The role of ozone flux and antioxidants in the suppression of ozone injury by elevated CO₂ in soybean

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Abstract

The projected rise in atmospheric CO₂ concentration is expected to increase growth and yield of many agricultural crops. The magnitude of this stimulus will partly depend on interactions with other components of the atmosphere such as tropospheric O₃. Elevated CO₂ concentrations often lessen the deleterious effects of O₃, but the mechanisms responsible for this response have received little direct examination. Previous studies have indicated that protection against O₃ injury by elevated CO₂ can be attributed to reduced O₃ uptake, while other studies suggest that CO₂ effects on antioxidant metabolism might also be involved. The aim of this experiment was to test further the roles of O₃ flux and antioxidant metabolism in the suppression of O₃ injury by elevated CO₂. In a two-year experiment, soybean \textit{Glycine max} (L.) Merr. was exposed from emergence to maturity to charcoal-filtered air or charcoal-filtered air plus a range of O₃ concentrations in combination with ambient or approximately twice-ambient CO₂ concentrations in open-top field chambers. Experimental manipulation of O₃ concentrations and estimates of plant O₃ uptake indicated that equivalent O₃ fluxes that suppressed net photosynthesis, growth, and yield at ambient concentrations of CO₂ were generally much less detrimental to plants treated concurrently with elevated CO₂. These responses appeared unrelated to treatment effects on superoxide dismutase, glutathione reductase, and peroxidase activities and glutathione concentrations. Total ascorbic acid concentration increased by 28–72% in lower canopy leaves in response to elevated CO₂ and O₃ but not in upper canopy leaves. Increasing concentrations of atmospheric CO₂ will likely ameliorate O₃ damage to many crops due to reduced O₃ uptake, increased carbon assimilation, and possibly as yet undetermined additional factors. The results of this study further suggest that elevated CO₂ may increase the threshold O₃ flux for biomass and yield loss in soybean.

Key words: Antioxidants, ascorbic acid, carbon dioxide, conductance, flux, \textit{Glycine max}, ozone, soybean, starch, yield.

Introduction

Increasing concentrations of atmospheric CO₂ from pre-industrial levels have been accompanied by an increase in the frequency and duration of tropospheric O₃ episodes (Prather et al., 2001; Prentice et al., 2001). Atmospheric concentrations of both trace gases are expected to increase in the 21st century as global industrialization and emissions of CO₂ and O₃ precursors continue to grow (Prather et al., 2001; Prentice et al., 2001). Because increases in atmospheric CO₂ and O₃ concentrations have demonstrable effects on crop growth, yield, and water use, there is significant concern about the possible effects of these gases on agricultural production and practices (Allen, 1990; Unsworth and Hogsett, 1996; Polle and Pell, 1999; Olszyk et al., 2000; Ainsworth et al., 2002; Fiscus et al., 2002, 2005; Fuhrer and Booker, 2003; Morgan et al., 2003; Ainsworth and Long, 2005).

Atmospheric CO₂ enrichment typically increases net photosynthesis, biomass, leaf area, and less consistently, yield, in a number of C₃ crop species, including soybean \textit{Glycine max} (L.) Merr.] (Allen, 1990; Kimball et al., 1993; Ainsworth et al., 2002; Ainsworth and Long, 2005). By contrast, current levels of tropospheric O₃ in many industrialized regions of the world suppress net photosynthesis, biomass accumulation, and yield of many C₃ crop plants, including soybean (Heagle, 1989; Fuhrer and
Booker, 2003; Morgan et al., 2003; Fiscus et al., 2005). In combination, however, elevated CO2 concentrations often prevent or delay the deleterious effects of O3 on soybean and other crop plants (Allen, 1990; Unsworth and Hogsett, 1996; Fiscus et al., 1997, 2002; McKee et al., 1997a; Polle and Pell, 1999; Olszyk et al., 2000; Morgan et al., 2003; Booker et al., 2005). Several explanations for this interaction have been suggested.

The most compelling reason is that elevated concentrations of CO2 lower O3 flux into leaves (Allen, 1990; Fiscus et al., 1997; McKee et al., 1997b, 2000; Cardoso-Vilhena et al., 2004). In many crop plants, stomatal conductance (gs) declines as atmospheric concentrations of CO2 increase. The resulting decrease in gs lowers the amount of O3 absorbed by the leaf and subsequent O3 injury. For example, Fiscus et al. (1997) estimated that twice-ambient CO2 concentration lowered midday O3 flux into upper canopy soybean leaves by an average of 35%. The reduction in O3 flux was associated with the lack of O3-induced reductions in yield in plants treated concurrently with elevated CO2 and O3. However, reduced gs in response to elevated CO2 concentrations is not observed in some tree species, although O3 injury is still diminished (Polle and Pell, 1999).

A second explanation for the protective effect of elevated CO2 against O3 injury involves increased photosynthetic availability that could be used for repair and detoxification processes (Allen, 1990; Rao et al., 1995; McKee et al., 1997b; Sehmer et al., 1998; Polle and Pell, 1999). Plants grown in elevated CO2 might also be able to increase or maintain pools of antioxidants that confer resistance to O3 injury (Polle and Pell, 1999). However, McKee et al. (1997b) found no significant effect of elevated CO2 on superoxide dismutase (SOD) activity and concentration or redox status of ascorbate and glutathione in wheat (Triticum aestivum L.) leaf tissue. Sgherri et al. (2000) found no significant effect of elevated CO2 on glutathione reductase (GR) activity and glutathione concentration in alfalfa (Medicago sativa L.), although ascorbate concentration was higher. Pritchard et al. (2000) found lower antioxidative enzyme activity in soybean treated with elevated CO2, possibly reflecting a decrease in oxidative stress resulting from growth in a CO2-enriched atmosphere.

A third proposal suggests that elevated CO2 concentrations shift the intercellular CO2 concentration (Ci) away from Rubisco-limited assimilation toward ribulose-1, 5-bisphosphate (RuBP)-regeneration-limited assimilation (McKee et al., 1995; McKee et al., 2000). This possibly lowers the suppressive effect of O3 on photosynthesis by moving the photosynthetically limiting assimilation process away from an O3-sensitive region (Rubisco activity) to the region where photosynthetic electron transport for RuBP-regeneration limits assimilation (McKee et al., 1995, 2000). Photosynthetic electron transport processes are less inhibited by O3 compared with carboxylation activity (McKee et al., 1995, 2000; Long and Naidu, 2002; Fiscus et al., 2005).

