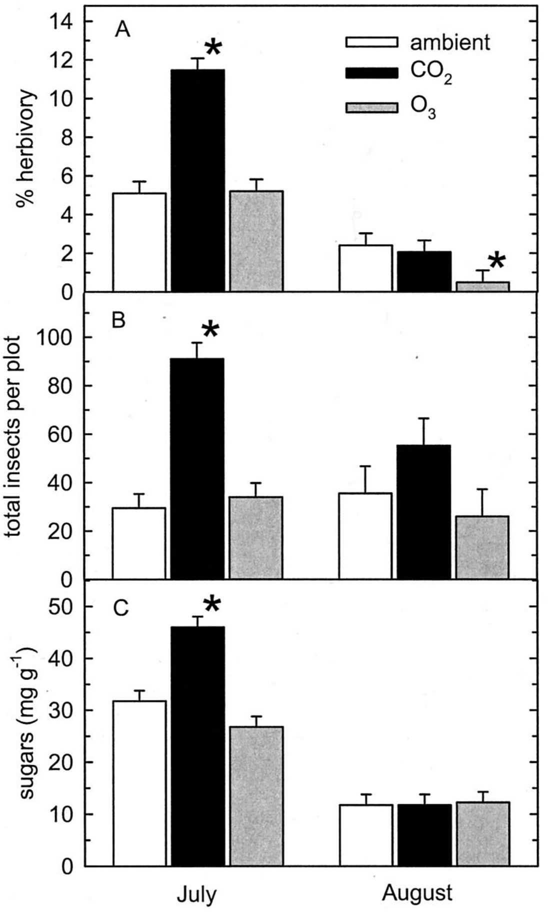

Fig. 1. Responses of soybean and insect herbivores to elevated [CO_2] or elevated [O_3]. (A) Percentage leaf area consumed by insect herbivores, (B) total numbers of insects per plot, and (C) leaf sugar concentrations. Sugar content was calculated as the sum of glucose, fructose, and sucrose. Data are means ± SE ($n = 4$). *Significant differences compared with ambient plots within a month as determined with a Bonferroni means separation test ($P \leq 0.05$).

and we did not detect an effect of elevated [CO_2] on herbivore damage. However, herbivory was significantly lower at elevated [O_3] ($F = 12.07$; df = 2,6; $P < 0.05$; Fig. 1A). In July, the total number of insects above ground was 66% greater at elevated [CO_2] ($F = 28.44$; df = 2,8; $P \leq 0.05$), and there was no effect of elevated [O_3] on insect abundance (Fig. 1B). In August, there was no significant effect of either elevated [CO_2] or elevated [O_3] on insect population densities (Fig. 1B). In July, the most common insects were the Japanese beetle, which accounted for 24, 55, and 35% of the total insect community in the control, elevated [CO_2], and elevated [O_3] plots, respectively, and the potato leafhopper (*Empoasca fabae*), which accounted for 56, 21, and 51% of the community in the control, elevated [CO_2], and elevated [O_3] plots, respectively. In August, there were very few Japanese beetles (2% of the total), and the majority of insects captured (61%) were western corn rootworm (*Di-*

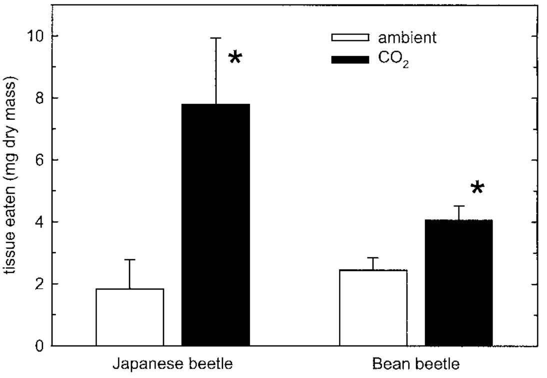

Fig. 2. Leaf mass eaten by Japanese beetle (*P. japonica*) and Mexican bean beetle (*E. varivestis*) in an arena feeding trial where the herbivores were offered a choice of foliage from soybeans grown at ambient or elevated CO_2 concentrations. Data are means ± SE (n = 4). Feeding trials were run for 24 h. *Significant differences within a species ($P \leq 0.05$).

abrotica virgifera), which were absent from the plots in July. At both sampling dates, no other insect taxa represented more than a few percent of the total, and there were no significant effects of elevated [CO_2] or elevated [O_3] on the combined densities of these other insect species.