There has been limited manipulative experimentation performed to test any of the previous hypotheses. In an experiment with a ‘wilky’ mutant of tomato (Lycopersicon esculentum Mill. flaccus) that has non-functional stomata, there was no statistically significant interaction between the elevated CO2 plus O3 treatment compared with the individual effects of elevated CO2 and O3 on plant dry mass (Olszyk and Wise, 1997). The study revealed that elevated CO2 did not suppress the deleterious effects of O3 on plant biomass. By inference, these results suggested that partial stomatal closure with elevated CO2 is an important plant-protection mechanism against O3 injury. A similar conclusion was reached in a CO2 plus O3 study conducted in growth chambers with wheat (Cardoso-Vilhena et al., 2004). However, manipulations of O3 concentrations to compensate for lower gs also suggested that elevated CO2 resulted in additional effects that alleviated the negative impacts of O3 on photosynthesis beyond that attributable solely to effects on O3 flux (Cardoso-Vilhena et al., 2004). The present study was designed to test some of these possibilities further.

The primary objective of this study was to test the role of O3 flux in the protective effect of elevated CO2 on biomass production and yield in O3-treated soybean. This was done by manipulations of chamber O3 concentrations in which O3 flux into upper canopy leaves of plants treated with elevated CO2 was equivalent to the O3 flux received by upper canopy leaves of plants treated with O3 at ambient CO2. The aim of this experiment was to determine if elevated CO2 protected against O3 injury when O3 concentrations were increased enough to obviate the effect of the lowered leaf conductance at elevated CO2. The results of the study should indicate whether the protective effects of elevated CO2 are mainly due to lower O3 flux. In addition, treatment effects on antioxidant metabolism were assayed to determine whether the protective effects of elevated CO2 against O3 injury might be linked to metabolic adaptations.

Materials and methods

Plant material and gas treatments

The experiments were performed at a site 5 km south of Raleigh, NC, USA (36° N, 79° W). Soybean (cv. Essex) was planted on 3 June 1998 and 1 June 1999 in pots containing 21 l of a 2:1:1 mixture of sandy loam soil:sand:Metro Mix 220 (Scotts Sierra Horticultural Products Co., Marysville, OH, USA) (pH 6). There were 16 potted plants in each chamber. Pot temperature fluctuation was moderated by a sleeve of 0.6 cm thick bubble wrap coated on both sides with aluminum (ReflectixTM; Reflectix, Inc., Markleville, IN, USA) fitted tightly around each pot. Plants were irrigated with drip tubes as needed to prevent visible signs of water stress and fertilized bweekly with an aqueous solution containing 2.5 g of soluble fertilizer (10-30-20, N-P-K) (Peters Professional; Scotts-Sierra Horticultural
Effects of elevated CO\textsubscript{2} on O\textsubscript{3} flux and injury

The initial fertilization included 0.31 g l\textsuperscript{-1} of a micro-nutrient formulation (STEM; Scotts-Sierra Horticultural Products Co.). Insects and mites were controlled with applications of acephate (Valent USA Corp., Walnut Creek, CA, USA), bifenthrin (Whitnire Micro-Gen Research Laboratories, Inc., St Louis, MO, USA), and abamectin (Syngenta Crop Protection, Inc., Greensboro, NC, USA). Plants were also treated with the fungicide mefenoxam (Syngenta Crop Protection, Inc.).

Plants were treated in 2.4 m tall \( \times \) 3 m diameter open-top field chambers (Heagle et al., 1979; Rogers et al., 1983) from germination to physiological maturity with reciprocal treatments of CO\textsubscript{2} and O\textsubscript{3}. Ozone was produced by electrostatic discharge in dry O\textsubscript{2} (model GTC-1A; Ozonia North America, Elmwood Park, NJ, USA) and monitored using UV photometric O\textsubscript{3} analysers (model 49; Thermo Environmental Instruments Co., Franklin, MA, USA). The CO\textsubscript{2} monitors were calibrated bi-weekly with CO\textsubscript{2} standards. The air 10 cm below the top of the plant canopy was monitored using infrared CO\textsubscript{2} analysers (model 6252; Li-Cor, Inc., Lincoln, NE, USA). The CO\textsubscript{2} monitors were calibrated bi-weekly with CO\textsubscript{2} standards. The air 10 cm below the top of the plant canopy was sampled in each chamber through Teflon tubing, and the concentration of CO\textsubscript{2} and O\textsubscript{3} in the air sample was measured every 30 min throughout the experiment.

The treatment combinations were: (i) charcoal-filtered (CF) air and ambient CO\textsubscript{2} (control); (ii) CF air plus 336 \( \mu \)mol CO\textsubscript{2} mol\textsuperscript{-1} (elevated CO\textsubscript{2}); (iii) CF air plus 1.5 times ambient O\textsubscript{3} and ambient CO\textsubscript{2} (elevated O\textsubscript{3}); and (iv) CF air plus 1.5 times ambient O\textsubscript{3} and 336 \( \mu \)mol CO\textsubscript{2} mol\textsuperscript{-1} (elevated CO\textsubscript{2} and O\textsubscript{3}) (Table 1).

In the elevated O\textsubscript{3} treatments, O\textsubscript{3} was added in a prescribed function based on the amount of midday leaf conductance (EOF) treatment, the O3 concentration was adjusted so that midday O\textsubscript{3} flux in elevated CO\textsubscript{2} matched that absorbed by leaves in the elevated O\textsubscript{3}–ambient CO\textsubscript{2} treatment. Daily changes of O\textsubscript{3} flux into upper canopy leaves. These calculations of O\textsubscript{3} flux into upper canopy leaves were based on measurements of midday leaf conductance. In the EOF treatment, the O\textsubscript{3} concentration was adjusted so that midday O\textsubscript{3} flux in elevated CO\textsubscript{2} matched that absorbed by leaves in the elevated O\textsubscript{3}–ambient CO\textsubscript{2} treatment at midday. Daily changes of O\textsubscript{3} concentration in the EOF treatment chambers were done by manually adjusting the O\textsubscript{3}-dispensing system.

**Leaf conductance measurements and O\textsubscript{3} flux calculation**

Ozone fluxes into upper canopy leaves were calculated using measurements of midday leaf conductance \((g_{w})\) and concurrent hourly O\textsubscript{3} concentrations. Midday leaf conductance was measured on the abaxial and adaxial surfaces of upper canopy leaves with a steady-state porometer (model 1600M; Li-Cor, Inc.) when weather conditions permitted (no precipitation after sundown on the previous day and photosynthetic photon flux density (PPFD) >800 \( \mu \)mol m\textsuperscript{-2} s\textsuperscript{-1}). Leaf conductance values were composed of stomatal conductance plus a standard boundary layer conductance imposed by the instrument (2.7 mol m\textsuperscript{-2} s\textsuperscript{-1}; Li-Cor, Inc., 1600M Instruction Manual, Revision 6, 1989). Four plants in each chamber were measured, and the mean \( g_{w} \) was used as the chamber replicate value. Midday \( g_{w} \) was measured on 37 and 34 days in 1998 and 1999, respectively, between 4 and 14 weeks after planting. Midday O\textsubscript{3} flux into the leaf was computed by dividing measurements of \( g_{w} \) by the ratio of the binary diffusivities for water vapour and O\textsubscript{3} in air (1.68) and multiplying by the O\textsubscript{3} concentration in the chamber at the time (Fiscus et al., 1997). The concentration of O\textsubscript{3} in the leaf interior was assumed to be zero (Laish et al., 1989; Moldau and Bichele, 2002).

**Net photosynthesis assay**

The net CO\textsubscript{2} assimilation rate (\( A \)) and intercellular CO\textsubscript{2} concentration (\( C_{i} \)) of the terminal main-stem leaf on two plants in each chamber were measured weekly from 9 to 14 weeks after planting (weather permitting). Measurements were made in the chambers at growth CO\textsubscript{2} and O\textsubscript{3} concentrations between 10.00 and 14.00 h EST when PPFD \( \geq 1000 \) \( \mu \)mol m\textsuperscript{-2} s\textsuperscript{-1}, using a portable photosynthesis system with a 1 l cuvette (Model 6200; Li-Cor, Inc.) and Version 6.2 software. The CO\textsubscript{2} analyser was calibrated at the beginning of each measurement occasion. Net photosynthesis was measured on 6 and 5 days in 1998 and 1999, respectively. Average PPFD and relative humidity in the cuvette during the measurements were 1681 \( \mu \)mol m\textsuperscript{-2} s\textsuperscript{-1} and 48%, respectively.

**Table 1. Seasonal 12-h (08.00–20.00 h EST) daily average CO\textsubscript{2} and O\textsubscript{3} concentrations in the two-year (1998 and 1999) field experiment**

Plants were treated with charcoal-filtered air (CF) or CF air plus O\textsubscript{3} in combination with ambient or elevated CO\textsubscript{2} concentrations. Values for SUM06, the sum of all hourly average O\textsubscript{3} concentrations \( \geq 60 \) \( \mu \)mol mol\textsuperscript{-1} for the 3 month period between 15 June to 15 September, are also shown. The treatments were: (i) charcoal-filtered (CF) air and ambient CO\textsubscript{2} (control); (ii) CF air and elevated CO\textsubscript{2} (elevated CO\textsubscript{2}); (iii) CF air plus O\textsubscript{3} and ambient CO\textsubscript{2} (elevated O\textsubscript{3}); and (iv) CF air plus O\textsubscript{3} and elevated CO\textsubscript{2} (elevated CO\textsubscript{2} and O\textsubscript{3}). In the equal O\textsubscript{3} flux (EOF) treatment, O\textsubscript{3} concentrations were increased so that O\textsubscript{3} flux in elevated CO\textsubscript{2} was equivalent to that in the elevated O\textsubscript{3}–ambient CO\textsubscript{2} treatment. Seasonal meteorological conditions (maximum temperature \( T_{\text{max}} \), average temperature \( T_{\text{avg}} \), daily photosynthetic photon flux density (PPFD) integrals, and relative humidity (RH) are daytime averages (PPFD \( \geq 80 \) \( \mu \)mol m\textsuperscript{-2} s\textsuperscript{-1}). Values shown are means \( \pm \) standard error.

<table>
<thead>
<tr>
<th>Year</th>
<th>Treatment</th>
<th>CO\textsubscript{2} (( \mu )mol mol\textsuperscript{-1})</th>
<th>O\textsubscript{3} (nmol mol\textsuperscript{-1})</th>
<th>SUM06 (( \mu )mol mol\textsuperscript{-1} h)</th>
<th>( T_{\text{max}} ) (°C)</th>
<th>( T_{\text{avg}} ) (°C)</th>
<th>PPFD (mol m\textsuperscript{-2} d\textsuperscript{-1})</th>
<th>RH (%)</th>
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<td>42±1</td>
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<td>EOF</td>
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<td>112±1</td>
<td>119±0.3</td>
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<tr>
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<td>55±1</td>
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<td>37±1</td>
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Growth assays

The Essex soybean cultivar is a determinate growth variety and terminates its growth on the main stem with a terminal node bearing a trifoliolate leaf. Mid-canopy (leaf 8) and terminal (leaf 14) main-stem leaves were initiated within a 1-week period around 5 and 8 weeks after planting, respectively, in all the treatments. At 14 weeks after planting (time of maximum vegetative biomass), three plants per chamber were destructively sampled. Tissue samples (2 cm diameter leaf discs) from main-stem leaves 8 and 14 (counting acropetally) were pooled by leaf position and chamber, and immediately frozen in liquid N2. Upon physiological maturity, five plants in each chamber were harvested for measurements of yield.

Antioxidant assays

Tissue samples (approximately 0.5 g FW) for enzyme assays were ground to a powder in liquid N2 and mixed with 50 mg of polyvinylpolypyrrolidone. Each tissue preparation was then mixed with 4 ml of 100 mM TRIS–HCl buffer (pH 7) containing 1 mM EDTA and 0.25% (w/v) ascorbic acid (AA). Samples were centrifuged (15 000 g for 10 min, and each supernatant was filtered through a 0.45 µm nylon filter. Filtered supernatants (2.5 ml) were desalted using gel-filtration (Sephadex G-25, PD-10 columns; Sigma Chemical Co.) employing 100 mM TRIS–HCl buffer (pH 7). Protein concentration in the desalted extract was determined using the Bio-Rad assay with BSA as a standard (Bradford, 1976).

SOD activity was assayed in 2 ml of 100 mM TRIS–HCl buffer (pH 7.2) containing 0.2 mM NADPH, 5 mM glutathione disulphide (GSSG), and 100 µl of plant extract (Anderson, 1996). The change in absorbance at 340 nm was recorded at 25 °C in a spectrophotometer. Enzyme activity was based on the oxidation rate of NADPH using an extinction coefficient of 6.2 mM−1 cm−1.

SOD activity was assayed by measuring the inhibition of nitroblue tetrazolium reduction (Beyer and Fridovich, 1987). A 50-µl aliquot of plant extract was mixed with 1 ml of assay medium containing 50 mM K2PO4 buffer (pH 7.8), 0.1 mM EDTA, 10 mM methionine, 57 µM nitroblue tetrazolium, and 0.025% (w/v) Triton-X 100. The solution was then mixed with 10 µl of 117 µM riboflavin in the dark. Absorbance of the preparation was measured at 560 nm before and after a 5 min illumination by two 15 W cool-white fluorescent lamps suspended 10 cm above cuvettes containing the reaction mixture on a tube rocker. SOD activity was expressed as units cm−2 leaf area where a 50% decrease in absorbance change per minute equalled one unit of activity. Maximum absorbance change was measured in a control preparation containing buffer in place of plant extract that was run in parallel with the other samples.