In feeding choice trials, both the Japanese beetle and the Mexican bean beetle preferred foliage from plants grown at elevated [CO_2] (F = 8.46; df = 1,97; $P \leq 0.05$; Fig. 2). Beetles consumed 75 and 38%, respectively, more foliage from elevated CO_2-grown plants compared with ambient-grown plants.

The chemical composition of the leaves sampled in July and August and of those used in the feeding trial (July only) varied considerably between treatments and with sampling time (Table 1). In July, growth at elevated [CO_2] significantly reduced the specific leaf area by 17% and leaf water content by 4% (Tables 1 and 2) but had no effect on leaf C or N content or C:N ratio. At this time, however, the levels of glucose, fructose, sucrose, and starch all increased markedly (59, 19, 39, and 70%, respectively) in leaves grown at elevated [CO_2] (Tables 1 and 2). The total sugar content was also greater in elevated CO_2 in July (Fig. 1C). Growth at elevated [O_3] reduced both the glucose and fructose content of leaves, but had no effect on sucrose or starch in July (Tables 1 and 2, Fig. 1C). In August, there was no significant effect of [CO_2] or [O_3] on leaf composition. Nitrogen and carbohydrate concentrations typically were lower in August than in July, and values of SLA and percent water were greater (Tables 1 and 2; Fig. 1).

Table 2. Three-way ANOVA of the effects of date, treatment (elevated CO_2 or O_3), and the date by treatment interaction (date × treatment) on SLA, percent H_2O, C:N, and the contents of N, C, glucose, fructose, sucrose, and starch in mature soybean leaves

Factor	Source	F	df	P
SLA	Date	86.41	1,17	<0.0001
	Treatment	4.80	2,17	0.0222
	Date × treatment	6.87	2,17	0.0065
H_2O	Date	127.05	1,17	<0.0001
	Treatment	0.24	2,17	0.7919
	Date × treatment	10.15	2,17	0.0013
C:N	Date	3.35	1,17	0.0848
	Treatment	1.01	2,17	0.3861
	Date × treatment	1.41	2,17	0.2723
N	Date	11.85	1,17	0.0018
	Treatment	1.53	2,17	0.2332
	Date × treatment	2.39	2,17	0.1095
C	Date	0.52	1,17	0.4783
	Treatment	0.34	2,17	0.7177
	Date × treatment	0.33	2,17	0.7244
Glucose	Date	362.19	1,17	<0.0001
	Treatment	27.99	2,17	<0.0001
	Date × treatment	35.29	2,17	<0.0001
Fructose	Date	613.79	1,17	<0.0001
	Treatment	14.64	2,17	0.0002
	Date × treatment	8.08	2,17	0.0034
Sucrose	Date	35.18	1,17	<0.0001
	Treatment	1.82	2,17	0.1924
	Date × treatment	1.55	2,17	0.2413
Starch	Date	23.28	1,17	0.0002
	Treatment	7.97	2,17	0.0036
	Date × treatment	5.01	2,17	0.0195

All data except water content were on a dry mass basis.