Peroxidase (POD) activity was assayed in 2 ml of 100 mM K2PO4 buffer (pH 6.3) containing 40 mM guaiacol, 10 mM H2O2, and 25 µl of plant extract at 25 °C in a spectrophotometer at 436 nm. Activity was based on the rate of tetraguaiacol production using an extinction coefficient of 25.5 mM−1 cm−1 (Polle et al., 1990).

Tissue samples (approximately 0.25 g FW) for AA and glutathione assays were ground to a powder in liquid N2 and extracted three times with 1 ml of 2% (w/v) metaphosphoric acid, 2 mM EDTA, and 5% 5-sulphosalicylic acid (Anderson, 1996). Extracts were pooled by sample, centrifuged (12 000 g) for 3 min, and each supernatant was filtered through a 0.45 µm nylon filter.

Total AA was assayed in 1 ml of 100 mM K2PO4 buffer (pH 7) containing 0.25 mM dithiothreitol and 100 µl of plant extract (Okamura, 1980). The solution was incubated at 25 °C for 15 min. Afterwards, 50 µl of 0.5% (w/v) N-ethylmaleimide, 500 µl of dipyridyl colour reagent (Okamura, 1980), and 100 µl of 3% FeCl3 were added to the solution and mixed. The solution was incubated at 37 °C for 60 min, and absorbance at 522 nm was measured using a spectrophotometer. Reduced AA was assayed by omitting dithiothreitol and N-ethylmaleimide. AA concentration was determined from a standard curve.

For the glutathione assays (glutathione plus homoglutathione), a 190-µl aliquot of plant extract was mixed with 4 µl of 2-xyvinyl pyridine and 6 µl of neat triethanolamine for determination of GSSG (Anderson, 1996). A second 190 µl aliquot of plant extract was mixed with 10 µl of neat triethanolamine for determination of total glutathione. Samples were incubated at 25 °C for 30 min. Glutathione was assayed in 2 ml of 100 mM Tris–HCl buffer (pH 7.5) containing 0.6 mM of 5,5′-dithio-bis(2-nitrobenzoic acid), 0.2 mM NADPH, 5 units of glutathione reductase, and 20 µl of plant extract at 25 °C in a spectrophotometer at 412 nm. The change in absorbance per minute was used to determine glutathione concentration based on a standard curve.

Carbohydrate and chlorophyll assays

For the carbohydrate assays, tissue samples were freeze-dried and ground to pass a 0.5 mm mesh screen. Starch and soluble sugars were determined enzymatically by the UV method (R-Biopharm, Inc., Marshall, MI, USA). To solubilize starch, tissue samples (50 mg) were each mixed with 2.4 ml of dimethylsulphoxide and 600 µl of 8 N HCl in sealed polypropylene tubes for 60 min at 60 °C. Samples were then neutralized with 600 µl of 8 N NaOH and diluted to 15 ml with 112 mM citrate buffer (pH 4). Solutions were filtered, and 50 µl aliquots were assayed according to kit instructions. Results were expressed as D-glucose equivalents.

For the chlorophyll assay, tissue samples (approximately 0.17 g FW) were extracted twice with 3 ml of 95% ethanol overnight at 4 °C. Extracts were pooled by sample and absorbance at 649 nm and 665 nm was measured. Chlorophyll concentration was calculated as previously described (Lichtenthaler and Wellburn, 1983).

Statistics

The experiment consisted of five treatments, and the treatments were assigned to chambers in a randomized complete block design. There were three replicate chambers per treatment (n = 15) in each year of the study. Results from 1998 and 1999 experiments were combined for analysis in this study. Data were checked for homogeneity of variance prior to statistical comparisons. An ln transformation was applied to gn, O3 concentration, O3 flux, and A data prior to statistical analysis. Treatment effects and means for periodic plant response variables were estimated using a repeated measures model in which chambers constituted the whole plots and sampling period was the repeated factor (SAS Proc Mixed; Littell et al., 1996; SAS Institute, 2001). If a significant sampling period by main effect interaction was detected, data were analysed separately for each sampling period using a randomized complete block model. If a significant main effect was detected within a sampling period, pair-wise comparisons were made among treatments to identify significant differences. Treatment effects and means for seasonal averages, biomass components, and biochemical assays were estimated using a mixed model (SAS Proc Mixed; Littell et al., 1996; SAS Institute, 2001).

Results

Gas treatments

Seasonal, 12 h (08.00–20.00 h EST) average CO2 concentration in the elevated CO2 treatments was nearly twice (1.9 times) the ambient CO2 concentration (Table 1). Seasonal, 12 h O3 concentration in the elevated O3 treatments averaged 2.4 times that in the control treatment and 1.3
times that in ambient air. In the EOF treatment (O3 flux in elevated CO2 equivalent to that in the elevated O3–ambient CO2 treatment), the 12 h O3 concentration averaged 1.6 times that in the elevated O3–ambient CO2 treatment. The SUM06 in the EOF treatment was 1.6 and 2.3 times that in the elevated O3–ambient CO2 treatment in 1998 and 1999, respectively.

**Leaf conductance (g\textsubscript{w}), O3 flux, and yield**

In the control and elevated O3 treatments, g\textsubscript{w} increased and then decreased as the growing season progressed (Fig. 1A). In the elevated CO2 and EOF treatments, g\textsubscript{w} was stable until 9–11 weeks after planting when it declined. Average midday g\textsubscript{w} was 43% lower in the elevated CO2 treatments compared with the control (Table 2). The calculated midday O3 flux was 41% lower in the combined gas treatment compared with the elevated O3 treatment. Midday O3 flux was marginally lower in the EOF treatment compared with the elevated O3 treatment even though average midday O3 concentration in the EOF treatment was more than 43% higher. Midday O3 concentrations in the EOF treatment reached their highest level at 9–11 weeks after planting to compensate for the steep decline in midday g\textsubscript{w} in the elevated CO2 treatments that occurred during this time period and thereafter in the experiment (Fig. 1B, C).

Pod number and husk dry mass increased in the elevated CO2 treatment compared with the control, but the effect on seed yield was not statistically significant (Table 2). In the elevated CO2 and O3 treatment, only husk mass showed a statistically significant increase. Unfilled pods had little influence on yield measurements since they constituted <1.3% of total pod mass per plant among all treatments and were excluded from determinations of seed and husk mass. Pod number, seed and husk dry mass, and mass per seed were 15–30% lower in the elevated O3 treatment compared with the control treatment. By contrast, pod number and seed and husk dry mass were at most 10% less in the EOF treatment compared with the control, and the differences were not statistically significant. Harvest index was lower in the elevated CO2 and combined gas treatments compared with the control.

**Biomass production and partitioning**

Biomass production was 24% higher in the elevated CO2 treatment and 28% lower in the elevated O3 treatment by 14 weeks after planting (time of maximum vegetative biomass) (Table 3). Elevated CO2, however, had no statistically significant effect on leaf area in the elevated CO2 treatments compared with the control treatment, although leaf area was 29% lower in the elevated O3 treatments. Biomass production and partitioning were similar in the elevated CO2 and combined gas treatments. Biomass production in the EOF treatment was not significantly different from the control, with the exception of pod mass, which was 18% higher in the EOF treatment. However, leaf area in the EOF treatment was lower than the control by 18%.

Biomass partitioning to stems, roots, and pods was not significantly affected by elevated CO2 (Table 3). Biomass partitioning to stems was lower in the elevated O3 treatment while allocation to pods increased 16%. In the EOF treatment, allocation to pods increased 8%. There were no statistically significant treatment effects on root:shoot ratios.

**Net photosynthesis (A) and intercellular CO2 concentration (C\textsubscript{i})**

At 9–11 weeks after planting, A was 9–14% higher in the elevated CO2 and combined gas treatments compared with A for the control (Fig. 2A). Net photosynthesis was not significantly different among the elevated O3, EOF, and control treatments. At 12–14 weeks after planting, however, A was 20–26% higher in the elevated CO2 and combined gas treatments compared with the control. Net photosynthesis in elevated O3 was 39% lower than the control, while A in the EOF treatment was 14% higher than the control.

Average C\textsubscript{i} in the elevated CO2, combined gas, and EOF treatments was 2.1 times higher than in control plant leaves (Fig. 2B). In the elevated O3 treatment, C\textsubscript{i} was 9–15% higher than in the control.

**Antioxidant enzymes and metabolites**

Glutathione reductase and SOD activities in midstem (leaf 8) and terminal (leaf 14) leaf tissue samples were not significantly different among treatments (Fig. 3A, B). Peroxidase activity was higher in leaf 14 tissue samples from the elevated O3 treatment compared with controls at 14 weeks after planting (Fig. 3C).

Total AA was increased 27% in tissue samples from leaf 8 in the elevated CO2 treatment at 14 weeks after planting compared with the control (Fig. 4A). It was increased by 56% in the elevated O3 and combined gas treatments, and by 72% in the EOF treatment. Total AA concentration in leaf 14, however, was not significantly different among treatments. Concentrations of glutathione in tissue samples were higher in most of the elevated O3 treatments and lower in some of the elevated CO2 treatments compared with the control (Fig. 4B). More than 72% of the AA and glutathione was in its reduced form, and the redox state of the AA and glutathione pools was generally not significantly different among treatments (data not shown).

Total soluble protein concentration in extracts from leaf 8 in the elevated CO2 treatment at 14 weeks after planting compared with the control (Fig. 4C). It was increased by 22–29% lower than in the respective control plant leaves. In the elevated O3 and EOF treatments,
soluble protein concentration in leaves 8 and 14 was 41–50% lower than in the respective samples from the control.

Chlorophyll concentration in leaves at both positions was lower in almost all treatments compared with the control treatment at 14 weeks after planting (Fig. 4D).

Starch and total soluble sugar concentrations in tissue samples from leaves 8 and 14 were much higher in the elevated CO2 treatment compared with the control treatment at 14 weeks after planting (Fig. 5A, B). Starch and sugar concentrations in the other treatments were generally not significantly different from the control.

**Discussion**

The objective of this study was to test the hypothesis that the protective effect of elevated CO2 against O3 damage was primarily attributable to decreased O3 flux resulting from CO2-induced and possibly O3-induced decreases in $g_w$. Previous studies have found that lower $g_w$ at elevated CO2 correlated with lower O3 flux and less growth and yield suppression from O3 (Fiscus et al., 1997, 2002, 2005; McKee et al., 1997b, 2000; Reid and Fiscus, 1998; Polle and Pell, 1999; Olszyk et al., 2000; Morgan et al., 2003; Cardoso-Vilhena et al., 2004; Booker et al., 2005). In the present study, the hypothesis that decreased O3 flux at elevated CO2 protected plants from O3 damage was tested by increasing the O3 concentration in the EOF treatment so that O3 flux in upper canopy leaves of plants treated with elevated CO2 was equivalent to that in the elevated O3–ambient CO2 treatment. If the protective effect of elevated CO2 against O3 injury was due solely to reduced O3 flux, then plant responses to O3 should be similar between the elevated O3 and EOF treatment. In the EOF treatment, high O3 concentrations compensated for the reduced $g_w$ induced by elevated CO2. Results indicated that the CO2-induced reduction in O3 flux was an important component in the prevention of O3 injury, but the protective effect of elevated CO2 against O3 damage involved more than lowered O3 flux. For example, O3 flux was lower in the elevated CO2 and O3 treatment compared with the elevated O3 treatment,

**Fig. 1.** Midday leaf conductance to water vapour ($g_w$) (A), O3 concentration (B), and O3 flux (C) at four sampling periods between 4 and 14 weeks after planting (WAP) in the two-year (1998 and 1999) experiment. Results from the 1998 and 1999 experiments were combined for analysis in this study. The treatments were: (i) charcoal-filtered (CF) air and ambient CO2 (Control); (ii) CF air and elevated CO2 (Elev CO2); (iii) CF air plus O3 in combination with ambient CO2 (Elev O3); and (iv) CF air plus O3 in combination with elevated CO2 (Elev CO2 & O3). In the EOF treatment, plants were treated with O3 concentrations in elevated CO2 that provided an O3 flux equal to that in the Elev O3 treatment (see Table 2). Values are means ± standard error. Statistically significant differences among treatments (Trt), sampling period (WAP), and their interaction are indicated as $P <0.001$ (**). Statistically significant differences among treatments at each sampling period are indicated by a different letter ($P <0.05$).
The elevated O₃ treatment were lower than the control. Approximately 50% of soluble protein is estimated to consist of Rubisco in soybean leaves (Campbell et al., 1988; Reid et al., 1998; FL Booker, unpublished results). By contrast, both A and soluble protein concentration in the elevated O₃ treatment were lower than the control.