Discussion

Under open-air conditions and exposure to the full insect community, an increase of atmospheric [CO_2]

Table 1. Specific leaf area (SLA) and chemical composition of mature soybean leaves grown in ambient conditions (Ambient), CO_2-enriched air (CO_2), and ozone enriched air (O_3), harvested from the top of the canopy in July and August

	July			August		
	Ambient	CO_2	O_3	Ambient	CO_2	O_3
SLA (cm^2/g)	246.8 (9.8)^a	205.8 (6.7)^b	233.0 (7.2)^a	275.7 (5.2)^a	278.4 (7.6)^a	271.8 (3.2)^a
H_2O (%)	73.4 (0.9)^a	70.20 (0.7)^b	72.3 (0.7)^a	78.3 (1.6)^a	82.2 (0.7)^a	79.1 (0.6)^a
C:N	9.3 (0.3)^a	10.6 (0.4)^a	9.2 (0.2)^a	10.7 (0.3)^a	11.8 (0.3)^a	18.1 (6.3)^a
N (mg g^{-1})	51.1 (1.6)^a	44.8 (1.9)^a	51.8 (1.2)^a	44.6 (1.4)^a	40.1 (0.8)^a	32.6 (5.7)^a
C (mg g^{-1})	471.6 (1.5)^a	471.4 (1.0)^a	473.8 (1.6)^a	422.3 (53.8)^a	472.9 (1.9)^a	463.5 (17.4)^a
Glucose (mg/g)	15.1 (1.3)^a	24.0 (0.3)^b	10.5 (0.9)^c	4.0 (0.5)^a	3.2 (1.1)^a	4.1 (0.4)^a
Fructose (mg/g)	5.4 (0.3)^a	6.4 (0.3)^b	4.5 (0.3)^c	0.8 (0.3)^a	1.2 (0.3)^a	1.1 (0.0)^a
Sucrose (mg/g)	11.1 (1.7)^a	15.4 (0.3)^b	11.9 (0.4)^a	6.9 (1.3)^a	7.2 (2.1)^a	7.0 (0.9)^a
Starch (mg/g)	65.0 (5.6)^a	110.5 (17.2)^b	54.6 (5.7)^a	37.5 (2.7)^a	48.7 (6.9)^a	45.2 (2.6)^a

Data are means ± SE of four replicate FACE rings. With the exception of water content, which was calculated on a fresh mass basis, data are expressed on a dry mass basis. Different letters within a given month indicate a significant difference ($P < 0.05$) between treatments as determined with a Bonferroni means separation test.

to levels predicted for the year 2050 (Houghton et al. 2001) increased the susceptibility of soybean to herbivores. This increase in susceptibility was limited to July, when populations of the invasive Japanese beetle were highest. Plants grown at elevated [O_3] did not show increased herbivory; however, in August when the loss of leaf tissue to herbivory was low, soybeans grown in elevated [O_3] had a reduced level of herbivory. This reduction may be related to the small reduction in leaf N content for plants grown in elevated [O_3], but it is also possible that cumulative leaf damage over the growing season or accelerated leaf senescence in the elevated O_3 plots deterred herbivores (Morgan et al. 2003).

The insecticide application, which was required to protect the plants for the benefit of the overall SoyFACE experiment, resulted in a short-term cessation of herbivory. Consequently, our estimate of percentage increase in herbivory represents a minimum because we could not quantify subsequent herbivore damage that would have occurred had the plots not been sprayed. Nevertheless, our results indicate a significant increase in the susceptibility of soybean leaves to herbivores when grown at elevated [CO_2]. Furthermore, this increase was even greater when measured as the mass of tissue removed because of the 17% decrease in SLA under elevated CO_2.

Compensatory feeding (Lincoln et al. 1984, Lincoln 1993, Bezemer and Jones 1998, Cannon 1998, Coviella and Trumble 1999, Hunter 2001) was probably not the cause of increased herbivore damage in our study. First, when given a choice, both Japanese beetles and Mexican bean beetles preferred foliage grown in elevated [CO_2] to ambient-grown foliage. Second, although we measured an increase in herbivory, there was no evidence that elevated [CO_2] decreased N content or increased C:N ratio. Finally, mobile insects, such as adult beetles, might be expected to avoid plants with low nutritive value; instead, Japanese beetles were found to be twice as abundant in July in CO_2-enriched plots as in ambient plots. Also inconsistent with the mechanism of compensatory feeding, other free-air studies found a decrease in herbivory under elevated [CO_2] (Stiling et al. 2002, 2003, Hamilton et al. 2004). One reason compensatory feeding might not have been a factor in this system is that leaf nitrogen levels in our managed N-fixing soybean crop were relatively high (>4 mg/g) compared with wild species. Thus, nitrogen content might not be limiting herbivores in this system.