Elevated O₃ is known to decrease soluble protein and Rubisco content in a number of plants, including soybean (Reid et al., 1998; Chemikova et al., 2000). Increased A at elevated CO₂ should help counter the effects of O₃ on Rubisco content by increasing net carbon fixation per unit of Rubisco carboxylation capacity, with no stomatal or RuBP regeneration limitations. Thus, the elevated CO₂–ambient CO₂ treatment. Values are means ± standard error. Values followed by a different letter are statistically significantly different (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Midday gₑw (nmol H₂O m⁻² s⁻¹)</th>
<th>Midday [O₃] (nmol mol⁻¹)</th>
<th>Midday O₃ flux (nmol m⁻² s⁻¹)</th>
<th>Pod number</th>
<th>Seed mass (g plant⁻¹)</th>
<th>Husk mass (g plant⁻¹)</th>
<th>Mass per seed (g seed⁻¹)</th>
<th>Harvest index (g seed⁻¹ aerial biomass)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>628 ± 17 a</td>
<td>35 ± 1 a</td>
<td>13.1 ± 0.3 a</td>
<td>517 ± 17 a</td>
<td>183 ± 7 a</td>
<td>76 ± 4 a</td>
<td>0.17 ± 0.01 a</td>
<td>0.56 ± 0.01 a</td>
</tr>
<tr>
<td>Elevated CO₂</td>
<td>359 ± 10 b</td>
<td>37 ± 2 b</td>
<td>8.0 ± 0.2 b</td>
<td>572 ± 17 b</td>
<td>195 ± 7 a</td>
<td>98 ± 4 b</td>
<td>0.18 ± 0.01 b</td>
<td>0.50 ± 0.01 bc</td>
</tr>
<tr>
<td>Elevated O₃</td>
<td>586 ± 16 a</td>
<td>82 ± 1 c</td>
<td>28.4 ± 0.7 c</td>
<td>438 ± 17 c</td>
<td>128 ± 7 b</td>
<td>53 ± 4 c</td>
<td>0.14 ± 0.01 b</td>
<td>0.57 ± 0.01 a</td>
</tr>
<tr>
<td>Elevated CO₂ and O₃</td>
<td>342 ± 9 b</td>
<td>82 ± 1 c</td>
<td>16.8 ± 0.4 d</td>
<td>529 ± 17 ab</td>
<td>176 ± 7 a</td>
<td>93 ± 4 b</td>
<td>0.18 ± 0.01 a</td>
<td>0.49 ± 0.02 b</td>
</tr>
<tr>
<td>EOF</td>
<td>344 ± 9 b</td>
<td>128 ± 2 d</td>
<td>26.1 ± 0.7 e</td>
<td>496 ± 17 a</td>
<td>165 ± 7 a</td>
<td>75 ± 4 a</td>
<td>0.17 ± 0.01 a</td>
<td>0.53 ± 0.01 ac</td>
</tr>
</tbody>
</table>

Table 2. Seasonal midday leaf conductance to water vapour (gₑw), midday O₃ concentration, O₃ flux and plant yield components in the two-year (1998 and 1999) experiment

The 1998 and 1999 experiments were combined for analysis in this study. The treatments were: (i) charcoal-filtered (CF) air and ambient CO₂ (control); (ii) CF air and elevated CO₂ (elevated CO₂); (iii) CF air plus O₃ and ambient CO₂ (elevated O₃); and, (iv) CF air plus O₃ and elevated CO₂ (elevated CO₂ and O₃). In the equal O₃ flux (EOF) treatment, O₃ concentrations were increased so that O₃ flux in elevated CO₂ was equivalent to that in the elevated O₃–ambient CO₂ treatment. Values are means ± standard error. Values followed by a different letter are statistically significantly different (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf mass (g plant⁻¹)</th>
<th>Stem mass (g plant⁻¹)</th>
<th>Root mass (g plant⁻¹)</th>
<th>Pod mass (g plant⁻¹)</th>
<th>Total mass (g plant⁻¹)</th>
<th>Leaf area (m² plant⁻¹)</th>
<th>Stem:total mass ratio</th>
<th>Root:total mass ratio</th>
<th>Pod:total mass ratio</th>
<th>Root:shoot mass ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>70 ± 2 a</td>
<td>118 ± 9 a</td>
<td>43 ± 3 a</td>
<td>135 ± 5 a</td>
<td>366 ± 15 a</td>
<td>1.7 ± 0.1 a</td>
<td>0.32 ± 0.01 a</td>
<td>0.12 ± 0.01 a</td>
<td>0.37 ± 0.01 a</td>
<td>0.14 ± 0.01 a</td>
</tr>
<tr>
<td>Elevated CO₂</td>
<td>85 ± 2 b</td>
<td>154 ± 9 b</td>
<td>52 ± 3 b</td>
<td>166 ± 5 b</td>
<td>457 ± 15 b</td>
<td>1.6 ± 0.1 ab</td>
<td>0.34 ± 0.01 a</td>
<td>0.11 ± 0.01 a</td>
<td>0.37 ± 0.01 a</td>
<td>0.13 ± 0.01 a</td>
</tr>
<tr>
<td>Elevated O₃</td>
<td>53 ± 2 c</td>
<td>79 ± 9 c</td>
<td>27 ± 3 c</td>
<td>117 ± 5 c</td>
<td>276 ± 15 c</td>
<td>1.2 ± 0.1 c</td>
<td>0.28 ± 0.01 b</td>
<td>0.10 ± 0.01 a</td>
<td>0.43 ± 0.01 b</td>
<td>0.11 ± 0.01 a</td>
</tr>
<tr>
<td>Elevated CO₂ and O₃</td>
<td>75 ± 2 c</td>
<td>142 ± 9 ab</td>
<td>51 ± 3 b</td>
<td>166 ± 5 b</td>
<td>434 ± 15 bd</td>
<td>1.5 ± 0.1 ab</td>
<td>0.32 ± 0.01 a</td>
<td>0.12 ± 0.01 a</td>
<td>0.38 ± 0.01 a</td>
<td>0.14 ± 0.01 a</td>
</tr>
<tr>
<td>EOF</td>
<td>69 ± 2 a</td>
<td>129 ± 9 ab</td>
<td>39 ± 3 a</td>
<td>159 ± 5 b</td>
<td>396 ± 15 ad</td>
<td>1.4 ± 0.1 b</td>
<td>0.32 ± 0.01 a</td>
<td>0.10 ± 0.01 a</td>
<td>0.40 ± 0.01 c</td>
<td>0.11 ± 0.01 a</td>
</tr>
</tbody>
</table>

Table 3. Biomass components (organ and total dry mass, leaf area, and mass ratios) of soybean plants at 14 weeks after planting (time of maximum vegetative biomass) in the two-year (1998 and 1999) experiment

Results from the two-year experiment were combined in the analysis. The treatments were: (i) charcoal-filtered (CF) air and ambient CO₂ (control); (ii) CF air and elevated CO₂ (elevated CO₂); (iii) CF air plus O₃ and ambient CO₂ (elevated O₃); and (iv) CF air plus O₃ and elevated CO₂ (elevated CO₂ and O₃). In the equal O₃ flux (EOF) treatment, O₃ concentrations were increased so that O₃ flux in elevated CO₂ was equivalent to that in the elevated O₃–ambient CO₂ treatment. Values are means ± standard error. Values followed by a different letter are statistically significantly different (P ≤ 0.05).

and A, biomass, and yield were suppressed in the elevated O₃ treatment, but not in the combined gas treatment (Fig. 2A; Tables 2, 3). However, O₃ flux in the elevated O₃ and EOF treatments averaged 27 ± 1 nmol m⁻² s⁻¹, but pod number, seed yield, mass per seed, and total biomass were only significantly different from the control in ambient, not elevated, CO₂. Net photosynthesis in the EOF treatment was higher than the control at 12–14 weeks after planting, but it was suppressed in the elevated O₃ treatment (Fig. 2A). These results suggest that factors in addition to decreased O₃ flux are involved in the protective effect of elevated CO₂ against O₃ damage (Allen, 1990; Fiscus et al., 1997; McKee et al., 2000; Cardoso-Vilhena et al., 2004).

One likely factor is the increase in A at elevated CO₂ that could compensate for the inhibitory effects of O₃ on carbon assimilation. Net photosynthesis in the EOF treatment was not significantly different from the elevated CO₂ treatment (Fig. 2A). This occurred despite an average 48% decrease in soluble protein compared with the control (Fig. 4C). Approximately 50% of soluble protein is estimated to consist of Rubisco in soybean leaves (Campbell et al., 1988; Reid et al., 1998; FL Booker, unpublished results). By contrast, both A and soluble protein concentration in the elevated O₃ treatment were lower than the control.
increasing carbon assimilation over that at ambient CO₂. This would be effected at elevated CO₂ by increased photo-assimilation and decreased photorespiration (McKee et al., 1997b; Booker et al., 1997; Long and Naidu, 2002).

There were, however, indications in the present experiment that the protective effect of elevated CO₂ against O₃ damage in the EOF treatment was less than complete. Soluble protein levels were lowest in leaves from the elevated O₃ and EOF treatments (Fig. 4C). Pod number, total biomass, and starch concentration in the EOF treatment were not significantly different from the control treatment, whereas they were increased in the elevated CO₂ treatment (Fig. 5A; Tables 2, 3). Likewise, Reid and Fiscus (1998) found that A was higher in Essex soybean treated with both elevated CO₂ and O₃ compared with control plants, but elevated CO₂ in the presence of high O₃ did not prevent an early decline in A, A max, and carboxylation efficiency during reproductive growth. This suggests that CO₂ did not completely alleviate the accelerated senescence due to O₃ damage (Reid and Fiscus, 1998). However, effects of the relatively high O₃ concentrations on biomass in the EOF treatment in the present study were not nearly as severe as those caused by an equal O₃ flux in ambient CO₂.

Estimates of O₃ flux were derived from measurements of midday g w in upper canopy leaves and midday O₃ concentrations. The measurements comprised a relatively limited sample of daily upper canopy g w and O₃ concentrations. The steady-state porometer used to make the g w measurements also imposes an artificial boundary layer condition that might alter the O₃ flux compared with in situ conditions (Fiscus et al., 1997). However, the calculated midday O₃ flux values were considered indicative of integrated O₃ flux based on previous studies that showed a threshold relationship between midday O₃ flux and yield of Essex soybean and hybrid rice (Oryza sativa L.) treated with mixtures of CO₂ and O₃ in open-top chambers (Fiscus et al., 1997, 2002). The approach in this study was also supported by results from an experiment with soybean that showed that whole-plant transpiration was lowered by elevated CO₂ and O₃, and the trends persisted throughout the day and the growing season (Booker et al., 2004). Thus, measurements of midday g w and O₃ concentrations were considered a practicable way to characterize O₃ flux.

Similar conclusions regarding the protective effect of elevated CO₂ against O₃ injury, based on photosynthetic responses, were drawn from a CO₂ plus O₃ study conducted in growth chambers with wheat (Cardoso-Vilhena et al., 2004). In that study, elevated CO₂ reduced the extent of O₃-induced suppression of A and V c max when cumulative O₃ uptake was similar in leaf 4 of plants treated with and without elevated levels of CO₂. Explanations for the protective effects of elevated CO₂ against O₃ injury included decreased O₃ flux, increased availability of carbohydrates for detoxification and repair processes, and changes in leaf morphology with elevated CO₂ that affected O₃ diffusion. All of these explanations could apply to the present findings.

Although elevated CO₂ did not alter GR, SOD, or POD activities in ways that appeared instrumental in O₃ detoxification processes, AA concentrations were increased in leaf 8 in response to both elevated CO₂ and O₃ (Figs 2, 3). However, there were no statistically significant treatment effects on AA concentration in leaf 14. By contrast to Rao et al. (1995), McKee et al. (1997b) concluded that amelioration of O₃ injury by elevated CO₂ was not primarily related to effects on antioxidant metabolism, but rather...
decreased O₃ flux. Responses of antioxidant metabolism to elevated CO₂ are variable but it appears that growth at elevated CO₂ reduces oxidative stress, although it is unclear whether the accompanying metabolic changes affect plant susceptibility to oxidative stress from additional factors such as O₃ or drought (Azevedo et al., 1998; McKee et al., 1997b; Polle and Pell, 1999; Pritchard et al., 2000; Sgherri et al., 2000; Schwanz and Polle, 2001; Wustman et al., 2001). In addition, the decrease in glutathione concentration with elevated CO₂, which was observed in some of the elevated CO₂ treatments in the present study, could be linked to a lower mineral:C content because glutathione functions as a reserve of reduced S (Bergmann and Rennenberg, 1993; Kurz et al., 1998). Foliar concentrations of mineral nutrients tend to decrease at elevated CO₂ because nutrient uptake efficiency declines (Rogers et al., 1999). Kurz et al. (1998) and Schwanz and Polle (2001) found decreased glutathione levels in leaves of oak (Quercus spp.) saplings exposed to elevated CO₂, but McKee et al. (1997b) and Sgherri et al. (2000) found no statistically significant effect of elevated CO₂ on glutathione concentrations in wheat and alfalfa, respectively. Schwanz and Polle (2001) also found a lower level of AA, but Kurz et al. (1998) and Sgherri et al. (2000) found higher AA concentrations at elevated CO₂. Increased AA concentration was possibly the result of greater carbohydrate supply at elevated CO₂ (Polle and Eiblmeier, 1995; Kurz et al., 1998; Sgherri et al., 2000).

In the present study, increased AA and glutathione concentrations and POD activity in the elevated O₃ treatment, along with decreased soluble protein and chlorophyll concentration, can be interpreted as responses to oxidative stress imposed by O₃ (Hausladen and Alscher, 1993; Chernikova et al., 2000). However, the lack of statistically significant changes in the activities of the antioxidative enzymes GR and SOD in the elevated O₃ treatment, which was also observed in other O₃ studies (Azvedo et al., 1998; McKee et al., 1997b), differed from studies where activities of these enzymes increased in response to O₃ (Hausladen and Alscher, 1993; Chernikova et al., 2000).