Predictions concerning compensatory feeding typically have been based on the assumption that nitrogen is a primary limiting nutrient for insect growth. In mature insects, such as beetles, calories to fuel activity instead of nitrogen to build body tissues may be more important. Simple sugars such as glucose, fructose, and sucrose, however, are known feeding stimulants for Japanese beetle (Potter and Held 2002), and a relationship between insect feeding and leaf carbohydrate content has been observed before (Bezemer and Jones 1998). Our results suggest that the marked increase in these sugars in the leaves of soybeans grown at elevated [CO_2] may have caused increased susceptibility to herbivory. Rather than feeding in an effort to compensate for poor nutritional value, it seems that elevated sugar concentrations stimulated Japanese beetles to increase ingestion of leaves grown at elevated [CO_2]. Although the results from our study support the general prediction of increased herbivory under elevated CO_2, our findings point to an alternative underlying mechanism.

Growth under elevated CO_2 alters many aspects of leaf chemistry potentially affecting herbivory, including secondary plant compounds (Bezemer and Jones 1998, Hartley et al. 2000). Isoflavones and trypsin inhibitors have been implicated as resistance factors to arthropods in soybeans (Liu et al. 1992, Carraopanizzi and Kitamura 1995, Zhao et al. 1996), but how the content and effectiveness of these compounds may be altered by changes in tropospheric chemistry currently is unknown.

The effects of elevated [CO_2] on Japanese beetles in soybean would likely not have been detected except in an open-air FACE experiment, by virtue of its ability to allow free access to the complete community of naturally occurring insect herbivores. Japanese beetles have only recently come to the Midwest, having been introduced accidentally to North America in 1916 (Potter and Held 2002). Atmospheric changes may have greater consequences for interactions between native and introduced species than for those between species with long co-evolutionary histories. Thus, global biotic change in the form of invasive species (Pimentel et al. 2000) may interact with global climate change in a synergistic manner.

There was no evidence of differences in total insect counts between elevated CO_2 and control plots later in the season (August). There were very few Japanese beetles (partially because of the July spraying, but primarily because of the phenology of the beetle) and the majority of insects captured were western corn rootworm. That the western corn rootworm is not a major leaf-feeding herbivore of soybean explains the overall low levels of leaf damage in August. However, as a prevalent part of the soybean fauna (60%), it is nonetheless important because populations of this major corn pest in some parts of the Midwest are undergoing an evolutionary shift in behavior that circumvents crop rotation as a control method—they lay eggs in soybean fields that, in a typical two-crop rotation for rootworm management, will be planted in corn the following year (O'Neal et al. 2002). The attractiveness of soybeans grown at elevated [CO_2] to this insect may further exacerbate this problem over time.

There is increasing awareness of the direct impacts of increased concentrations of the anthropogenic pollutants CO_2 and O_3 on plants and food production. However, indirect effects, such as altered levels of herbivory, also have the potential to affect agricultural productivity. Historically, only $\approx$1% of Midwestern soybean acreage required treatment for insects (Suguiyama and Carlson 1985). Although soybean is typically considered to be tolerant of defoliation, the effects of leaf loss on yield depend on a number of factors and can often be significant (Haile et al. 1998). The limitations of working in the SoyFACE site did not allow us to let herbivory continue uncontrolled, but our study indicates that growth at elevated [CO_2] has the potential to increase crop susceptibility to pests, particularly those stimulated by sugars, thereby reducing potential agricultural gains from elevated CO_2 and increasing the need for pest management.

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