The variability in the responses of antioxidant metabolism to elevated CO₂ and O₃ among studies probably reflects differences in the magnitude of the perceived

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Fig. 3. Activities of glutathione reductase (GR) (A), superoxide dismutase (SOD) (B), and guaiacol peroxidase (POD) (C) in extracts of mid-stem (leaf 8) and terminal (leaf 14) leaf tissues at 14 weeks after planting. The treatments were: (i) charcoal-filtered (CF) air and ambient CO₂ (Control); (ii) CF air and elevated CO₂ (Elev CO₂); (iii) CF air plus O₃ in combination with ambient CO₂ (Elev O₃); and (iv) CF air plus O₃ in combination with elevated CO₂ (Elev CO₂ & O₃). In the EOF treatment, plants were treated with O₃ concentrations in elevated CO₂ that provided an O₃ flux equal to that in the Elev O₃ treatment (see Table 2). Values are means ± standard error. Statistically significant differences among treatments at each sampling period are indicated by a different letter (P <0.05).
oxidative stress, the species-specific mechanisms involved in responses to changes in redox status, mineral nutrition, and the plant’s capacity to cope with additional oxidative stress. Added to this are the confounding treatment effects of elevated CO2 and O3 on plant ontogeny that can shift development forward (Fiscus et al., 1997; Reid and Fiscus, 1998; Reid et al., 1998; Booker et al., 2005), although in the present study leaves of similar ages were used in the biochemical assays. Also, variability arises from different experiment protocols and environmental conditions among studies. Clearly, further study of the effects of elevated CO2 and O3 on antioxidant enzyme regulation as well as glutathione and ascorbate substrate availability, turnover rates, cellular localization, and interactions with other changes in cellular metabolism is needed to understand the responses of antioxidant metabolism to elevated CO2 and O3.

In the present study, increased biomass production with elevated CO2 included increased pod number and pod dry mass, but it did not extend to increased seed yield, by contrast with previous studies (Kimball et al., 1993; Ainsworth et al., 2002; Jablonski et al., 2002; Ainsworth and Long, 2005). In some of the previous studies, however, control plants were grown in ambient air, and yields could have been suppressed by O3 or another stressor that was lessened by elevated CO2 (Fiscus et al., 2002, 2005; Morgan et al., 2003). In the present study, increased pod dry mass in the elevated CO2 treatments was due primarily to increased allocation to husks. Other studies at the same location using the same soybean cultivar and similar treatment methods as in the present study found that seed yield ranged from −7% to 30% in elevated CO2 and CF air for plants grown in large pots and in the ground compared with ambient CO2 and CF air (Fiscus et al., 1997; Heagle et al., 1998; Booker et al., 2004, 2005). The underlying reasons for this variability in yield response to elevated
CO₂ in CF air are unclear. Possibly a borderline nutrient deficiency during pod fill in the elevated CO₂ treatments resulted in an accumulation of starch in the husks at the expense of sucrose transport to the seeds, which limited seed production in the present study. The lower glutathione and soluble protein, as well as higher non-structural carbohydrate concentrations (Figs 4B, C, 5A, B) in leaf tissues from the elevated CO₂ treatments suggest that mineral nutrients were lower than the control during pod-fill (Bergmann and Rennenberg, 1993; Kurz et al., 1998; Stitt and Krapp, 1999). The aforementioned confounding treatment effects of elevated CO₂ and O₃ on plant ontogeny, as well as the experimental variability inherent in multi-year field experiments, are likely to have had an influence on yield estimates as well.

Shifts in biomass allocation toward reproductive organs occurred in the elevated O₃ and EOF treatments (Table 3). Similar changes have been observed in previous studies with soybean (Cooley and Manning, 1987; Miller et al., 1998). During reproductive growth, assimilate is partitioned to pods at the expense of leaves, stems, and roots. In O₃-treated plants, pods apparently became stronger sinks for photosynthate than in the control, possibly due to altered levels of plant growth regulators or carbohydrates (Andersen, 2003). Elevated CO₂ counteracted the O₃ effects in the combined gas treatment, but only partially in the EOF treatment. The lower starch concentrations in the EOF and combined gas treatments in comparison with the elevated CO₂ treatment suggests that carbohydrates were being metabolized at a higher rate in O₃-treated plants (Fig. 5).

The findings of this and other chamber experiments relating to the manner in which plants respond to simultaneous increases in ground-level concentrations of both CO₂ and O₃ will require verification under field conditions. Open-top chambers decrease PPFD by 10–20%, increase air temperature by 0.5–2.5 °C, and alter the saturation vapour pressure deficit compared with outside the chamber (Heagle et al., 1979; Allen et al., 1992; Manning and Krupa, 1992; Kimball et al., 1997). Mean leaf boundary layer conductance is relatively constant in open-top chambers, which differs from field conditions (Grunhage and Jager, 1994). These and other environmental factors would be expected to modify the O₃ flux responses found in the present study, but not to the extent that would negate the major trends observed. Comparative studies of crop plants treated in open-top chambers and free-air CO₂ enrichment systems found similar trends in the responses of A, gs, biomass, and yield to elevated CO₂ for the two methodologies, although there were quantitative differences among responses between the two systems (Kimball et al., 1997, 2002; Ainsworth and Long, 2005). However, within the context of the present experiment, differences in environmental conditions between open-top chambers and the field are not really pertinent. The objective of the present study was to test the role of O₃ flux and antioxidants in the protection against O₃ damage by elevated CO₂ through experimental manipulations of O₃ concentrations in a system that maintained otherwise similar environmental conditions.

Overall, elevated CO₂ concentration counteracted the detrimental effects of O₃ on growth and yield. This response has been observed in previous studies with soybean and other crop species (Unsworth and Hogsett, 1996; Olszyk et al., 2000; Ainsworth et al., 2002; Fiscus et al., 2002, 2005; Morgan et al., 2003; Cardoso-Vilhena et al., 2004; Booker et al., 2005). Although, exceptions to this generalization have been reported when, for example, O₃ concentrations are relatively low compared with plant sensitivity (no O₃ interaction; e.g. Fangmeier and Bender, 2002), or conversely when cultivars are highly sensitive to O₃ stress (no CO₂ protection; e.g. Heagle et al., 2002, 2003).

Increasing concentrations of tropospheric O₃ will suppress, to varying degrees, the increase in growth and yield resulting from rising atmospheric CO₂ concentrations. On the other hand, O₃ injury is lessened by elevated CO₂ due to reduced O₃ uptake, increased carbon assimilation, and to as yet undetermined additional factors. In addition, the present findings have implications regarding exposure–response relationships for O₃ as atmospheric CO₂ concentrations continue to rise. Fiscus et al. (1997) estimated that the threshold midday O₃ flux for yield loss ranged between 20 and 30 nmol m⁻² s⁻¹ for soybean in ambient CO₂. The present study suggests that this threshold midday O₃ flux for biomass and yield loss in soybean may be increased by elevated CO₂. The potential for increasing concentrations of atmospheric CO₂ to modify threshold O₃ flux concentrations will need to be evaluated for possible incorporation into O₃ flux models used to predict plant injury from ambient O₃.

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